COMPOSITION AND FUNCTION OF INTERMEDIATE FILAMENTS IN AVIAN MUSCLE CELLS AND ERYTHROCYTES

Thesis by

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To my parents

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ABSTRACT

Intermediate filaments comprise a family of morphologically similar cytoplasmic structures whose individual subunits are biochemically and immunologically distinguishable, and largely cell-type specific. This thesis is an investigation of the intermediate filaments in avian smooth and skeletal muscle, and avian erythrocytes.

Conditions have been found under which sheets of interconnected Z-discs can be generated from skeletal muscle. The stability of these sheets demonstrates that the Z-discs of adjacent myofibrils are firmly linked to one another. Desmin, the intermediate filament subunit characteristic of muscle, encircles each Z-disc and thereby forms an insoluble, two-dimensional scaffold at right angles to the fiber axis within each Z-plane. Desmin may thus be responsible for maintaining the cross-striated appearance of skeletal muscle fibers, and may function to mechanically integrate the contractile actions of their constituent myofibrils. Vimentin, the intermediate filament subunit characteristic of mesenchymal cells, coexists with desmin at the periphery of the Z-disc, and demonstrates that a terminally differentiated cell can possess more than one class of intermediate filament subunit.

A 230,000 dalton polypeptide, named synemin, copurifies with desmin from smooth muscle. It coexists and colocalizes with desmin and vimentin in skeletal muscle at all stages of differentiation, from fusing myoblasts to mature fibers. Nondenaturing conditions under which synemin can be separated from desmin and vimentin have not been found.

Detection of synemin in avian erythrocytes has led to the realization that this cell might be a relatively simple model system for the study of intermediate filaments. A fraction of the intermediate filaments in avian erythrocytes is stably associated with the plasma membrane, but can be selectively removed from it with water; this results in preparations consisting predominantly of vimentin and synemin. Immunoelectron microscopy reveals that vimentin forms the bulk of the core filament

in these cells, and synemin exists at regular intervals along this core. The axial periodicity of synemin appears to change during erythropoiesis, perhaps in accordance with some structural or functional change in the filaments. Synemin appears to crosslink the filaments through self-association, and may thus regulate the rigidity or dispersion of the intermediate filament network in erythrocytes as well as in muscle cells.

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Chapter 1

Introduction

Identification and Characterization of Intermediate Filaments

Electron Microscopic Identification

Twenty years ago, electron microscopists noted bundles of 8-10 nm filaments in thin sections of many cells of higher vertebrates (e.g., DePetris, Karlsbad and Pernis, 1962; Tanaka, 1964); many of these cells were non-epithelial in origin, indicating that the cytoplasmic filaments were different than the 8-10 nm tonofilaments prominent in the keratinizing cells of the epidermis (see Rudall, 1952; Brody, 1959). These filaments were first recognized as comprising a distinct class of cytoplasmic filaments in embryonic skeletal muscle (Heuson-Stiennon, 1965), where they were termed "intermediate filaments" because their average diameter was greater than the thin (actin) filaments and less than the thick (myosin) filaments and microtubules (Ishikawa, Bischoff and Holtzer, 1968; Kelly, 1969). This morphological classification scheme was also found to hold true for filaments in cardiac (Rash, Biesele and Gey, 1970a) and smooth muscle (Campbell et al., 1971; Cooke and Chase, 1971; Uehara, Campbell and Burnstock, 1971). Intermediate filaments had previously been seen in thin sections of muscle, but had been misinterpreted as being actin filaments (e.g., Allen and Pepe, 1965; Obinata, Yamamoto and Maruyama, 1966; Przybylski and Blumberg, 1966; Fischman, 1967) or myosin filaments in the process of assembling (Heuson-Stiennon, 1965; Firket, 1967) or disassembling (Lowy and Small, 1970). However, even though the intermediate filaments of both muscle and non-muscle cells were found not to bind heavy meromyosin (HMM) as would the actin filaments (Ishikawa, Bischoff and Holtzer, 1969), interconversion of actin filamaents and intermediate filaments was still considered by some to be a possibility (Ishikawa et al., 1969; Rash, Shay and Biesele, 1970b). Remarkably, this issue was settled only recently with the publication (Buckley, Raju and Stewart, 1978) and subsequent retraction (Buckley et al., 1981) of a claim that intermediate filaments have an HMM-decorable core of F-actin that is revealed by brief trypsinization.

In the nervous system, filaments roughly 10 nm in diameter had been noted in neuronal processes and in astroglia, where they were known as neurofilaments and glial filaments, respectively (Peters and Vaughn, 1967; Schmitt, 1968; Wuerker and Palay, 1969; Peters, Palay and Webster, 1970; Wuerker, 1970; Wuerker and Kirkpatrick, 1972). The selective staining of neurons with various silver compounds used by light microscopists since the beginning of this century now appears to be a function of the neurofilaments that pack their axons and dendrites (Gambetti, Autilio-Gambetti and Papasozomenos, 1981). Neurofilaments have been studied most readily and to great advantage in the giant axons of certain marine invertebrates (Schmitt, 1950, 1968; Davison and Taylor, 1960; Gilbert, 1972, 1975; Metuzals, 1969), and have been studied in mammals most intensively because of their apparent proliferation in certain natural and induced states of disease and neuronal degeneration (Terry, 1963; Terry and Peña, 1965; Wisniewski and Terry, 1967; Wisniewski, Shelanski and Terry, 1968; Wisniewski, Terry and Hirano, 1970; Schlaepfer, 1971; Selkoe et al., 1979). Similarly, glial filaments are a particularly prominent cytoplasmic component of gliosed scar tissue that results from injuries to, and a variety of pathological states of, the central nervous system (Eng et al., 1971; Goldman, Schaumburg and Norton, 1978; Skoff, 1976; Bignami, Dahl and Rueger, 1980). As with intermediate filaments in other cell types (see above) neurofilaments were found not to bind HMM (Burton and Kirkland, 1972; Chang and Goldman, 1973), thus distinguishing them from actin filaments.

Exposure of cultured cells to antimitotic, microtubule-depolymerizing drugs such as colchicine, colcemid and vinca alkaloids was found to result in the appearance of bundles of cytoplasmic filaments (Robbins and Gonatas, 1964). Morphologic examination of such filaments led to their identification as intermediate filaments, and also led to the realization that this behavior was characteristic of intermediate filaments in many different cell types, such as muscle cells (Ishikawa et al., 1968; Holtzer

et al., 1975), PtKl cells (Brecher, 1975), BHK cells (Holmes and Choppin, 1968; Goldman, 1971; Goldman and Knipe, 1973), macrophages (Bhisey and Freed, 1971), osteoclasts (Holtrop, Raisz and Simmons, 1974), and neurons (Wisniewski et al., 1968; Wisniewski and Terry, 1967; Schlaepfer, 1971; Daniels, 1973). The aggregation and apparent increase in intermediate filaments that followed depolymerization of the microtubules caused by these agents suggested that the filaments were breakdown products of the tubules (Wisniewski et al., 1968); this was supported by the observed inverse relationship between the numbers of microtubules and filaments in differentiating neurons and astrocytes (Peters and Vaughn, 1967). Although most electron microscopic data could not be interpreted as demonstrating a direct interconversion of intermediate filaments and microtubules, the possibility that they were nevertheless composed of the same protein subunits remained (Ishikawa et al., 1968; Robbins and Gonatas, 1964; Schlaepfer, 1971; Holtrop et al., 1974; Johnson and Sinex, 1974). This issue was resolved conclusively only when their respective subunits were characterized biochemically and immunologically (see below). However, indirect evidence against microtubule-intermediate filament interconversion came from cultured endothelial cells, whose intermediate filaments are normally present in a perinuclear ring. During mitosis, there is no obvious disassembly of these filaments, contrary to what might be expected if their subunits were needed for spindle microtubule assembly (Blose and Chacko, 1976; Blose, 1979). The aggregation of intermediate filaments caused by agents such as colcemid was found not to be dependent on protein synthesis (Goldman and Knipe, 1973; Croop and Holtzer, 1975; Blose and Chacko, 1976; Albertini and Kravit, 1981), indicating that the apparent proliferation of the filaments was not the result of synthesis of new subunits (although there is some evidence that this may not hold true for all cell types; see DeBrabander et al., 1975). The relative size of the soluble subunit pools from which filaments might be assembled appears to be low (see Hynes and Destree, 1978), but remains to be quantitated or even examined

in most cell types. Also, the relative kinetics of tubulin paracrystal induction by vinblastine and the appearance of cytoplasmic intermediate filament bundles argued against interconversion (Tomlinson and Bennett, 1976).

Subunit Identification and Distribution—Electrophoretic and Immunologic Approaches

Further characterization of intermediate filaments required techniques other than electron microscopy. Even though the stereotypic intermediate filament was long, smooth, unbranched, somewhat wavy, not consistently associated with other cellular structures, aggregatable by colcemid, most visible when in bundles, and with a diameter of about 10 nm, most intermediate filaments were identified as such because of what they did not appear to be, i.e., actin filaments, myosin filaments or microtubules. Filament diameter was the least reliable criterion, since reported diameters for the same filaments in different laboratories could differ by a factor of two or three. This was a major obstacle in the initial recognition of intermediate filaments as a new type of cytoplasmic filament, as described above. Differences in preparative techniques for electron microscopy seem to be responsible for much of this variability in diameters (see Rash et al., 1970; Eriksson and Thornell, 1979), and has precluded any sort of easy subclassification of intermediate filaments based on ultrastructural morphology.

Clearly, the next steps toward understanding the properties and relationships of intermediate filaments was to isolate their subunits, investigate their solubility and reassembly properties, explore their immunologic similarities to each other, and determine their respective cell and tissue distributions. Unfortunately, some of the initial attempts along these lines were careless and resulted in a considerable amount of misinformation and confusion in the intermediate filament field.

A prime example concerns the neurofilament subunits. Since vertebrate neurofilaments were known from electron micrographs to be abundant in axons, a technique was devised for isolating axons from brain (Shelanski et al., 1971). This technique

involved initial centrifugation of brain homogenates in a 0.85 M sucrose solution; segments of myelinated axon floated under these conditions, and were thus separated from the soluble proteins and cellular debris present in the whole homogenate (DeVries, Norton and Raine, 1972). Hypotonic lysis then stripped the myelin away, and the presumed axon contents were pelleted and analyzed. When analyzed by SDS-polyacrylamide gel electrophoresis, such preparations appeared to be composed almost exclusively of a 51-54,000 dalton polypeptide, which was assumed to be the neurofilament subunit (Shelanski et al., 1971). Antisera raised against this polypeptide were seen to crossreact with glial filaments (Yen et al., 1976), neuroblastoma cells (Jorgensen et al., 1976), endothelial cells and cardiac muscle cells (Blose, Shelanski and Chacko, 1977). This led to the belief that intermediate filaments in different cell types were quite similar if not identical.

Other lines of evidence, however, began to indicate that neurofilaments were not composed of this particular polypeptide subunit. Indirect evidence came from studies of axoplasmic transport, in which five polypeptides were seen to comprise the slowest component of anterograde flow. Two of these polypeptides were microtubule subunits (α and β tubulin), and the other three were postulated to be subunits of neurofilaments (Hoffman and Lasek, 1975). Isolation of neurofilaments from peripheral nerve, as well as more careful isolation from spinal cord and brain, revealed that neurofilaments were indeed composed of a "triplet" of polypeptides with molecular weights of roughly 70,000, 160,000 and 200,000 daltons (Anderton, Ayers and Thorpe, 1978; Schlaepfer and Freeman, 1978; Mori and Kurokawa, 1979; Micko and Schlaepfer, 1978; Thorpe et al., 1979; Delacourte et al., 1980; Shecket and Lasek, 1980). The lower molecular weight polypeptides in these and earlier preparations have since been attributed to contaminating tubulin, vimentin, glial filament protein and neurofilament degradation products (see also Shelanski and Liem, 1979; Day, 1980; Czosnek and Soifer, 1980; Czosnek Soifer and Wisnieswki, 1980; Davison and Jones, 1981;

Brown et al., 1981; Runge, Schlaepfer and Williams, 1981). The original neurofilament preparations of Shelanski et al. (1971) were probably composed predominantly of glial filaments (Liem et al., 1978), since neurofilaments are typically solubilized or degraded under the conditions used (Schlaepfer, 1978; Schlaepfer and Micko, 1979); the "neurofilament polypeptide" of Johnson and Sinex (1976) was evidently mostly tubulin, as its peptide map had indicated. Even when the neurofilament triplet was occasionally observed in early axon preparations, the preponderance of contaminating glial protein led to the conclusion that the triplet polypeptides were not of neurofilamentous origin (Davison and Winslow, 1974).

Neurofilaments from marine invertebrates were found to have a polypeptide composition different from that of vertebrates (Huneeus and Davison, 1970; Lasek, Krishnan and Kaiserman-Abramof, 1979; Roslansky et al., 1980; Zackroff and Goldman, 1980) yet it became evident in these systems also that proteolysis was a significant source of error and confusion in identifying the native filament subunits (Gilbert, Newby and Anderton, 1975).

Despite the inadvertent isolation of glial filaments in early attempts to isolate neurofilaments, contemporary attempts to isolate the glial filament subunit resulted instead in the isolation of a stable proteolytic fragment of this polypeptide. Multiple sclerosis plaques and brain tissue scars composed predominantly of filament-packed fibrous astrocytes yielded a 40,000 dalton polypeptide termed "glial fibrillary acidic protein" (GFAP; Eng et al., 1971). Antibodies to GFAP selectively stained astrocytes in tissue sections (Bignami et al., 1972; Schachner et al., 1977), as well as in culture (Antanitus, Choi and Lapham, 1975). Realization that this polypeptide was a soluble proteolytic fragment of GFAP, and that undegraded GFAP was insoluble and had a molecular weight of 54,000 (Dahl, 1976; Rueger, Dahl and Bignami, 1978a,b) came at about the same time the neurofilament triplet was beginning to be accepted; however, reports that neurofilaments had a component that comigrated with intact

GFAP persisted (Benitz et al., 1976; Dahl and Bignami, 1976; Goldman et al., 1978; Schachner, Smith and Schoonmaker, 1978). Even though highly purified neurofilament preparations from central and peripheral nerves usually do not exhibit a polypeptide of this size, the identity of such a polypeptide in some preparations of peripheral nerve remains unresolved, but may be tubulin or vimentin (see Dahl and Bignami, 1976; Davison and Hong, 1977; Schlaepfer and Micko, 1978; Shelanski and Liem, 1979; Davison and Jones, 1981). It also became evident that tubulin in particular, was a major comigrating contaminant in glial filament protein preparations (Liem and Shelanski, 1978), but was electrophoretically separable from GFAP if alternative polyacrylamide gel systems were used (Rueger et al., 1978b).

Muscle cell intermediate filaments were first seen in developing skeletal muscle (Ishikawa et al., 1968), but were first characterized biochemically in smooth muscle. Extraction of actin and myosin from smooth muscle with high salt was found to leave an insoluble residue rich in 10 nm filaments (Cooke and Chase, 1971). This residue was enriched in a 50-55,000 dalton polypeptide that would reassemble into 10 nm filaments after disassembly in urea or acetic acid (Cooke, 1976; Small and Sobieszek, 1977). Antibodies to this polypeptide, named desmin, specifically labeled muscle cells, and showed that this antigen was present in skeletal and cardiac muscle as well as in smooth muscle cells (Lazarides and Hubbard, 1976).

Muscle provided a convenient system in which intermediate filaments could be studied during differentiation in vivo as well as in vitro; whereas smooth muscle intermediate filaments seemed to be present throughout the cytoplasm at all stages of differentiation (Uehara et al., 1971), striated muscle desmin was found to alter its distribution from an initially dispersed state to an ultimately Z-line-associated state (Lazarides, 1978; Gard and Lazarides, 1980). The wealth of ultrastructural studies on myogenesis and muscular contraction provided a firm basis for the exploration of the molecular morphogenesis of this new type of filament (see Chapter 7).

Treatment of cultured chick fibroblasts with the nonionic detergent Triton X-100 was found to leave an insoluble cytoskeleton composed predominantly of 10 nm filaments and a corresponding 52,000 dalton polypeptide (Brown, Levinson and Spudich, 1976). An autoimmune rabbit serum was found to have activity against a similar polypeptide in cultured mammalian cells, and stained in immunofluorescence a cytoplasmic network of wavy filaments that could be induced to aggregate with colcemid (Gordon, Bushnell and Burridge, 1978). A human autoantibody seemed to have a similar binding activity (Kurki et al., 1977). An antiserum produced specifically against the analogous polypeptide from murine fibroblast detergent cytoskeletons confirmed that it was the major subunit of their intermediate filaments (Hynes and Destree, 1978). This polypeptide, named vimentin, was found to have a widespread distribution, occurring in most cells of mesenchymal origin (Franke et al., 1978a). Avian vimentin and mammalian vimentin were found to differ slightly in isoelectric point and molecular weight as were avian and mammalian desmin (Lazarides and Balzer, 1978). In retrospect, it is evident that the first isolation of vimentin was performed using cultured BHK cells, naturally-occurring or colcemid-induced perinuclear bundles or "caps" of intermediate filaments could be mechanically dissociated from the cells for biochemical analysis (Starger and Goldman, 1977; Starger et al., 1978). These caps consisted primarily of two similar polypeptides, which were later shown to be desmin and vimentin (Gard, Bell and Lazarides, 1979; Tuszynski et al., 1979).

Epithelial tonofilaments have come to be regarded as another class of intermediate filament because of their diameter and ultrastructural morphology, and characteristic insolubility in physiologic buffers and nonionic detergents (see Fraser, MacRae and Rogers, 1972). These filaments are composed of subunits of the α-keratin class of polypeptide, a diverse family of polypeptides ranging in molecular mass from 40,000 to 70,000 daltons (see Franke et al., 1981). Whereas the functions of the other classes of intermediate filaments are largely enigmatic (see Discussion), the

function of epithelial keratin filaments seems clear: interlinking adjacent cells and imparting mechanical strength and structural integrity and continuity to epithelia. Antibodies raised against epidermal keratins were found to label most epithelia in vivo as well as cultured epithelial cells in vitro (Franke et al., 1978b; Freudenstein et al., 1978; Sun and Green, 1978a; Sun, Shih and Green, 1979; Franke et al., 1979e). The only epithelioid cells or cells of epithelial origin in which keratins have not been found are vascular endothelial cells (Franke et al., 1979d), Sertoli cells (Franke, Grund and Schmid, 1979) and lens cells (Ramaekers et al., 1980; Geisler and Weber, 1981c); these cells all contain vimentin as their sole intermediate filament subunit. The different keratin polypeptides are related biochemically and immunologically, but most have been found to be distinct gene products rather than posttranslationally-modified derivatives of each other (Fuchs and Green, 1978; 1979). A given cell usually contains around six different keratin polypeptides, but different cell types can contain different sets or combinations, and these can change during differentiation (Lee, Kubilus and Baden, 1979; Fuchs and Green, 1980; Franke et al., 1981; Milstone, 1981; Lane, 1982). Epithelial cells that would express different sets of keratin polypeptides in vivo express similar sets when cultured in vitro (Lee et al., 1979; Fuchs and Green, 1980). The terms prekeratin and cytokeratin have been used to distinguish those subunits in the cytoplasms of non-cornified epithelia from the disulfide-bonded keratins in the nonliving stratum corneum (see Maltotsy, 1964; Franke et al., 1978b).

Two major conclusions that emerged from the immunological characterization of intermediate filaments were (a) that there are immunologically distinguishable classes of intermediate filaments, and (b) that these classes are largely cell type specific (Franke et al., 1978a; Lazarides and Balzer, 1978; Bennett et al., 1978; Schmid et al., 1979; Lazarides, 1980). These generalizations are currently valid, with few exceptions, and define five classes of intermediate filaments (as alluded to above): keratin filaments in epithelial cells; neurofilaments in neurons; glial

filaments in astrocytes; desmin filaments in muscle; and vimentin filaments in many undifferentiated and fibroblast-like cells, and cells of mesenchymal origin.

Within a given class of filament, immunologic crossreactivity may or may not be observed between different polypeptides, different isoelectric variants, and different species, depending on such factors as the tolerance of the animal immunized and the state of the immunogen, as well as on antigenic similarities and differences. Limited immunologic crossreactivity between different classes of filament has been reported on many occasions, but the significance of these reports is nearly impossible to assess because of the difficulties in determining if one is detecting true crossreactivity or merely seeing the result of an impure immunogen preparation. Most polyclonal antisera appear by most assays to be specific to one class of intermediate filament, but crossreacting antibodies may go undetected because of their relative paucity.

However, monoclonal IgM antibodies have recently been found that bind to most or all of the intermediate filament subunits, implying that these polypeptides indeed share antigenic determinants that are not normally detected with polyclonal sera (Pruss et al., 1981; Dellagi et al., 1982). This supports the concept of an evolutionary relationship or common origin of the five classes of filament. The discovery of a monoclonal IgM that binds to both vimentin and tropomyosin (Blose, Matsumura and Lin, 1982), however, urges caution in the interpretation of these results (see Lane and Koprowski, 1982).

Antisera that are specific for given classes of intermediate filament have recently disclosed that certain cells or cell types simultaneously exhibit in their cytoplasms two or more different filament subunits. Many epithelial cells in culture (but not in vivo) simultaneously express vimentin as well as keratin filaments; these two classes of filament can be distinguished not only by their own characteristic immunofluorescence patterns, but also by their differential response to colcemid: the vimentin filaments readily aggregate, whereas the keratin filaments remain

dispersed (Sun and Green, 1978a; Franke et al., 1978a, 1979b,e; Osborn, Franke and Weber, 1980). Developing and mature skeletal muscle fibers contain both vimentin and desmin (Gard et al., 1979; Granger and Lazarides, 1979; Gard and Lazarides, 1980). The ratio of desmin to vimentin increases during myogenesis (Gard and Lazarides, 1980), yet, in chickens at least, vimentin persists into adulthood (Granger and Lazarides, 1979; Mikawa et al., 1981). Some vascular smooth muscle cells appear to possess both desmin and vimentin, but the relative proportion of each in the aorta, for example, has not yet been resolved (Johnson and Yun, 1980; Berner et al., 1981; Frank and Warren, 1981; Gabbiani et al., 1981b; Osborn, Caselitz and Weber, 1981; Schmid et al., 1982). Fibroblast-like BHK cells in culture contain both desmin and vimentin (Gard et al., 1979; Tuszynski et al., 1979); cultures of chick muscle fibroblasts appear to synthesize small amounts of desmin in addition to vimentin (Gard et al., 1979), but the identity of these cells is uncertain (Virtanen et al., 1981). Some astroglia in vivo and in vitro simultaneously express vimentin and glial fibrillary acidic protein (Chiu, Norton and Fields, 1981; Schnitzer, Franke and Schachner, 1981; Shaw, Osborn and Weber, 1981; Tapscott et al., 1981b; Yen and Fields, 1981; Yokoyama, Mori and Kurokawa, 1981) as do cultured glioma cells (Paetau et al., 1979). Neurons in vivo briefly possess both neurofilaments and vimentin filaments during differentiation when there is a transition from replicating neuroepithelial cells containing exclusively vimentin to mature neurons containing only neurofilaments (Tapscott, Bennett and Holtzer, 1981; Tapscott et al., 1981b); both of these filament classes seem to be present in cultured embryonic neurons (Jacobs, Choo and Thomas, 1982), although some studies have detected only neurofilaments (Bennett et al., 1981; Shaw et al., 1981). Recent reports of desmin in neurons (Eriksson et al., 1980), desmin in astrocytes (Dahl and Bignami, 1982) and GFAP in fibroblasts (Bock et al., 1977) are based solely on serology and seem likely to be erroneous.

The presence of more than one type of filament subunit in a given cell raises the question of whether the subunits copolymerize (form heteropolymers) or exist as separate filament networks (form homopolymers). The situation seems clear in epithelial cells that exhibit distinct keratin and vimentin filament networks, but is unresolved in other cell types. This issue will be considered more fully in the next section.

The immunochemical distinction of the various intermediate filament classes has recently proven to have clinical application. Immunofluorescence has been used to diagnose the origin of tumors, particularly those of epithelial origin (e.g., Bannasch et al., 1980; Schlegel et al., 1980; Franke et al., 1981; Gabbiani et al., 1981a) and glial origin (Duffy, Graf and Rapport, 1977; Delpech et al., 1978; Eng and Rubinstein, 1978; Jacque et al., 1978; Velasco et al., 1980). Antibodies to keratin have been used to study Mallory bodies in the livers of alcoholics (Denk et al., 1979; Franke et al., 1979c). Neurofibrillary tangles that develop during normal aging processes, and are especially prevalent in senile dementias such as Alzheimer's disease, have been studied with antibodies to the intermediate filament proteins of the central nervous system; however, repercussions from earlier mistakes in this field, as well as a general failure to adequately characterize antiserum specificities, has led to conflicting conclusions regarding the molecular basis of some of these pathologies (see Dahl et al., 1982).

Reconstitution

Each of the five classes of intermediate filament has now been disassembled and reassembled in vitro. In each case, conditions have been found in which the dissociated subunits can be reconstituted into long, smooth, unbranched filaments around 10 nm in diameter, with X-ray diffraction patterns similar to the native filaments.

Keratin polypeptides were first extracted from epidermis with concentrated solutions of urea; removal of the urea by dialysis resulted in the formation of short filaments (Steinert, 1975). If this were done in the presence of a thiol reducing agent, the filaments would become much longer (Steinert, Idler and Zimmerman, 1976). This was consistent with the natural formation of epidermal tonofilaments in the reducing environment of the living cells, followed by oxidative crosslinking (disulfide formation) in the nonliving stratum corneum during terminal differentiation (Sun and Green, 1978b). Keratin could also be solubilized with a low pH citrate buffer (Maltotsy, 1964). The six keratin polypeptides of bovine hoof epidermis are not all required for filament formation, but most combinations of two or three of the polypeptides will polymerize into filaments with precise molar ratios of 1:2 or 1:1:1, indicating a fundamental three-chained unit structure (Skerrow, 1974; Steinert et al., 1976).

Smooth muscle intermediate filaments, composed primarily of desmin, were found to reassemble in vitro after solubilization from high salt cytoskeletons with urea (Cooke, 1976) or acetic acid (Small and Sobieszek, 1977; Hubbard and Lazarides, 1979). These extracts contained significant amounts of proteinaceous material other than desmin (most notably actin), yet chromatographically pure desmin from both smooth and skeletal muscle was also found to be capable of assembling into 10 nm filaments (Huiatt et al., 1980; O'Shea et al., 1981). Avian desmin is composed primarily of two isoelectric variants (Izant and Lazarides, 1977), whereas mammalian desmin is predominantly one variant with a slightly higher molecular weight than the avian variants (Lazarides and Balzer, 1977); the different variants seem to copolymerize with equal efficiency (Hubbard and Lazarides, 1979; Huiatt et al., 1980; O'Shea et al., 1981).

Vimentin was first reassembled in vitro in association with desmin, since these two polypeptides coexist in BHK cells in culture. As described above, BHK cell

intermediate filaments form compact, birefringent, perinuclear caps during spreading or after exposure to colcemid, which allows them to be physically isolated and thereby partially purified (Starger and Goldman, 1977; Starger et al., 1978). These caps will disassemble in solutions of very low ionic strength, and then reassemble into 10 nm filaments when the ionic strength is increased (Starger et al., 1978; Zackroff and Goldman, 1979). Similar results have been obtained with aortic endothelial cells (Milstone and McGuire, 1981). Urea-solubilized vimentin from cytoskeletons of CHO cells, whose sole intermediate filament subunit seems to be vimentin, will polymerize into 10 nm filaments in vitro (Cabral et al., 1981), as will chromatographically purified vimentin from both cultured cells and lens cells (Geisler and Weber, 1981c; Renner et al., 1981).

GFAP purified by immunoaffinity chromatography can assemble into 10 nm filaments; GFAP is soluble in low ionic strength solutions, and has a tendency to form disulfide-bonded dimers under oxidizing conditions, which apparently interferes with filament assembly (Rueger et al., 1979).

In each of the above cases, 10 nm filaments were polymerized from polypeptides in the 40,000 to 70,000 dalton molecular mass range. Neurofilaments appeared anomalous, since they seemed to consist of two high molecular weight polypeptides in addition to a polypeptide in this lower molecular weight range. Immunofluorescence showed that the neurofilament triplet polypeptides were present together in neurons, implying that they were components of the same filaments (Shaw and Weber, 1981). Reconstitution studies using each of the three polypeptides purified by chromatography in urea demonstrated that the 70,000 dalton polypeptide was capable of forming filaments on its own; the two high molecular weight polypeptides were not required for this polymerization and were not capable of forming filaments themselves, but would become incorporated into filaments if the low molecular weight polypeptide were also present (Moon et al., 1981; Geisler and Weber, 1981a; Liem and Hutchison, 1982;

Zackroff et al., 1982). Ultrastructural studies using antibodies specific for each of these three polypeptides suggested that the core filament polymer was formed by the 70,000 dalton polypeptide, while the other two members of the triplet were peripheral, associated polypeptides (Willard and Simon, 1981; Sharp, Shaw and Weber, 1982). The antibody decoration patterns indicated that the 200,000 dalton polypeptide was wrapped helically around the filament with an axial periodicity of 100 nm, whereas the 160,000 dalton polypeptide was more uniformly distributed along the filament.

The following generalizations can be made regarding the solubility properties of intermediate filaments in higher vertebrate cells, based on the isolation and reconstitution studies cited above. Intermediate filaments are insoluble under physiologic conditions, as well as in solutions of high salt and nonionic detergents (this is one reason they have come to be regarded as "cytoskeletal" elements). In the absence of multivalent cations, they are also soluble in slightly alkaline solutions of low ionic strength (see also Schlaepfer, 1978; Zackroff and Goldman, 1979; Huiatt et al., 1980; Steinert et al., 1981b; Granger, Repasky and Lazarides, 1982; Traub and Nelson, 1982). The filaments are soluble in (and can be reconstituted from) solutions of SDS and urea; there is no evidence of covalent crosslinking of the subunits other than disulfide formation between the subunits of keratin and GFAP (desmin and vimentin may also form disulfide linked tetramers; unpublished observations); however, there is evidence that desmin, at least, can act as a substrate for covalent linkage by transglutaminase (Gard and Lazarides, 1979), much as the epidermal keratins do during terminal differentiation (Abernethy, Hill and Goldsmith, 1977).

Intermediate filaments have been studied extensively only in mammalian and avian cells and tissues, and in squid and marine worm nervous systems. Significantly, neurofilaments from these marine invertebrates have solubility properties (as well as subunit molecular weights) considerably different than vertebrate filaments. A major difference is their solubility in high salt, a property that has been utilized

for their disassembly and reassembly (Gilbert et al., 1975; Krishnan, Kaiserman-Abramof and Lasek, 1979; Zackroff and Goldman, 1980). Possible structural similarities to vertebrate neurofilaments have not been extensively explored, although it was Myxicola filaments that were used for the first demonstration that neurofilament subunits were fibrous (Day and Gilbert, 1972) rather than globular (Schmitt, 1968; Wuerker, 1970).

The ability to reconstitute intermediate filaments in vitro has allowed the study of potential copolymerization of different classes of subunit. Even though data concerning native filament reconstitution and immunofluorescence localization are consistent with the copolymerization of desmin and vimentin in BHK cells and muscle cells (Starger et al., 1978; Gard and Lazarides, 1980), the alternative possibility of strict homopolymers cannot be ruled out with this evidence. Copolymerization studies using desmin and vimentin that have been denatured during purification indicate that desmin and vimentin can (and prefer to) form heteropolymers in vitro (Steinert et al., 1981b), but further work is needed to determine the relevance of this finding to the normal situation in vivo.

Limited proteolysis and X-ray diffraction studies have led to the formulation of a model for the molecular structure of the fundamental building block of intermediate fialments (Steinert, Idler and Goldman, 1980): a three-chained unit with two domains of coiled-coil α-helix interspersed with regions of non-α-helix. This model is consistent with the idea that different subunits might have conserved domains (e.g., the α-helical regions) responsible for polymer formation and general structure of the filament, as well as non-conserved, diverse domains (e.g., the non-α-helical regions) that would account for differences in subunit size, antigenicity and, presumably, function (Lazarides, 1980). It is also consistent with the finding that desmin, vimentin and the neurofilament core polypeptide have regions of homologous amino acid sequence (Geisler and Weber, 1981b; Geisler, Plessman and Weber, 1982), and thus supports the concept of an

evolutionary relationship between these proteins. The assembly and packing of these three-chained units into 10 nm filaments has not yet been detailed; there is little agreement on the number of monomer polypeptides in a filament cross-section or on the number or arrangement of possible sub-filaments or protofilaments in a native 10 nm filament. There is evidence for a fundamental axial periodicity of 20 nm, based on X-ray diffraction (Fraser, MacRae and Suzuki, 1976) and electron microscopy (Milam and Erickson, 1981; Henderson, Geisler and Weber, 1982), but also evidence for a 31 nm periodicity, based on optical diffraction (Small and Sobieszek, 1977) and monoclonal antibody decoration (Blose et al., 1982). Hopefully, elucidation of the structure of the filaments will simplify our understanding of how the cell regulates polymer formation.

A number of polypeptides "copurify" or "copolymerize" with intermediate filaments; deciding which of these "associations" are physiologically or functionally meaningful can be a difficult task. The problems concerning the "copurification" of neurofilaments, glial filaments and microtubules have already been addressed; conversely, neurofilaments have been shown to be a contaminant in axonal microtubule preparations (Berkowitz et al., 1977; Runge, Detrich and Williams, 1979). Ironically, a currently-popular method of obtaining neurofilaments is based on a microtubule isolation protocol (Delacourte et al., 1980). Smooth muscle actin copurifies with desmin through repeated cycles of polymerization and depolymerization using acetic acid (Hubbard and Lazarides, 1979); whether actin interacts with desmin or any of the other intermediate filament proteins in vivo is still enigmatic. A class of cationic proteins, termed filaggrin, has been isolated from epidermis and shown to cause specific aggregation of intermediate filaments (Steinert et al., 1981a). Antibodies against creatine phosphokinase bind to intermediate filaments (Eckert et al., 1980), and monoclonal antibodies specific for 95,000 and 210,000 dalton myofibrillar polypeptides of unknown identity label intermediate filaments in a variety

of cell types in culture (Lin, 1981). A high molecular weight protein occasionally copolymerizes with BHK desmin and vimentin filaments (Starger et al., 1978; Zackroff and Goldman, 1979), and polypeptides related to the high molecular weight microtubule associated proteins (MAPs) seem to copurify with some intermediate filaments under certain conditions (Pytela and Wiche, 1980).

A 230,000 dalton polypeptide copurifies with desmin from avian smooth muscle, and colocalizes at all stages of differentiation with desmin and vimentin in skeletal muscle (Granger and Lazarides, 1980). This polypeptide, named synemin, is also found in association with vimentin in avian erythrocytes, where it exhibits an axial periodicity of roughly 200 nm on the filament core (Granger et al., 1982; Granger and Lazarides, submitted for publication). When a variety of avian tissues are assayed by immunofluorescence of frozen sections, synemin seems to be present in significant amounts only in erythrocytes and muscle cells (B. Granger, unpublished observations); this suggests that synemin can coexist with either desmin or vimentin, but not with other classes of intermediate filament subunit. The expression of synemin appears to parallel the expression of desmin, but not necessarily correlate with the expression of vimentin. The study of synemin constitutes a major part of this thesis (see below and Chapters 4-6).

A 280,000 dalton polypeptide, distinct from synemin (and named paranemin), is also found with desmin and fimentin filaments in muscle, but disappears during myogenesis (Breckler and Lazarides, 1982); it colocalizes with intermediate filaments in several types of non-muscle cells that are devoid of synemin (M. Price, personal communication).

Thus, a number of polypeptides in addition to the five types of core subunit seem to be permanent or transient components of intermediate filaments. Determination of their cell and tissue distributions, and their effect on the structure of the filaments, may reveal roles for intermediate filaments that studies of the core subunits have failed to demonstrate or even predict.

SCOPE OF THIS THESIS

My initial work in the field of intermediate filaments concerned the purification and properties of desmin from avian smooth muscle. My attention turned to skeletal muscle with the chance observation of an unusual fragmentation pattern that this muscle would undergo after glycerination and actomyosin solubilization. Study of the sheets of Z-discs that were generated indicated that Z-discs of adjacent myofibrils were firmly linked to one another by structures resistant to high salt and nonionic detergent. The observation of interlinking filaments and desmin immunofluorescence at the Z-disc periphery strongly suggested that desmin-containing intermediate filaments encircled the discs and formed planar networks or scaffolds at right angles to the fiber axis (see Chapter 2).

Concurrently, the intermediate filament protein vimentin was being characterized in several other laboratories. The recognition of vimentin's position on a two-dimensional gel led to its identification as a component of skeletal myofibrils. Specific antibodies revealed that vimentin co-localized with desmin in adult chicken skeletal muscle, and demonstrated for the first time that a fully differentiated cell could contain more than one class of intermediate filament protein (see Chapter 3). It also highlighted and supported the possibility that different classes of intermediate filament subunit might copolymerize (see also Gard et al., 1979; Gard and Lazarides, 1980).

A large polypeptide that consistently copurified with chicken gizzard smooth muscle desmin was investigated as a possible new intermediate filament component. It was hoped that this polypeptide could be utilized as a tool for determining the intrinsic polarity of intermediate filaments, much as myosin was used for determining actin filament polarity (Huxley, 1963) and dynein was used for determining microtubule polarity (Haimo, Telzer and Rosenbaum, 1979). This goal has not yet been reached by this or any other means, yet this polypeptide has fortuitously revealed other, unexpected information about intermediate filaments. This polypeptide coexists,

colocalizes and copurifies with desmin and vimentin filaments in muscle, and was therefore named "synemin" to signify that it is found "with filaments" (see Chapter 4).

Comparison of synemin's electrophoretic mobility and antigenicity with other well characterized high molecular weight polypeptides (including spectrin) led to the discovery that synemin was also present in avian erythrocytes. Anticipating that the avian erythrocyte might be a simpler system than muscle for the study of intermediate filaments (in accordance with the philosophy of Loewy, 1952), I began investigating these cells and made the following observations: synemin and vimentin are the predominant intermediate filament polypeptides in avian erythrocytes; a significant fraction of the filaments is stably associated with the plasma membrane of mechanically enucleated cells, yet can be selectively released from the membranes upon treatment with certain low ionic strength solutions; and synemin and vimentin colocalize in intermediate filaments when visualized by immunofluorescence (see Chapter 5). Synemin is thus the first polypeptide unambiguously shown to specifically associate with two different classes of intermediate filaments in vivo.

Immunoelectron microscopy was employed to study the relationship of synemin to vimentin at the ultrastructural level. A technique that allowed clear visualization of erythrocyte intermediate filaments (Chapter 5), was coupled with the technique of antibody decoration to demonstrate that vimentin is the primary subunit of the filaments, and synemin is most likely a peripherally-associated polypeptide (see Chapter 6). Synemin appears to form bridges or crosslinks between some of the vimentin filaments as a result of self-interaction; this is the first indication of a possible role for synemin, a role that may apply to synemin in muscle as well. The axial periodicity of synemin along the filament was found to differ between adult erythrocytes and embryonic erythroid cells, suggesting that intermediate filaments might undergo fundamental structural changes during differentiation in some systems. This is consistent with topographic and antigenic changes that intermediate filaments

undergo during myogenesis (Gard and Lazarides, 1980; Danto, Lazarides and Fischman, 1981). How these studies relate to the functions of intermediate filaments is discussed in Chapter 7.

A project that may ultimately relate to intermediate filament-membrane interactions is not represented in this thesis. It involves the demonstration that α-spectrin is present along the plasma membranes of a variety of non-erythroid cells (Repasky, Granger and Lazarides, 1982). This is contrary to prevailing dogma, which holds that spectrin is found only in erythrocytes. If avian erythrocyte intermediate filaments are anchored to the spectrin network that lines the plasma membrane, then a similar mechanism may exist in other cell types as well. Of particular interest is the periodic distribution of spectrin that lines the plasma membrane of skeletal muscle, since it has a repeat distance equal to that of the underlying sarcomeres.

Many of the techniques and protocols that have been developed and refined in this laboratory over the past few years have been compiled in an appendix (Chapter 8).

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Chapter 2

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The Existence of an Insoluble Z Disc Scaffold in Chicken Skeletal Muscle

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Summary

Extraction of glycerinated chicken skeletal muscle with 0.6 M potassium iodide leaves a framework of insoluble components within each muscle fiber. This framework is composed primarily of planes of in-register Z discs that have been thickened by the accumulation of material on both sides of each disc during extraction. Membrane vesicles, presumably remnants of the T system, remain surrounding the Z discs. When the framework is sheared in a blender, it is preferentially cleaved between Z planes, resulting in the formation of large sheets of interconnected, closely packed Z discs in a honeycomb-like array. Cleavage occurs in regions formerly occupied by the A bands, which have been weakened by the removal of myosin. The existence and stability of these planar Z disc arrays demonstrate the presence and strength of connections between adjacent myofibrils.

SDS-polyacrylamide gel electrophoresis reveals that this framework consists primarily of actin and desmin, with lesser amounts of a few proteins including α-actinin, myosin and tropomyosin. Z disc sheets and KI-extracted myofibrils provide a distinct face-on view and side view, respectively, of the Z disc. In indirect immunofluorescence, these two views have revealed that desmin is present at the periphery of each Z disc, forming a network of proteinaceous collars within the Z plane. α-Actinin is localized within each disc, giving a face-on fluorescence pattern that is complementary to that of desmin. Actin is present throughout the thickened Z plane, while myosin and tropomyosin exist only in the insoluble residue that coalesces on both faces of each disc.

We conclude that desmin, perhaps in conjunction with actin, is responsible for interlinking Z discs of adjacent myofibrils, and may thus serve as a mechanical and structural integrator of muscle fibers. Its hydrophobic nature and coincident distribution with the T system suggest that it may also be responsible for mediating filament-membrane interactions and anchoring the triad to the Z disc. Its collar-like distribution suggests that it may aid in maintaining the structural integrity of the Z disc and the actin filaments inserted into it.

Introduction

Perhaps the most striking feature of skeletal muscle is the microscopic pattern of cross-striations resulting from the alignment of sarcomeres in neighboring myofibrils. This cross-banding is preserved regardless of the state of contraction of the muscle fiber. Such uniformity and perfection in the juxtaposition of adjacent Z lines, M lines and other sarcomeric units suggest the existence of some sort of connection between them. Surprisingly, however, there is little evidence for such connections in vertebrate muscle. Thin-sectioned tissue rarely, if ever, shows fibrillar links when viewed by transmission electron microscopy. The presence of the triad (the transverse tubular system bounded by terminal cisternae of sarcoplasmic reticulum) at the level of the Z line (Porter and Palade, 1957; Franzini-Armstrong and Porter, 1964; Mendell, 1971) might be responsible for obscuring any filamentous connections in these thin sections. Instability in commonly used fixatives or inadequate staining properties might also prevent them from being salient muscle features, a supposition that is plausible in view of the detrimental effects of some fixation procedures on actin filaments (Maupin-Szamier and Pollard, 1978) and microtubules (Ledbetter and Porter, 1963; Luftig et al., 1977). Some of the earliest electron micrographs of vertebrate muscle suggested that such connections might indeed exist (Pease and Baker, 1949; Bergman, 1958) despite the poor ultrastructural preservation and the ambiguous appearance of these links. One study of avian muscle concluded that such links were merely strands of SR, with little tensile strength or importance in transmitting tension in contracting fibers (Bennett and Porter, 1953). The best electron microscopic evidence for these connections has come from the honeybee, in which interfibrillar "Z bridges" have been demonstrated in the indirect flight muscles (Garamvölgyi, 1965). The nature of these bridges, however, has not been elucidated.

We present evidence that such connections exist in chicken skeletal muscle, that they are filamentous in nature, resistant to solubilization by glycerol and 0.6 M KI, and mechanically strong and elastic. Furthermore, those at the Z line appear to be composed of desmin, the major subunit of smooth muscle 100 Å filaments (Lazarides and Hubbard, 1976).

Potassium iodide is a chaotropic agent capable of solubilizing actin, myosin and a variety of other muscle proteins (Dubuisson, 1950; Szent-Györgyi, 1951). When glycerinated skeletal muscle is incubated in a solution containing 0.6 M KI, nearly all of its protein mass is solubilized and extracted. What remains is an insoluble residue that maintains its original three-dimensional structure and consequently presents new possibilities for the elucidation of skeletal muscle cytoarchitecture. We have

found that simple shearing of this residue results in the production of sheets of interconnected Z discs which present a face-on view of a transverse slice of the myofibril lattice. (Z discs are those regions between the I bands of successive sarcomeres that appear as Z lines in longitudinal thin-sections of myofibrils.) These sheets are analogous to the "Z networks" produced by homogenization of KCl-extracted bee muscles (Garamvölgyi, 1962).

We have investigated the distribution of residual proteins in KI-extracted chicken skeletal muscle by indirect immunofluorescence. By examining side views of extracted myofibrils as well as face-on views of Z disc sheets, we have circumvented the problem of superimposition of fluorescent and nonfluorescent structures, and have determined more precisely the spatial arrangements of certain residual proteins. Desmin, actin, α-actinin, myosin and tropomyosin all give characteristic fluorescence patterns that shed new light on the extraction process, filament-membrane interactions, and the structural and functional integrity of the fiber.

Results

Cleavage Patterns of Skeletal Muscle Fibers

When glycerinated skeletal muscle is subjected to shearing forces in a blender, the fibers fragment along their long axes into individual myofibrils and small bundles of myofibrils of variable length. The basic unit of muscle structure, the sarcomere, is preserved throughout the length of the fibrils except at the ends, where breakage has occurred at or near the Z line. If, however, the muscle is first extracted extensively with 0.6 M KI to remove most of the actomyosin, a different cleavage pattern is obtained upon blending. Solubilization of A band material creates a new weakest link in the muscle fiber, so that fragmentation occurs in the A band region rather than longitudinally along the myofibril. Cleavage occurs preferentially along planes perpendicular to the long axis of the muscle fiber; adjacent Z discs remain attached to one another, so that planar arrays of Z discs are produced (Figure 1A). An overall honeycomb-like appearance is imparted by the tightly knit array of discs. These arrays are of variable thickness, often containing several planes of Z discs; when cleavage occurs on both sides of a given Z plane, a "Z disc sheet" is formed, which is thin enough to be suitable for immunofluorescence or whole-mount electron microscopy.

Evidence that these honeycomb-like arrays are really Z disc networks comes partly from scanning electron micrographs. When KI-extracted muscle is blended at low speed, it is broken primarily into large cylindrical pieces of whole fibers. Cleaved fibers always show planar ends with exposed surfaces at right angles to the length of the fiber, as shown in Figure 2. Often the cleavage plane drops down to subjacent levels, giving a step-like appearance to the exposed layers. The thickness of these layers corresponds to the inter-Z line distance visible on the side of the fiber. Some relief is visible on the faces of these layers (shown in Figure 2B) which resembles the phase-image arrays with units corresponding to individual Z discs that are approximately 1 μ in diameter.

Phase-contrast microscopy also demonstrates the nature of the cleavage process. Layers that have not completely separated from the fiber and multiple-layered sheets are occasionally oriented favorably on the slide, as in Figure 1B. These also show the planar nature of the Z disc sheets, perpendicular to the length of the fiber.

Thin sections of KI-extracted fibers reveal the Z planes with their associated residual material more clearly, and show how little material remains in the regions formerly occupied by the A bands. Longitudinal sections, as in Figure 3, exemplify the continuity of the Z planes across the extracted fiber and suggest an amorphous coalescence of material on either side of each Z plane. It is apparent that cleavage would tend to occur between Z planes to form Z disc sheets. Cross-sections of extracted fibers, as in Figure 4, reveal the honeycomb pattern of Z discs that is made evident by phase-contrast microscopy. The basket weave or square lattice pattern of the Z disc occasionally seen in unextracted muscle (for a discussion, see Ullrick et al., 1977b) has not been observed in these preparations.

Whole mounts of Z disc sheets can also be visualized by transmission electron microscopy. When drops of extracted muscle homogenates are applied to carbon-coated grids, sheets of Z discs occasionally adhere along one face to the carbon. Positive staining with uranyl acetate reveals an image similar to the phase-contrast image: dense peripheral material surrounding a less dense Z disc matrix (Figure 5A). Since these specimens are unfixed and air-dried, ultrastructure is not preserved and only the grosser structural features are apparent. Such images, however, do exemplify the thinness, sturdiness and uniformity of the sheets.

Myofibril Extraction

Myofibrils are easily prepared by vigorous blending of glycerinated skeletal muscle. When washed free of glycerol, they can be applied to glass coverslips onto which they will settle and adhere, making it possible to observe various extraction procedures as they actually occur. When 0.6 M KI is perfused under such a coverslip, the myofibrils undergo a

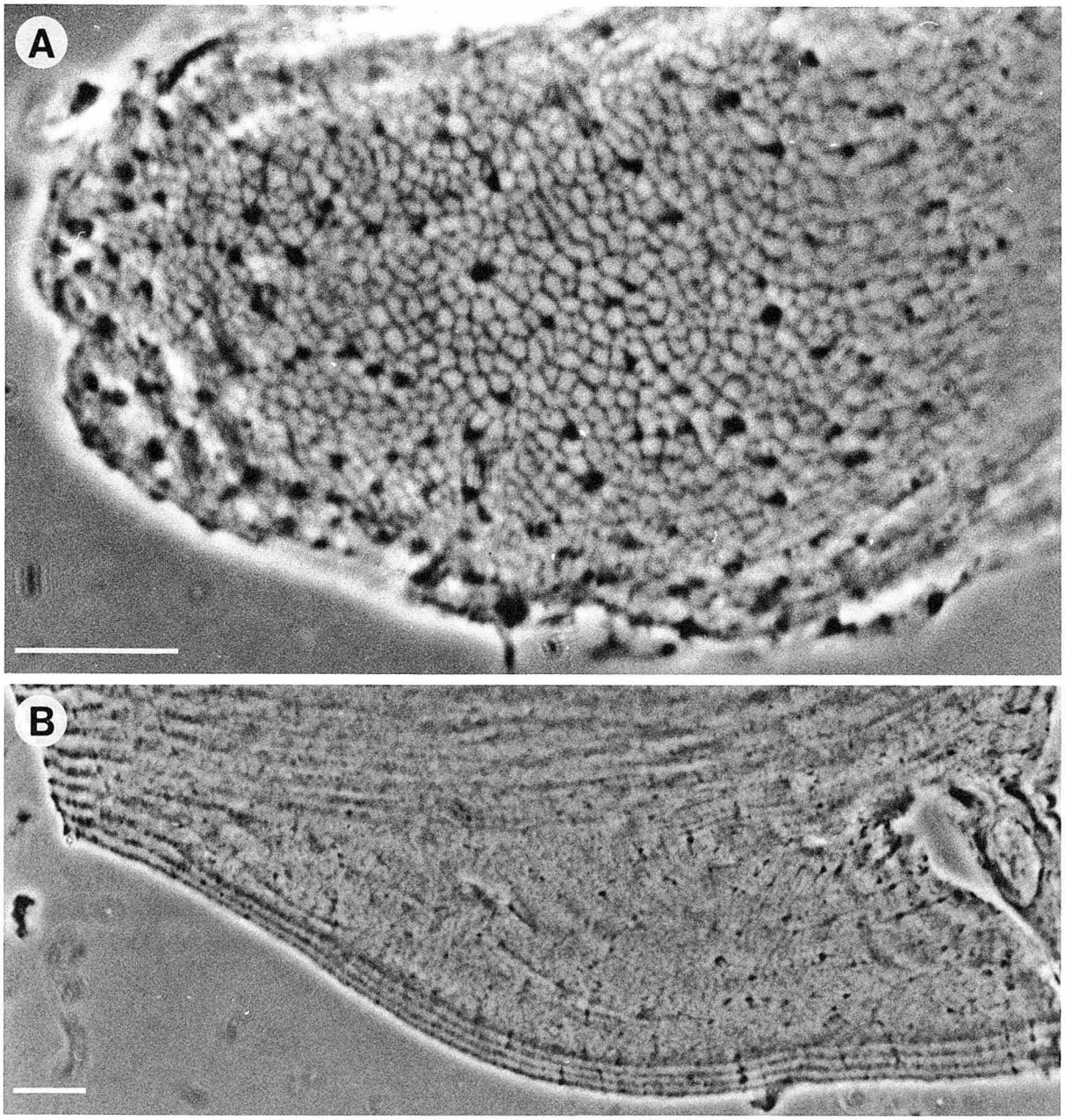


Figure 1. Phase-Contrast Micrographs of Z Disc Sheets Produced by Blending of KI-Extracted Muscle Fibers

(A) Multi-layered Z disc sheet representing a cross-section of a muscle fiber. Each Z disc is surrounded by phase-dense material. Note the radial arrangement of Z discs around some of the mitochondria (dark patches). (B) Stack of four Z planes partially split away from the mother fiber. If completely split off and viewed face-on, it would resemble the stack in (A). Bars = 10 μm.

dramatic change: the A bands swell and become phase-lucent, the Z lines thicken and become very dense, and the myofibril often shortens slightly in length (see Laki and Bowen, 1955). In a process that is complete within a few seconds, it appears that the thick filaments are solubilized by the KI, and that insoluble material then "collapses" or coalesces onto the Z line. The resultant "Z bands," composed of Z lines flanked by dense material, thus become the only structures visible by light microscopy. When KCl is used instead of KI, the A bands still disappear but the I band collapse does not seem to occur. The result is Z lines flanked by what appear to be I bands of intermediate density (Hanson and Huxley, 1955).

Myofibrils can also be extracted with KI directly on carbon-coated grids and then viewed by transmission electron microscopy. The Z line typically is

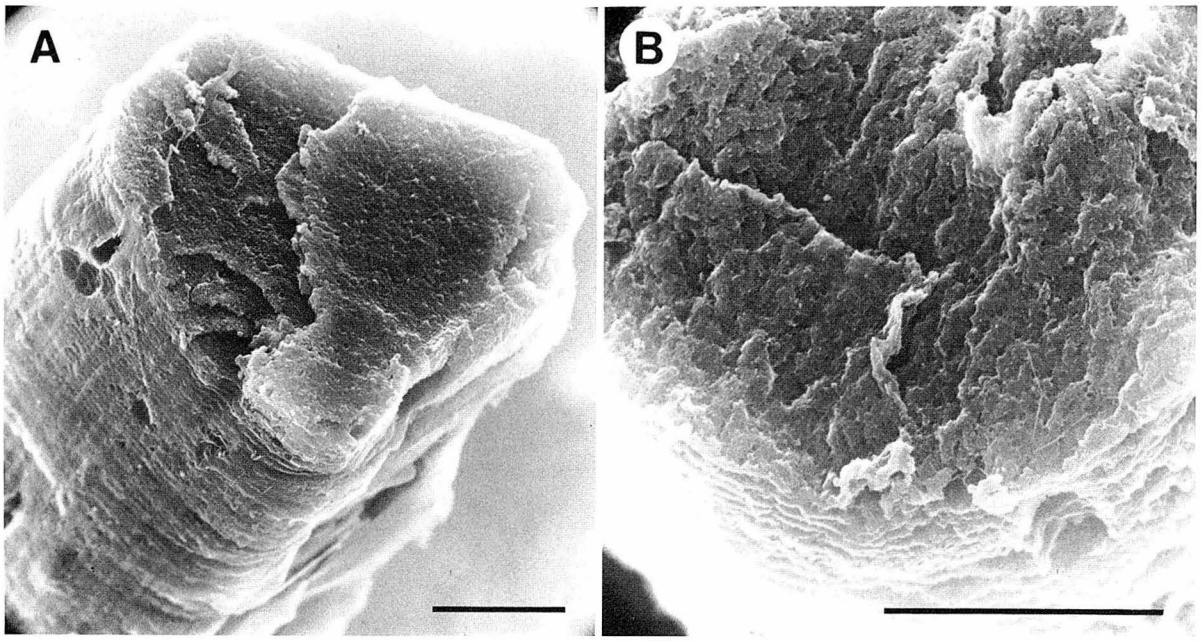


Figure 2. Scanning Electron Micrographs of Cleaved Ends of KI-Extracted Muscle Fibers

(A) Fragment of a single fiber. The cleavage planes are flat and at right angles to the muscle fiber axis. Five or six layers of Z disc sheets are exposed in this fiber. (B) Close-up of the end of a cleaved fiber. The individual Z discs are visible, and it is apparent that they can peel off in sheets. Bars = 10 μm.

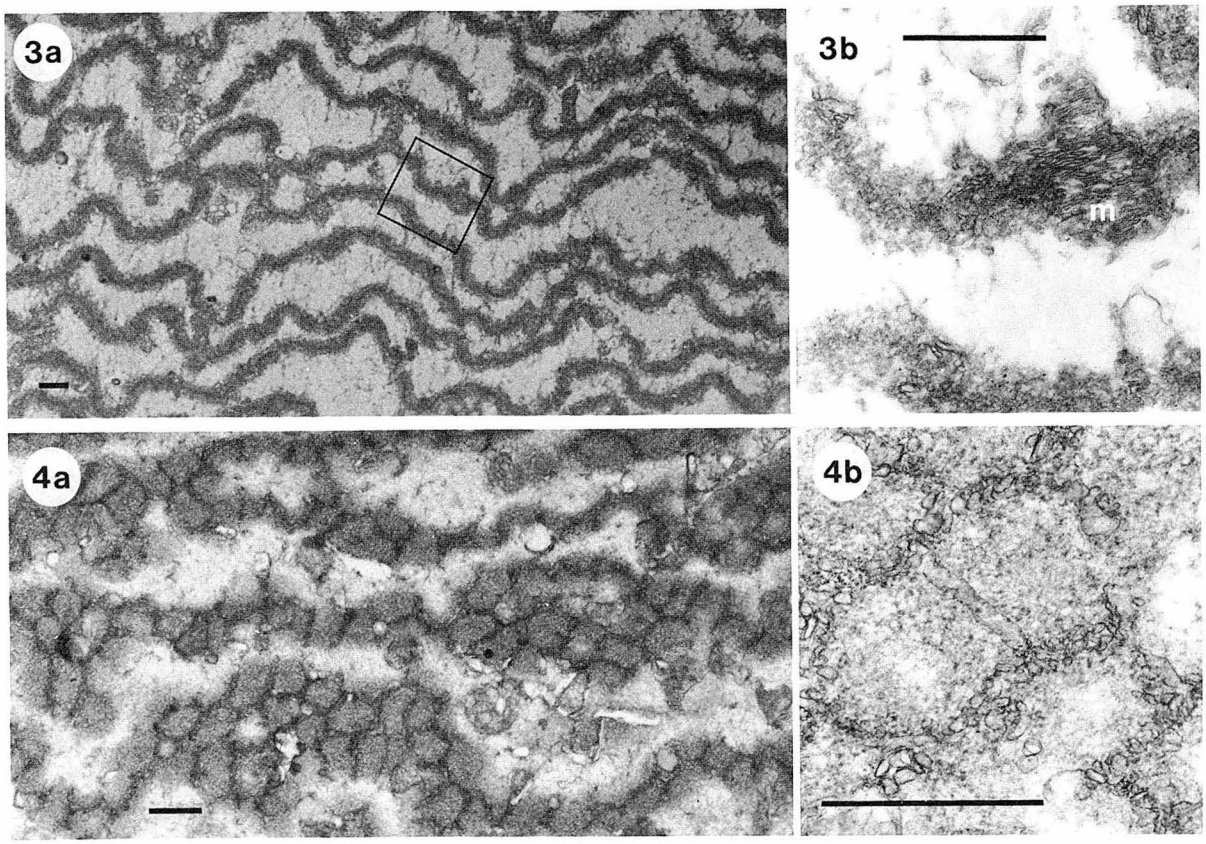
flanked by dense bands, and sometimes a small gap is present between each band and the Z line, as seen in Figure 5B. The position of this gap suggests that it may represent the area between the Z line and the N_1 line (Franzini-Armstrong, 1970). Myofibril bundles often show interfibrillar bridges at various points along the sarcomere. Filamentous Z bridges and M bridges are common, as in Figure 5C, and bridges at the level of the N_1 line are occasionally seen (not shown). It is not known whether their extreme state of extension exists before or results from the extraction process, but it does indicate a high degree of plasticity. These micrographs corroborate the postulated existence of interfibrillar bridges, particularly the Z bridges, that survive glycerination and KI extraction and hold adjacent Z discs together in a plane. The fate of the M and N bridges during Z disc sheet formation has not been determined, but they probably collapse onto the sheet and extend from Z disc to Z disc if they are not broken during blending. Residual intrafibrillar filaments spanning the sarcomere are also visible using this technique. These filaments may be undissolved actomyosin complexes or some sort of "gap filaments" (see Discussion), and are presumably broken to produce Z disc sheets.

Effect of Extraction on Membranes

Electron microscopy reveals an abundance of vesicularized membrane near the level of the Z line in extracted fibers, as in Figures 3b and 4b (see also Lazarides and Granger, 1978). These vesicles are most probably remnants of transverse (T) tubules and sarcoplasmic reticulum (SR) that have survived the disruptive effects of glycerol and KI. Longitudinal thin sections show an abundance of vesicles in the Z bands, and cross-sections show that they actually surround each Z disc, as does the T system. SR membranes that normally occupy the region adjacent to the A band probably coalesce at the peripheries of the Z discs during extraction. Nuclei resist the extraction procedure and are often seen floating free in the homogenates. Mitochondria also survive complete extraction and are generally aligned in rows along the length of the muscle fiber. Dark patches visible in phase-contrast micrographs of Z disc sheets (Figure 1A) are in fact mitochondria, which are usually more abundant near the surface of the fiber than at its center. Thin sections often suggest some sort of association between mitochondria and Z planes; the presence of mitochondria in Z disc sheets implies a firm association between the two.

Gel Electrophoretic Analysis of the Insoluble Residue

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and two-dimensional isoelectric focusing (IEF) SDS-PAGE demonstrate that the KI-insoluble



Figures 3 and 4. Thin Sections of Extracted Fibers

Figure 3. Longitudinal section showing the continuity of the thickened Z planes and the absence of A band material. (a) Low-power view; (b) high-power view of bracketed region in (a) showing a residual mitochondrion and membrane vesicles within the thickened Z plane.
Figure 4. Cross-sections showing Z disc arrays. (a) Low-power view; this slightly oblique section cuts through several Z planes and shows the close packing of the Z discs. (b) High-power view showing residual membrane vesicles surrounding the Z discs. Bars = 1 μm.

scaffold is composed of relatively few proteins, most notably actin and desmin (Figure 6). In addition to extracellular collagen, these gels show α-actinin, small amounts of myosin and tropomyosin, and several other unidentified proteins. Two major proteins can be seen to have molecular weights between those of actin (42,000 daltons) and desmin (50,000 daltons); one is more acidic, with a pI of about 5.3 (49,000 daltons), and the other is more basic, with a pI of about 6.2 (45,000 daltons). Proteins that may be present in small amounts but which have not been positively identified are the troponins, myosin light chains, extracellular coat proteins, mitochondrial proteins, nuclear matrix proteins (Berezney and Coffey, 1974) and proteins associated with residual sarcoplasmic membranes (see Lau, Caswell and Brunschwig, 1977). The nonionic detergent NP40 solubilizes several minor proteins from the KI-insoluble residue, as shown in Figure 6B, lane 2, suggesting that these may indeed be membrane-bound. There appears to be a nonequivalence of the proteins resolved by SDS-PAGE and IEF/SDS-PAGE as a result of differing protein solubilities in SDS and urea sample buffers, and because some proteins (such as myosin) do not isoelectric-focus in the pH range used. Notice in Figure 6B that SDS sample buffer solubilizes much more collagen than does urea sample buffer.

Immunofluorescence of Z Disc Sheets

Indirect immunofluorescence reveals that desmin surrounds each Z disc within a Z disc sheet but is apparently absent from the disc itself. At the level of resolution of the light microscope, as shown in Figure 7, desmin appears to be present throughout the space between adjoining discs, thereby forming a two-dimensional net within each Z plane.

Figure 8 shows that α-actinin is present within each Z disc but not between the discs. α-Actinin and desmin thus exist in nonoverlapping regions of the Z disc sheet and give complementary fluorescence patterns that cover the entire sheet.

In an occasional Z disc, desmin can be seen to occupy a small spot at the center of the disc or

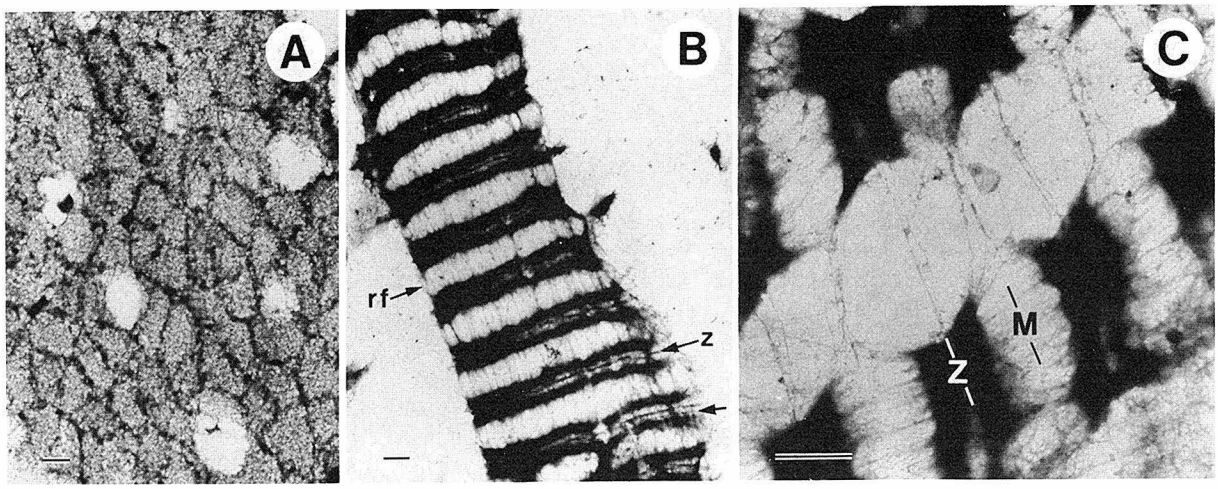


Figure 5. Transmission Electron Micrographs of Whole-Mounted Specimens

(A) Z disc sheet consisting of a single Z plane. (B and C) Myofibrils extracted after adhesion to a carbon-coated grid. (B) Bundle of myofibrils; note the apparent continuity of the Z lines (Z) across the bundle and the dense bands of accumulated material on either side. Arrow indicates probable N_1 line. Residual filaments (rf) remain in regions of extracted A bands. (C) Pair of myofibrils separated to reveal interfibrillar filaments at the level of the Z and M lines. (Z) Z line; (M) position of M line before extraction. Bars = 1 μm.

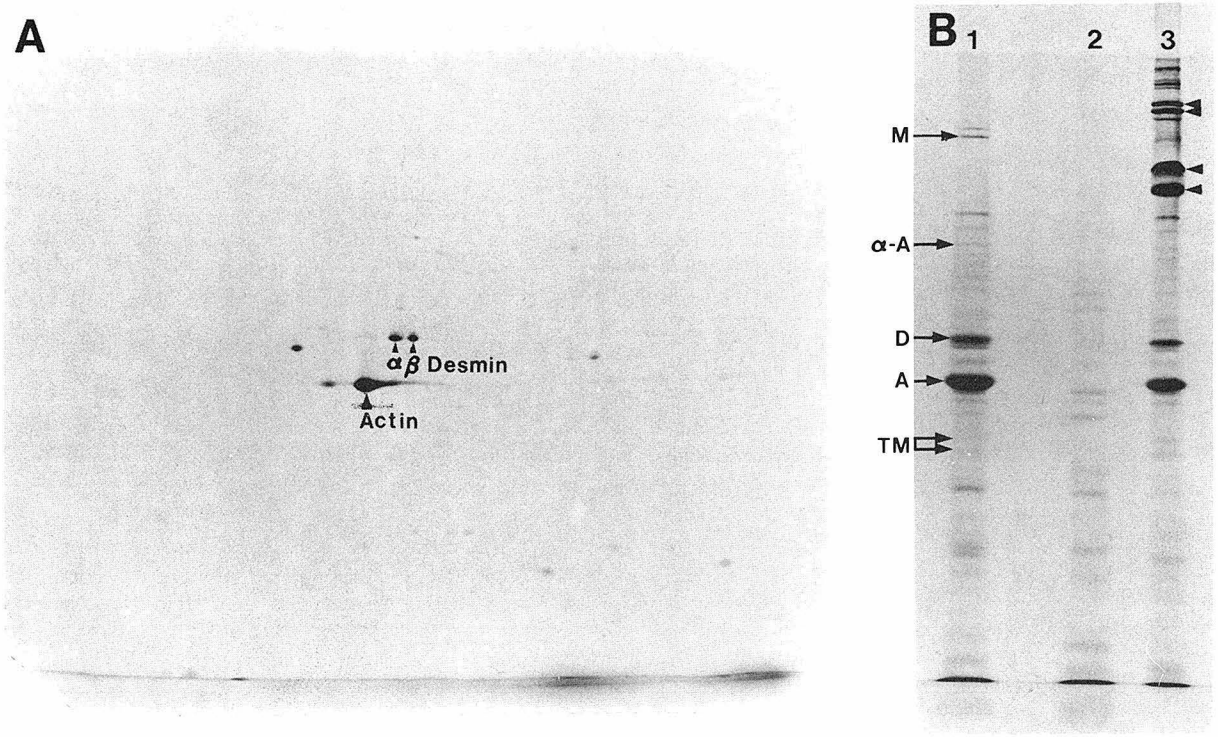


Figure 6. Two-Dimensional IEF/SDS-PAGE and One-Dimensional SDS-PAGE Gels

(A) The KI-insoluble residue was freed of salts by washing briefly in water and then extracted with 8 M urea-0.5% 2-mercaptoethanol-2% NP40 for 1 hr. The solubilized proteins were run on both the two-dimensional gel (A) and a one-dimensional gel (B1). (B2) Components of the KI-insoluble residue that are solubilized after 1 hr in 1% NP40. (B3) The KI-insoluble and NP40-insoluble material remaining after the extraction in (B2). This residue was extracted with SDS sample buffer rather than the urea solution used in (A) and (B1), so its protein composition is somewhat different. Most striking is the differing amount of collagen (arrowheads) solubilized by these two solutions. Note the presence of a wide band, presumably glycolipid, running ahead of the dye front in (B2) but not in (B3). (A) actin; (D) desmin; (α-A) α-actinin; (TM) tropomyosin; (M) protein that migrates at position of myosin.

along a radial segment extending outward from the center (not shown adequately in Figure 7). Conversely, α-actinin is absent from such regions. These are presumably the areas where the initial stages of the longitudinal subdivision of myofibrils that accompanies growth take place (see Discussion). Figure 9 shows that actin is present over the entire face of the Z disc sheet. Usually the sheet will appear uniformly fluorescent, but occasionally the discs are more intense. This might be explained in part by a large amount of actin in the layers of "collapsed" material on either side of the Z disc proper.

The distribution of tropomyosin appears to be the same as that of α-actinin when a stained Z disc sheet is viewed face-on (Figure 10). Figure 11 demonstrates that myosin also gives this fluorescence pattern, appearing only within the boundary of each disc. As is demonstrated below, the collapsed material on each face of each Z disc accounts for all of the myosin that is visualized by immunofluorescence, and for all or nearly all of the tropomyosin.

Staining with preimmune serum results in uniform, extremely weak fluorescence over each Z disc sheet, presumably due to a small amount of nonspecific adsorption. When photographed and printed as for the other antibodies, no fluorescence is visible (Figures 12 and 18). Staining with just the FITC-conjugated goat anti-rabbit antibody gave even fainter, barely detectable fluorescence.

The localization of different proteins in these sheets by immunofluorescence is most favorable when the sheets consist of a single layer of Z discs. Multiple layers result in unclear fluorescent images because superimposition occurs. There is not as much contrast between the Z disc matrix and peripheral material in single layers of Z discs, however, so that phase-contrast images of these thin sheets do not reveal the honeycomb pattern as well as do the multiple-layered sheets.

Immunofluorescence of Extracted Myofibrils

Indirect immunofluorescence was used to locate proteins in extracted myofibrils to complement the Z disc sheet data.

In KI-extracted "ghost" myofibrils, desmin is visualized as a single fluorescent line corresponding to the center of the thick Z band, as shown in Figure 13. Desmin is thus present only at the level of the Z line and does not occur in the collapsed material. This observation is in agreement with the localization of desmin along the Z line in unextracted muscle (Lazarides and Hubbard, 1976; Lazarides, 1978; Lazarides and Balzer, 1978), and in conjunction with the Z disc sheet data, it indicates that desmin encircles each Z disc, forming rings around each myofibril at the level of each Z line.

α-Actinin shows the same distribution in these ghost myofibrils as desmin when viewed from the side (Figure 14), corroborating the existence of α-actinin at the Z line in unextracted muscle (Masaki, Endo and Ebashi, 1967; Lazarides and Burridge, 1975). The Z disc sheet fluorescence, however, demonstrated that α-actinin and desmin actually have complementary distributions; α-actinin is thus present in matrix of each Z disc, but not between discs or in the collapsed material.

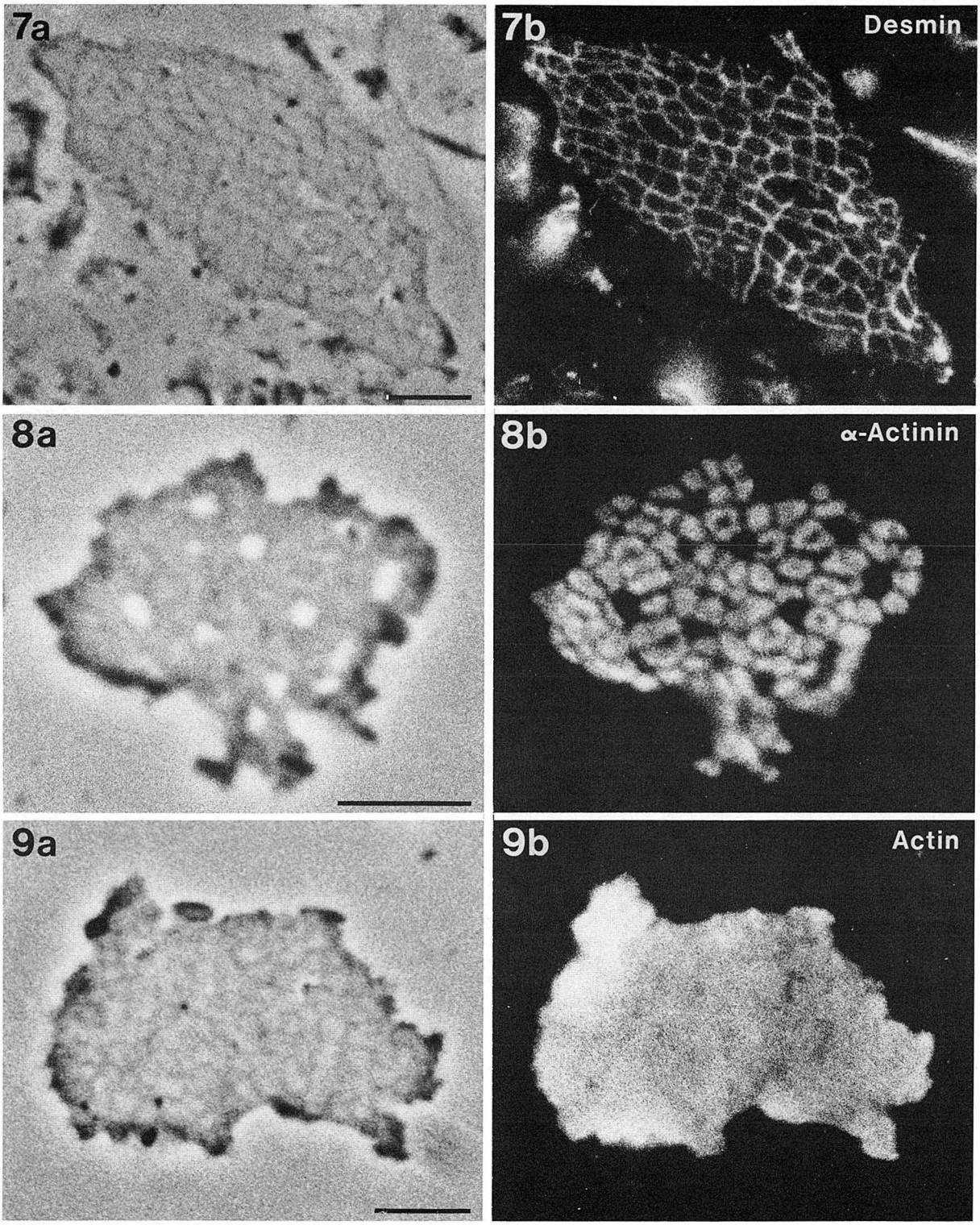
Actin is present over the entire Z band, both along the Z line itself and in the adjacent collapsed material, as shown in Figure 15. Its fluorescence pattern is a single wide band corresponding to the whole Z band of the myofibril ghost.

Myosin and tropomyosin are not present along the Z line itself, but appear in the collapsed material on either side of the Z line. A double fluorescent line constituting the edges of each Z band is therefore revealed, as shown in Figures 16 and 17. These lines, however, are not as easy to resolve for tropomyosin as for myosin. The myosin is present only in the collapsed material, and apparently only at its outer edges; tropomyosin is present throughout the collapsed material, and also in the region between this material and the Z line (in the N_1-Z region) which is occasionally evident. In the dense Z band, the tropomyosin fluorescence is separated only by the Z line, which is near the limit of resolution of the light microscope and difficult to resolve by photography. The myosin fluorescence is separated by a much greater distance, as is apparent in the micrographs. This pattern is reminiscent of, and perhaps indirectly related to, the "contraction bands" produced by the supercontraction of myofibrils (Hanson and Huxley, 1955; Tunik and Holtzer, 1961). The myosin and tropomyosin staining occasionally demonstrate thin strands in the region of the residual filaments which continue to span the sarcomere after extraction.

Discussion

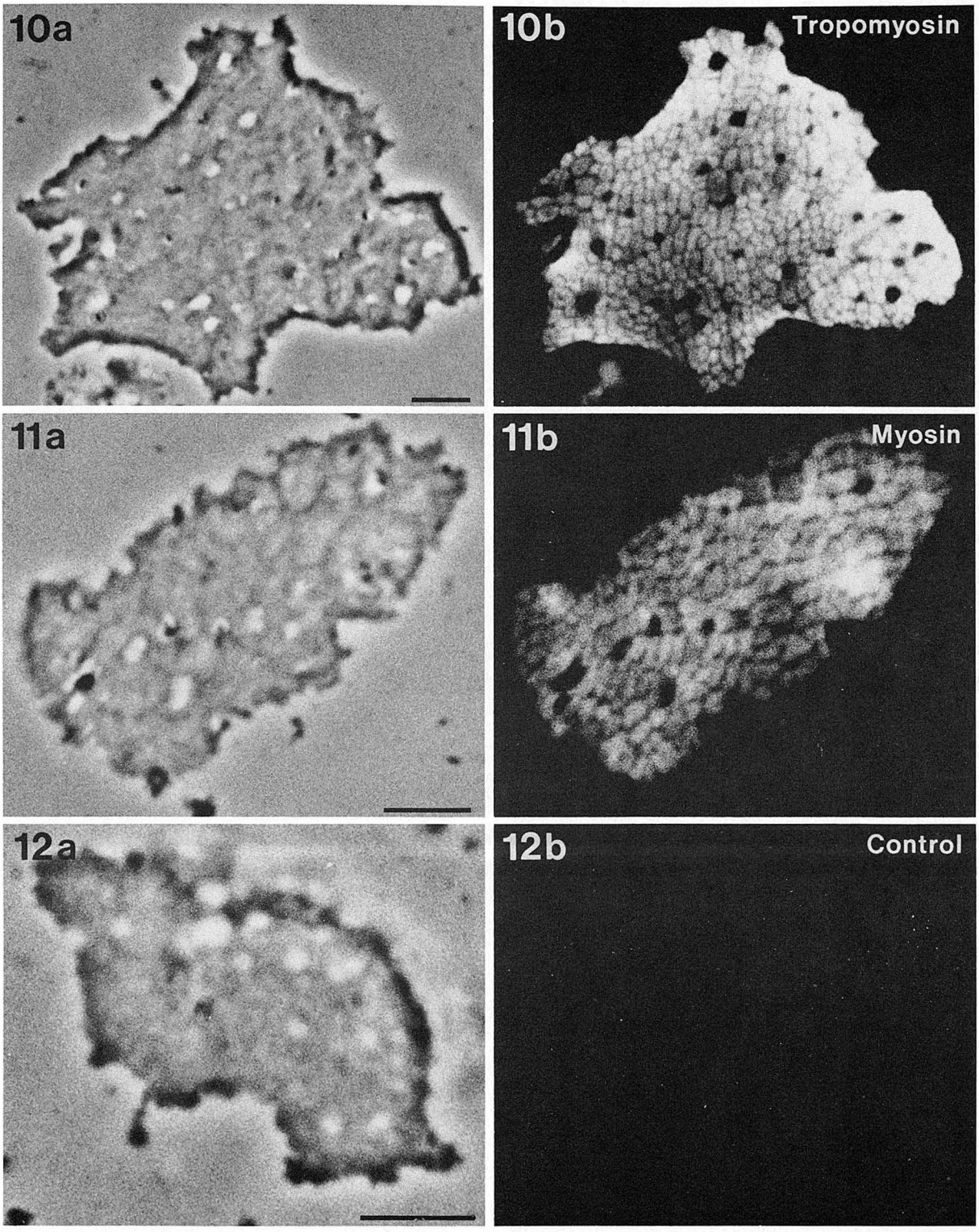
Extraction Process

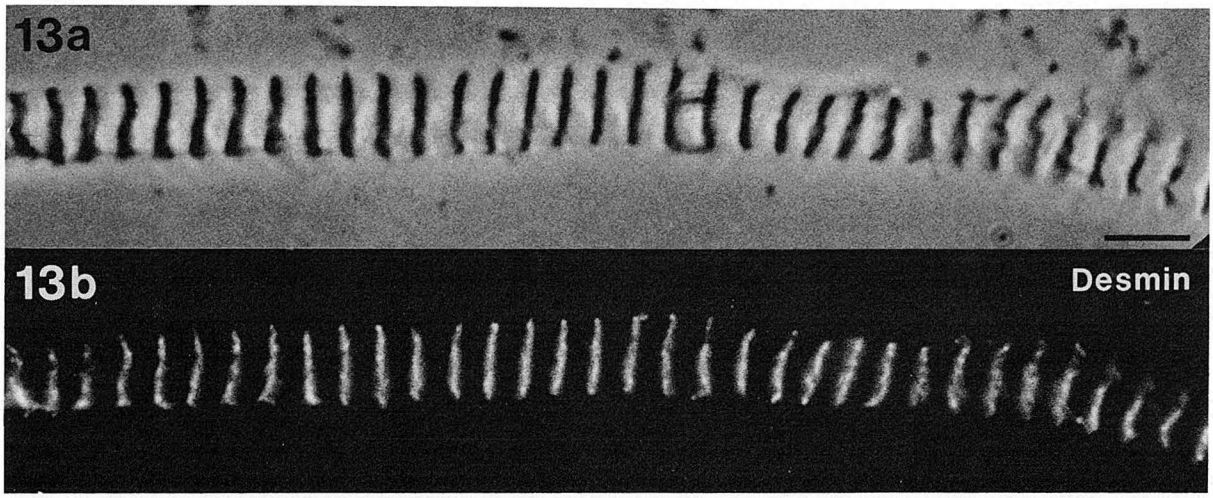
Glycerination removes soluble substances from muscle by disrupting membranes and allowing the substances to diffuse out. The membranes are not solubilized or removed, but they do become fragmented (see Ishikawa, Bischoff and Holtzer, 1969; Robson et al., 1970). Further extraction with 0.6 M KI solubilizes most of the myosin and actin, and appears to cause a "collapse" of insoluble material onto the Z discs (see Hanson and Huxley, 1955; Holtzer, 1959). This collapsed material may reinforce the Z disc, rendering it more stable to shear

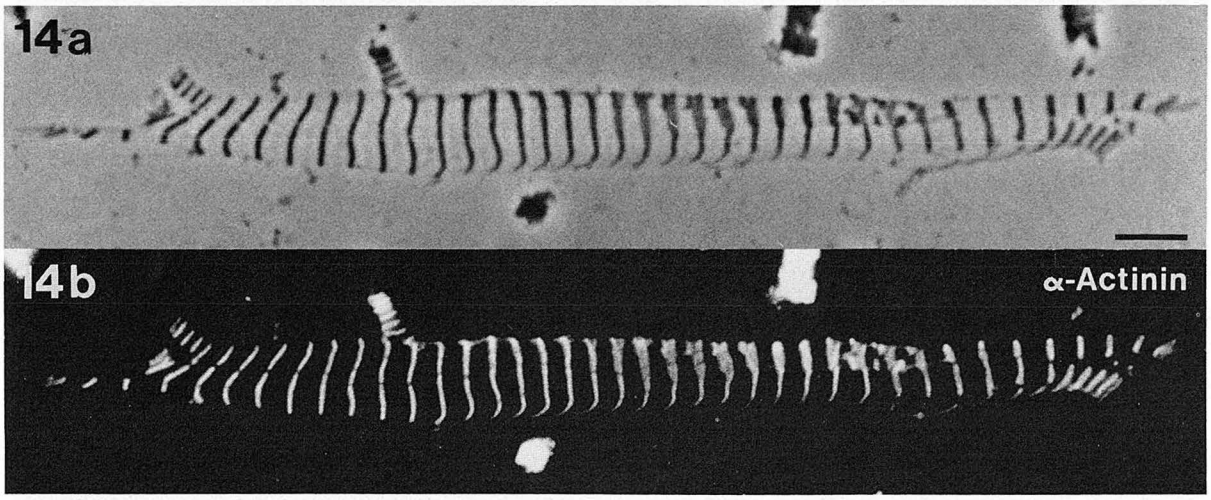


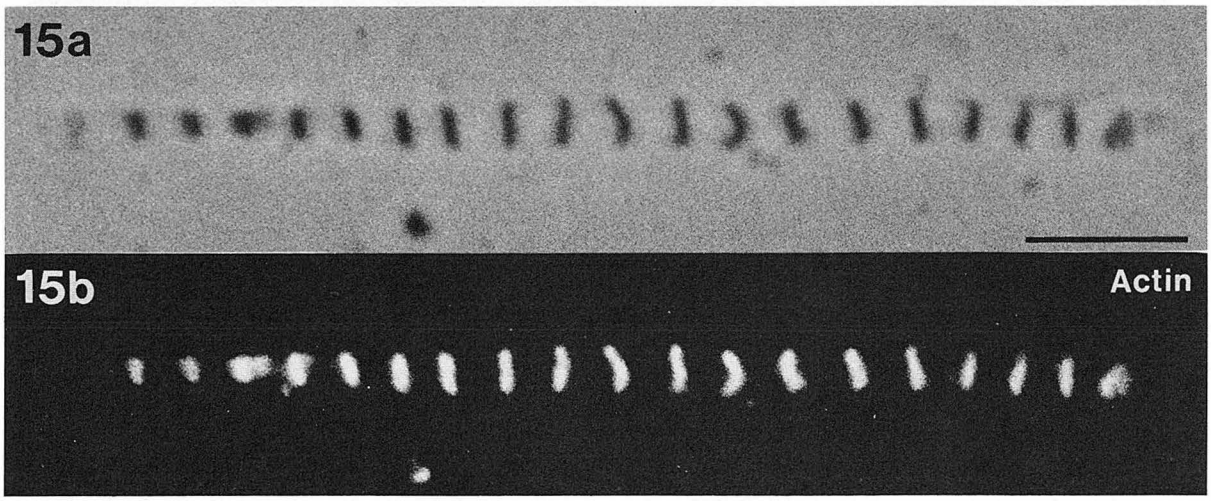
Figures 7–12. Indirect Immunofluorescence of Isolated Z Disc Sheets

(a) Phase-contrast image; (b) fluorescence image. The antibodies used are indicated in the figures. Note the complementarity of the desmin and α-actinin fluorescence patterns. Individual Z disc sheets are so thin that the honeycomb pattern of discs is often difficult to detect by phase-contrast microscopy. Bars = 5 μm.


10a
10b
Tropomyosin
11a
11b
Myosin
12a
12b
Control

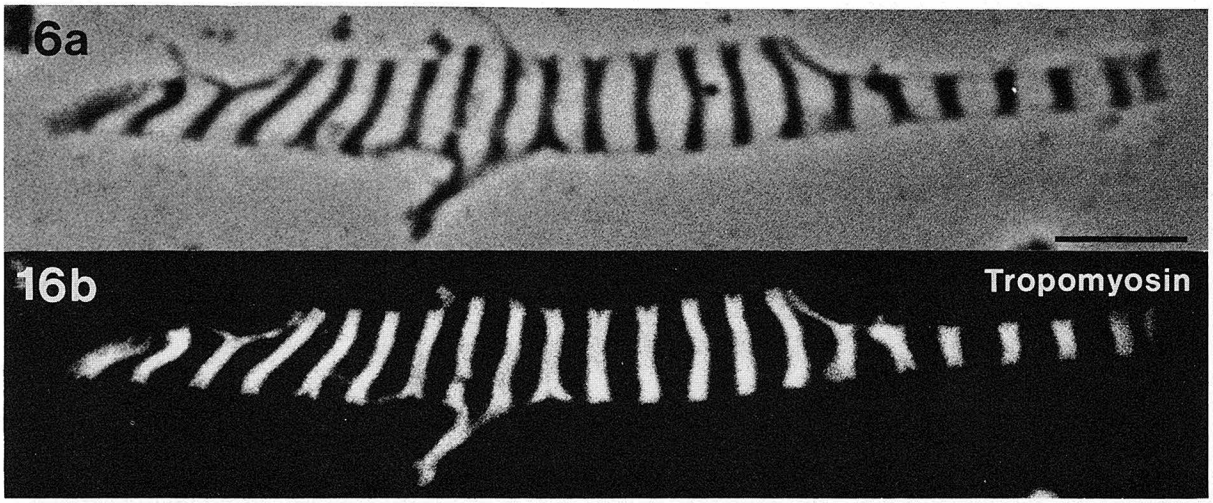


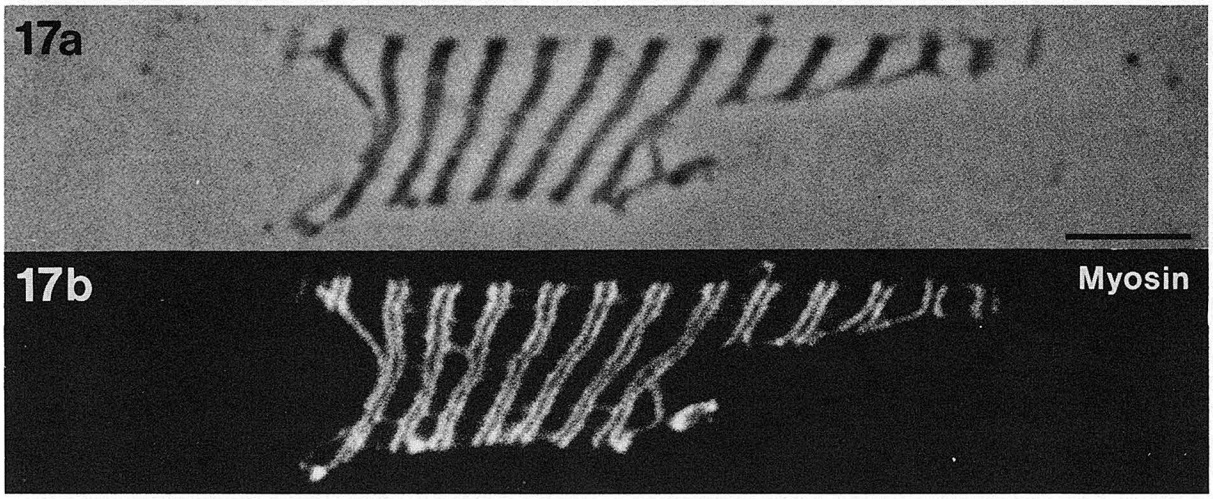


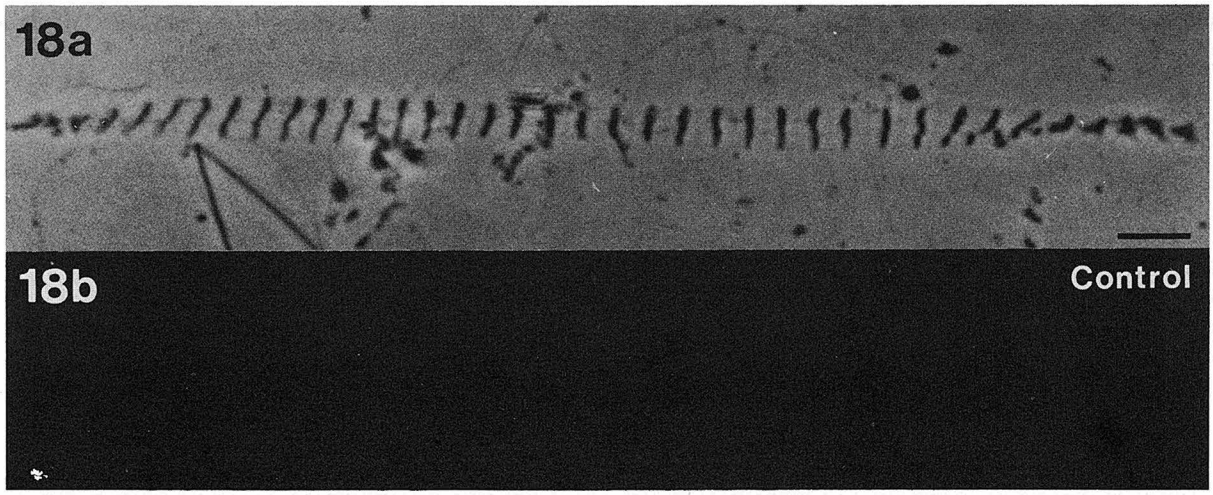


Figures 13–18. Indirect Immunofluorescence of KI-Extracted Myofibrils and Myofibril Bundles

(a) Phase-contrast image; (b) fluorescence image. The antibodies used are indicated in the figures. Note that some of the Z discs of the myofibril bundle stained for α-actinin are oriented so as to be visible face-on. The absence of tropomyosin within the Z line is difficult to resolve here. Bars = 5 μm.


16a
16b
Tropomyosin


17a
17b
Myosin


18a
18b
Control

forces during blending. This material is phase-dense and composed of at least actin, with smaller amounts of tropomyosin and myosin. Under phase-contrast microscopy, it usually makes the Z line appear to be a single, dark, wide band. Electron microscopy, however, shows that the collapsed material occasionally appears to have coalesced short of the Z line, leaving a small space on either side of it and suggesting that there may be something other than the Z line, perhaps the N_1 line, acting as the focal point or limiting boundary for this coalescence. The true mechanism of this coalescence and the reason for its resistance to further solubilization by KI are unknown. Since the extractions were carried out in nonreduced environments, disulfide bonding may be partially responsible for the insolubility of these proteins. It is also possible that impurities in the glycerol cross-linked some of these components and made them resistant to solubilization (Bello and Bello, 1976). Perhaps some of the Z disc proteins are made naturally insoluble by enzymatic cross-linking (Gard and Lazarides, 1979).

Immunofluorescence

Indirect immunofluorescence is useful for the localization of structural proteins, but is of little use in their quantitation. Differences in a protein's immunogenicity, number of antigenic sites, number of exposed antigenic sites in situ and accessibility of these sites to antibodies can all contribute to the observed differences in immunofluorescence intensity. These problems were apparent in this study and require some explanation.

There was little correlation between the amount of protein present, as determined by gel electrophoresis, and the intensity of staining. Actin is the most abundant protein, yet its fluorescence is relatively weak, presumably due to poor immunogenicity and masking by the many proteins with which it combines. Myosin and tropomyosin are present in almost negligible amounts, but immunofluorescence reveals their distributions very clearly: myosin at the outer edge of the collapsed material and tropomyosin throughout this material and up to the Z line.

Superimposition can be another problem: myofibril fluorescence reveals desmin along the Z line, but a face-on view shows that it really encircles the Z disc and is not present within it. It is thus necessary to view the stained specimen from different angles to ascertain the true distribution of the antigen.

Some proteins may redistribute themselves during extraction; perhaps the existence of interfibrillar actin is an artifact of the collapse that accompanies extraction with KI. If this were true, however, then myosin and tropomyosin would also be expected to show this distribution.

These are difficulties in the localization of any antigen by immunofluorescence and they are difficult to rule out without extensive experimentation. Until confirmed or denied, these difficulties should be taken into consideration but should not necessarily affect the validity of the results.

Intermediate Filaments and Membranes

In addition to thick and thin filaments, a class of intermediate-sized filaments has been shown to exist in developing muscle cells (Ishikawa, Bischoff and Holtzer, 1968; Kelley, 1969). These filaments, about 100 Å in diameter, can be seen throughout the cytoplasm at all stages of myofibrillogenesis, but they do not appear to be involved directly in myofibril formation. Kelley noted that they tend to become oriented at right angles to the myofibrils at the level of the Z disc. He surmised that they might have a cytoskeletal role in the myoblast or myotube, and then become a permanent transverse supportive component in the mature fiber.

This interpretation is in agreement with our data. The demonstration that transverse sheets of Z discs can be produced by blending KI-extracted muscle attests to the presence of a network of cross-links at the level of the Z disc. The major subunit of the 100 Å filaments is desmin, which is localized around the Z disc. The Z disc cross-links may therefore be composed of desmin-containing filaments; such filaments have already been shown to extend between adjacent Z lines in frayed-out, unextracted myofibril bundles (Lazarides and Hubbard, 1976) and to be insoluble in KI (Lazarides and Hubbard, 1976; Small and Sobieszek, 1977; Hubbard and Lazarides, 1979). The filamentous nature of the links is evident from electron micrographs of KI-extracted myofibril bundles, which show strands of interfibrillar filaments at the level of the Z line. 100 Å filaments are not clearly discernible in these links, however, perhaps because the desmin is in a different form, aggregated or complexed with other proteins.

Our micrographs occasionally show interfibrillar filament bridges at levels other than that of the Z line, most notably at the M line. Intrafibrillar M line filaments may hold the thick filaments together and in register, but in being attached to the residual filaments of the ghost myofibrils, they also appear to span the distance from one fibril to the next. Why they have not been observed in electron micrographs of thin sections is not clear; possible explanations include fixation-embedding lability or their being obscured by the SR. This difficulty may also be true of other interfibrillar bridges. It is interesting that our micrographs show interfibrillar

M bridges even after intrafibrillar M lines are gone, suggesting that the two might in fact be composed of different macromolecules. It is possible that they might interact with T-SR complexes in vertebrate muscles that have these structures at levels other than that of the Z line, or simply with the SR cisternae which predominate at these levels. It is also unknown what relation, if any, these other bridges have to desmin and intermediate filaments, and what their fate is when Z disc sheets are prepared by shearing.

The presence of both the interfibrillar Z bridges and triad in the small space between adjacent discs suggests a close association or interaction between the two. A possible connection between Z discs and membranes has been noted by several investigators (Bergman, 1962; Allen and Pepe, 1965; Ezerman and Ishikawa, 1967; Walker, Schrodt and Bingham, 1968), suggesting that at least some part of the triad is bound to the Z disc via a filament network. We believe that this network is related to or identical to the filamentous bridges that hold adjacent myofibrils together, and that it is composed of desmin.

Residual Sarcomeric Filaments

The nature of the structures that hold successive intrafibrillar Z discs together in vertebrate skeletal muscle after KI extraction is not known. The existence of filaments other than the thick (myosin) and thin (actin) filaments within the sarcomere has been debated for years (for example, see Hanson and Huxley, 1955; Locker and Leet, 1976; Ulrick et al., 1977a; dos Remedios and Gilmour, 1978). The arguments against such filaments are based largely on negative evidence. They would be difficult to characterize in electron micrographs because of their thinness and because embedding and staining procedures do not seem to enhance or preserve them adequately. The difficulty in distinguishing them from other types of filaments casts doubt on their identification as a separate entity. The widely accepted sliding filament model of muscle contraction (Huxley, 1969) postulates no role or need for such filaments, suggesting that evidence for their existence may be artifactual and based on unnatural manipulations. The fact is, however, that something filamentous remains joining intrafibrillar Z discs together after extraction of actomyosin with KI. Immunofluorescence reveals that these filaments are not composed of actin, tropomyosin or myosin (as evident in Figures 15–17), unless they are antigenically masked or different from their usual counterparts. Strands of myosin can be visualized occasionally in this region (see Figure 17), but these probably represent thick filaments that have not been completely extracted. Strands of tropomyosin are also seen sometimes, usually along the edge of the ghost myofibril (not shown). It is possible that there is some sort of interaction between these filaments and the SR which ensheaths each myofibril, such that peripheral myofibrillar filaments are not extracted as readily. Perhaps the filaments are composed of undissociated actin-myosin (thin filament-thick filament) complexes. Such complexes would not be typical, however, since KI has been shown to dissociate actomyosin (Holtzer, Wang and Noelken, 1960), and to depolymerize myosin (Tonomura, Sekiya and Imamura, 1962) and actin (Guba, 1950).

The preservation of these residual filaments in our micrographs is not adequate to allow their identification or characterization. Again, such insoluble complexes may be artifacts of an oxidizing environment or of chemical cross-linking during glycerination. Strong adhesion of filaments to the substrate may be responsible for making the former so apparent in electron micrographs of whole-mounted, extracted myofibrils.

Myofibril Subdivision

It has been postulated that myofibril proliferation is a direct result of radial growth, which ultimately results in a longitudinal subdivision of the myofibril after a maximum size is attained (Goldspink, 1971). This division allows the sarcoplasmic reticulum and T system to expand into the myofibril and complete the formation of new, smaller myofibrillar assemblies. Electron micrographs show that this process probably starts with the formation of a small fissure at the center of the Z disc (Goldspink, 1970, 1971; Shear, 1978) and proceeds outward radially.

These observations may explain an interesting feature of our immunofluorescence pictures. Labeling the Z discs with anti-desmin often reveals a small patch of desmin at the center of the disc, or a short radial segment of desmin reaching from the center to the edge. Conversely, α-actinin is absent from these locations. Thus desmin, SR and T tubules are most probably invading the larger myofibrils as they split into smaller units. This suggestion adds further support to the concept of a close structural relationship between desmin and membranous organelles.

Conclusions

Our results suggest that skeletal muscle fibers are in part composed of an internal scaffold of proteins which is resistant to solubilization by glycerol and 0.6 M KI. Simple mechanical disruption of this scaffold causes it to separate into sheets of interconnected Z discs, demonstrating the existence of strong connections between Z discs of adjacent

myofibrils. These links contain desmin and perhaps actin as well. An actin-desmin complex resistant to dissociation by KI and capable of assembling into intermediate-sized filaments has recently been isolated from chicken smooth muscle (Hubbard and Lazarides, 1979). A complex analogous to this may exist in skeletal muscle, such that the Z bridges or the membrane anchors are composed of an actin-desmin complex or made possible by an actin-desmin interaction. The coincident localization of desmin and the T system in chicken muscle suggests that desmin might be responsible for anchoring T tubules to the Z disc, or for guiding, orienting and stabilizing them during development. By forming a collar around each Z disc, desmin may help preserve the integrity of each Z disc and myofibril. As part of a network of interlinked collars, it may be responsible for maintaining the alignment of adjacent sarcomeres and unifying the contractile actions of all the separate myofibrils, and thus for structurally and mechanically integrating the entire fiber. The function of desmin indeed seems to justify its name, from the Greek noun, δεσμός = link, bond.

Experimental Procedures

Sample Preparation

Lower leg muscles were cut in strips from adult male leghorn chickens, tied to wooden applicator sticks to prevent contraction and stored in 50% glycerol, PBS, 1 mM EGTA at −20°C for at least 1 month [PBS contains 137 mM NaCl, 3 mM KCl, 2 mM KH_2PO_4 and 8 mM Na_2HPO_4(pH 7.5)]. Small strips of well glycerinated muscle were tied to glass rods and swirled in several changes of 0.6 M KI, 20 mM $Na_2S_2O_3$ (to prevent free iodine formation), PBS at 4°C for up to about 3 weeks, depending upon the size of the sample. These pieces were then blended in a Virtis "45" microhomogenizer at various speeds and for varying times. The effect of the blending was monitored by frequent examination of drops of the homogenate by phase-contrast microscopy; large thin sheets of Z discs were best produced by moderately fast blending for 10–20 sec. For indirect immunofluorescence, drops of homogenate were spread on glass coverslips and then immediately rinsed of nonadherent material by dipping in PBS. For scanning electron microscopy, large muscle fiber fragments were produced by slower blending speeds and shorter durations; these fragments were allowed to settle onto coverslips overnight.

Myofibrils were prepared by vigorous blending of glycerinated muscle in a Lourdes MM-1B homogenizer for 3–5 min at near-top speed. Blending and storage were in the 50% glycerol solution. The myofibrils were washed free of glycerol before use by 2 or 3 cycles of suspension in PBS followed by centrifugation. Myofibrils used for immunofluorescence were allowed to settle onto coverslips; extraction was effected by immersion in a reservoir of the 0.6 M KI solution for approximately 1 hr.

Transmission Electron Microscopy

Drops of myofibril suspension or scaffold homogenates were applied to carbon-coated 400 mesh copper grids and then rinsed of nonadherent material after 1 min. Whole myofibrils were extracted with KI solution directly on the grid by applying two or three changes of this solution over a 5 min period. After rinsing with distilled water, specimens were stained with 2% aqueous uranyl acetate for 2 min.

For thin sectioning, samples were fixed in 2.5% glutaraldehyde-0.1 M cadodylate buffer (pH 7.4; 1 hr at room temperature), post-fixed in 1% OsO_4-0.1 M cacodylate buffer (pH 7.4; 1 hr on ice) and stained with 1% uranyl acetate-0.1 M maleate buffer (pH 5.15; overnight at 4°C). Small pieces were excised, embedded in 1,2,7,8-diepoxyoctane/nonenyl succinic anhydride (NSA)/tridimethyl amino methyl phenol (DMP-30) in a volume ratio of 9/16/1, and cured at 60°C for 2 days. Sections were cut on a Reichert OmU2 microtome and post-stained for 1 min at room temperature with 0.2% lead citrate.

Specimens were examined with a Philips EM201c at 80 kV and photographed on 35 mm film.

Scanning Electron Microscopy

Coverslips were cleared of unbound material by dipping in PBS and then fixed by immersion in a reservoir of 1% glutaraldehyde, PBS for 30 min at room temperature. After a PBS rinse, the samples were dehydrated in ethanol and then critical point-dried. Coverslips were mounted on eluminum stages, coated with 100 Å of a gold-palladium alloy and examined in an ETEC SEM.

Polyacrylamide Gel Electrophoresis

One-dimensional SDS-PAGE was run according to the method of Laemmli (1970), as modified and described by Hubbard and Lazarides (1979).

Two-dimensional IEF/SDS-PAGE was run according to the method of O'Farrell (1975), as modified and described by Hubbard and Lazarides (1979). Analytical gels all contained 12.5% acrylamide and 0.1% N,N'-methylene bisacrylamide.

Samples were boiled for 5–10 min in 2 × SDS sample buffer (containing 2% SDS and 2% dithiothreitol) or incubated for approximately 1 hr on ice in urea sample buffer (8 M urea, 0.5% 2-mercaptoethanol, 2% NP-40).

Indirect Immunofluorescence

Coverslips with adherent samples were rinsed of KI by equilibration in PBS before antibody application. 50–100 λ of diluted antibody solution were applied to each coverslip as it lay face up in a humidified chamber. After incubating for 30 min at 37°C, the coverslips were immersed in a PBS reservoir for 1 hr to remove unbound antibodies. The coverslips were then drained and returned to the humidified chamber, and 75 λ of a 1:15 dilution of FITC-conjugated goat anti-rabbit IgG were applied. Again, incubation was for 30 min at 37°C; subsequent rinsing in PBS was usually for 2–3 hr. The coverslips were mounted face down with Elvanol; observation and photography were performed with a Leitz phase-contrast microscope equipped with epifluorescence optics, using an oil immersion, 100X N. A. 1.3 phase fluorescence lens and filter module H. Photographs were taken on Kodak Tri-X film and developed in Diafine.

Antibody Characterization

FITC-conjugated goat anti-rabbit IgG was purchased from Miles Laboratories. Rabbit anti-desmin was prepared from chicken smooth (gizzard) muscle, as described by Lazarides and Hubbard (1976), using preparative SDS-PAGE as a final purification step (Lazarides, 1976a). Rabbit anti-actin was prepared in a similar manner (see Lazarides, 1976b; Eckert and Lazarides, 1978). Rabbit anti-α-actin was raised against purified porcine skeletal muscle α-actinin (Lazarides, 1976b), and rabbit anti-tropomyosin was raised against purified chicken skeletal muscle tropomyosin (Lazarides, 1975).

Myosin was purified from chicken breast muscle by the method of Pollard, Thomas and Niederman (1974). Preparative SDS-PAGE was used as a final purification step, and the myosin heavy chain was used an antigen (Figure 19c). The immunization procedure was identical to that for α-actinin (Lazarides, 1976b). The anti-myosin forms strong precipitin lines with both native and SDS-denatured chicken breast myosin; it does not cross-react with crude chicken smooth muscle (gizzard) myosin. It stains only A bands of whole myofibrils (Figures 19a and 19b).

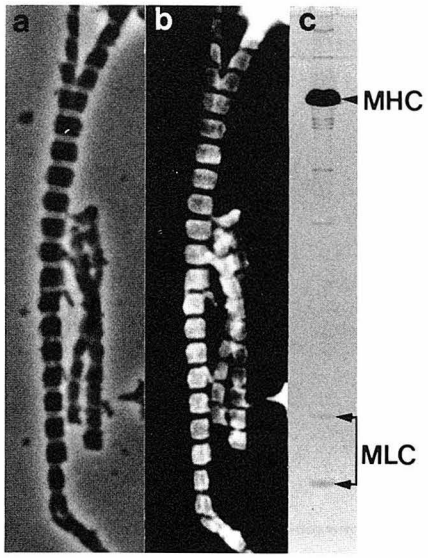


Figure 19. Anti-Myosin Characterization

(a and b) Phase and fluorescence images of whole myofibrils stained with the anti-myosin antibody. Only A band staining is apparent. (c) SDS-polyacrylamide gel showing purified myosin preparation; the myosin heavy chain band was cut from a preparative gel and used as antigen. (MHC) Myosin heavy chain; (MLC) myosin light chains.

All antisera were purified by ammonium sulfate fractionation and used at dilutions that gave optimal staining. All preimmune sera gave negligible fluorescence. The dilutions (in PBS) used were approximately: desmin, 1:10; actin, 1:15; α–actinin, 1:25; tropomyosin, 1:30; myosin, 1:30; preimmune serum, 1:10.

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Note Added in Proof

α-A is misidentified in Figure 6B. The arrow should be pointing to the denser band two bands above.

Chapter 3

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Desmin and Vimentin Coexist at the Periphery of the Myofibril Z Disc

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Summary

Two-dimensional gel electrophoresis has revealed that vimentin, the predominant subunit of intermediate filaments in cells of mesenchymal origin, is a component of isolated skeletal myofibrils. It thus coexists in mature muscle fibers with desmin, the major subunit of muscle intermediate filaments. Antisera to desmin and vimentin, shown to be specific for their respective antigens by two-dimensional immunoautoradiography, have been used in immunofluorescence to demonstrate that vimentin has the same distribution as desmin in skeletal muscle. Both desmin and vimentin surround each myofibril Z disc and form honeycomb-like networks within each Z plane of the muscle fiber. This distribution is complementary to that of α–actinin within a given Z plane. Desmin and vimentin may thus be involved in maintaining the lateral registration of sarcomeres by transversely linking adjacent myofibrils at their Z discs. This linkage would support and integrate the fiber as a whole, and provide a molecular basis for the cross-striated appearance of skeletal muscle.

Introduction

Intermediate filaments comprise a diverse class of cytoplasmic filaments whose diameter is intermediate to those of actin filaments and microtubules. Although similar in morphology, they are quite heterogeneous by chemical and immunological criteria (for review see Lazarides, 1979). Desmin is the major subunit of intermediate filaments from avian smooth muscle (Lazarides and Hubbard, 1976; Small and Sobieszek, 1977); it is electrophoretically identical to and immunologically cross-reactive with a protein in striated muscle that has been localized along Z lines (Lazarides and Hubbard, 1976; Izant and Lazarides, 1977; Lazarides, 1978). The relationship of skeletal muscle desmin to the Z disc was uncertain until a technique was developed for the visualization of Z discs face-on. If a skeletal muscle fiber is weakened by the extraction of its actomyosin in high salt, subsequent homogenization will cause it to fragment along planes perpendicular to the myofibrillar axis. Z discs are not liberated individually by this treatment, but remain interlinked in large sheets. These Z disc sheets are each derived from a transverse Z plane of the original muscle fiber (Granger and Lazarides, 1978). Indirect immunofluorescence has shown that desmin is present at the periphery of each disc, forming a honeycomb-like net within the Z plane. This distribution suggested that intermediate (desmin) filaments might be responsible for attaching neighboring Z discs to one another, acting perhaps as mechanical integrators of myofibrillar contraction. This view is supported by electron microscopic evidence of filaments surrounding Z discs in a variety of muscle types (see Discussion).

Vimentin, the major subunit of fibroblastic intermediate filaments, is found in most cells of mesenchymal origin (Bennett et al., 1978; Franke et al., 1978). It was recently found that cultured chick skeletal myotubes contain vimentin as well as desmin [Gard, Bell and Lazarides, 1979; vimentin is referred to as fibroblastic intermediate filament protein (F-IFP) in this paper]. Myotube vimentin is not merely a remnant from relatively undifferentiated myoblasts, however, since it is synthesized after fusion and throughout early myogenesis (Gard and Lazarides, 1980). This observation raised the question of the fate of vimentin in adult, fully differentiated skeletal muscle. In the present study, two-dimensional isoelectric focusing/polyacrylamide gel electrophoresis (IEF/SDS-PAGE) has revealed that vimentin is present in KI-insoluble Z disc scaffolds used to produce Z disc sheets. The possibility that this vimentin is contributed by nonmuscle cells present in whole muscle tissue has been ruled out by its presence on two-dimensional gels of purified myofibrils. Finally, an antiserum specific for vimentin stains the Z lines of myofibrils and, in particular, the peripheries of Z discs in isolated Z disc sheets, as does anti-desmin. Vimentin thus appears to have the same distribution as desmin in adult skeletal muscle, and may therefore have the same functions.

Results

Electrophoretic Analysis of Myofibrils

Mature chicken skeletal muscle gives a pattern of protein spots on two-dimensional gels (O'Farrell, 1975) that includes vimentin. Since whole muscle tissue contains a variety of cells known to be rich in vimentin, such as fibroblasts and vascular endothelial cells (Brown, Levinson and Spudich, 1976; Hynes and Destree, 1978; Starger et al., 1978; Blose, 1979; Franke et al., 1979; Gard et al., 1979), the possibility that muscle fibers themselves contain vimentin was investigated.

If fresh or glycerinated muscle is homogenized vigorously, myofibrils are released; these myofibrils can be isolated by differential centrifugation and purified on a sucrose gradient. Analysis of this material by IEF/SDS-PAGE reveals that vimentin is still present. Figure 1 shows the electrophoretic profile of glycerinated chicken pectoralis myofibrils that have been detergent-washed to remove membranous organelles, banded on a discontinuous sucrose gradient, and

briefly extracted with 0.6 M KI to remove actomyosin, whose high concentration in myofibrils would otherwise obscure minor protein components. In addition to small amounts of residual myosin, α-actinin and tropomyosin, actin, desmin and vimentin are present. The α and β variants of desmin (Izant and Lazarides, 1977) as well as two isoelectric variants of vimentin (O'Connor, Balzer and Lazarides, 1979; Gard et al., 1979) are clearly visible. The identity of the faint satellite spots above each of the desmin and vimentin variants is unknown; they are visible on nearly all gels of desmin and vimentin preparations from skeletal muscle, whether fresh, glycerinated or grown in tissue culture. Using light microscopy, no contaminating organelles other than occasional nuclei are visible in these preparations; IEF/SDS-PAGE of purified skeletal muscle nuclei suggests that they contain little or no vimentin, and cannot account for any of the proteins visible in Figure 1 (data not shown). Both desmin and vimentin appear on two-dimensional gels of all avian muscles examined, including peroneus longus, anterior latissimus dorsi (ALD) and posterior latissimus dorsi (PLD). The ratio of vimentin to desmin is generally greater in fresh than in glycerinated muscle, and greater in muscles of young chickens than older chickens.

Immunoautoradiographic Characterization of Vimentin and Desmin Antisera

To determine the distribution of vimentin in skeletal muscle, antiserum to embryonic chicken muscle vimentin was raised in a rabbit. Total leg and breast muscle from 14–16 day old embryos was homogenized and extracted with detergent and high salt to give cytoskeletons rich in desmin and vimentin. These cytoskeletons were electrophoresed on preparative SDS slab gels and stained briefly with Coomassie blue to visualize the different protein bands. The vimentin band was excised, homogenized and used as antigen. Reelectrophoresis of the antigen showed no detectable contaminants of different molecular weight or of the same molecular weight and different isoelectric point (not shown). Double immunodiffusion of this antiserum against whole muscle extract results in a single precipitin line, both before and after staining with Coomassie blue (not shown; see Experimental Procedures). Antiserum to desmin was raised against chicken gizzard desmin as described (Lazarides and Hubbard, 1976).

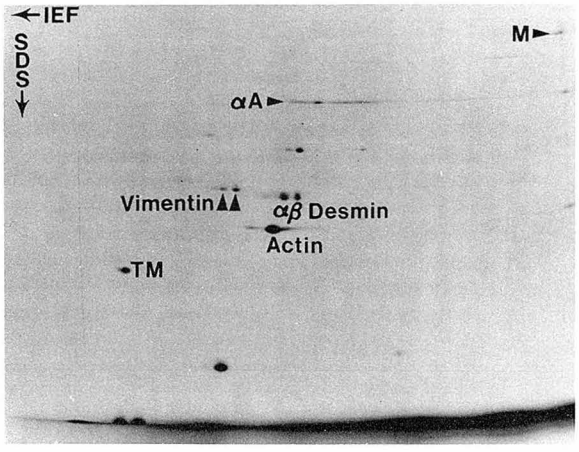


Figure 1. Two-Dimensional Gel Electropherogram of Myofibrils from Chicken Pectoralis Muscle

Isoelectric focusing (IEF) was performed from right to left (acid on the left) and SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed from top to bottom. Major proteins include vimentin, α- and β-desmin, actin and tropomyosin (TM; the α variant predominantes in this muscle). Myosin (M) and α-actinin (αA) do not focus well in this system.

In this study, it was essential to be certain that the desmin and vimentin antisera used were specific for their respective antigens. The similarity in the peptide maps of desmin and vimentin (Starger et al., 1978; Gard et al., 1979) raised the possibility of extensive immunologic cross-reaction, which would prevent accurate localization of these antigens by immunofluorescence.

To ascertain the specificity of the antisera, the technique of immunoautoradiography (Burridge, 1976) was used. Immunoautoradiography involves the detection of antigens in SDS-polyacrylamide gels by incubating the fixed gels first with a primary antiserum, then with a radioiodinated secondary antiserum or protein A (Adair, Jurivich and Goodenough, 1978), which binds to the Fc portion of IgG (Forsgren and Sjöquist, 1966; Kronvall et al., 1970), followed by autoradiography of the dried gel to detect the labeled bands. This method is extremely sensitive but is limited by the inherent resolution of one-dimensional gels. Several major structural proteins, such as actin, desmin, vimentin and tubulin, have similar mobilities in SDS-PAGE, making one-dimensional immunoautoradiography inadequate for the demonstration of the specificity of the corresponding antisera. For this reason we have used two-dimensional IEF/SDS-PAGE, which separates these proteins on the basis of charge as well as size and makes unambiguous identification of each protein possible. We have also modified the technique to minimize or eliminate nonspecific binding of antibodies to proteins in the gel.

We chose the material present in high speed supernatants of embryonic chick muscle to display on the gels to be immunoautoradiographed. These supernatants contain actin, desmin, vimentin and tubulin, as well as many unidentified proteins.

Figure 2a shows a gel that was treated with anti-vimentin and ^{125}I–protein A, and Figure 2b shows the corresponding autoradiogram. Vimentin is the predominant species labeled; there is clearly no cross-reaction with desmin, tubulin or actin. The diagonal band of spots more acidic and of lower molecular weight than vimentin that is labeled by the antiserum

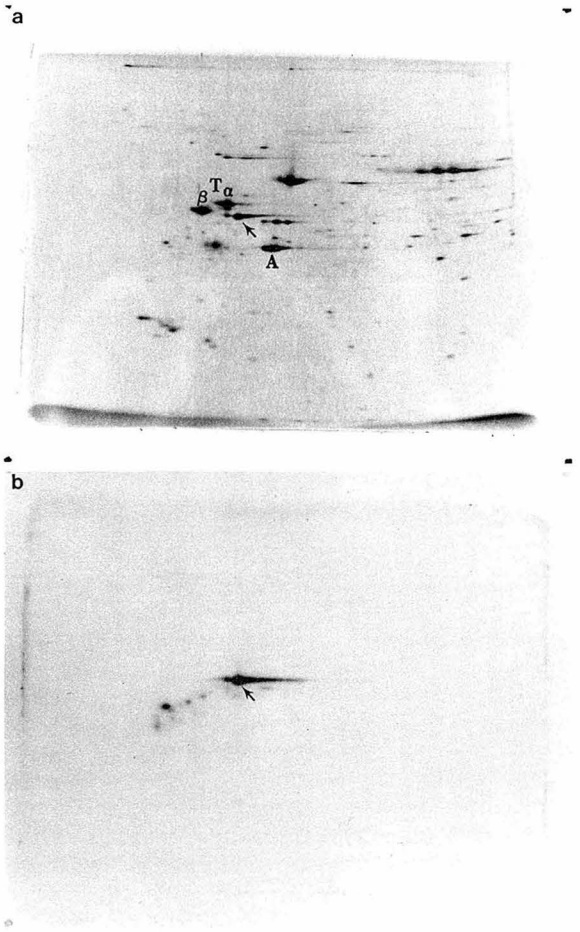


Figure 2. Two-Dimensional Immunoautoradiography Using Anti-vimentin Antiserum

(a) Dried two-dimensional gel of a high speed supernatant of a low ionic strength extract of embryonic chicken muscle. Actin (A) and α and β tubulin (T) are labeled. The arrow points to the major isoelectric variant of vimentin. (b) Corresponding autoradiogram showing specific labeling of vimentin and its degradation products with this antiserum. ^{35}S-methionine was mixed with india ink and used to make reference marks in the corners.

is believed to represent degradation products of vimentin. Most of these proteins are of sufficiently different molecular weight so as not to be contaminants of the band cut from the SDS gel and used as antigen in the immunization (see above). In addition, one-dimensional peptide maps of some of these polypeptides show identity to peptide maps of vimentin (D. Gard and E. Lazarides, unpublished observations). These peptides co-purify with vimentin in variable amounts, and are reduced in quantity when special precautions are taken to inhibit proteolysis (Gard et al., 1979; our unpublished observations). The faint spot below and to the right of vimentin on the autoradiogram does not coincide with the acidic satellite of α–desmin (Gard et al., 1979), but runs immediately beneath it. In immunofluorescence, this antiserum stains (not shown; Gard and Lazarides, 1980) chick embryo fibroblasts with a pattern characteristic of intermediate filaments (Hynes and Destree, 1978) and stains perinuclear aggregates of intermediate filaments in cells exposed to colcemid (Goldman and Knipe, 1973). These data demonstrate that the antiserum used for localizing vimentin in this study does not have detectable amounts of antibodies specific for proteins other than vimentin.

Figure 3a shows a gel treated with anti-desmin, and Figure 3b is its autoradiogram. Again, this antiserum appears to be highly specific. The three variants of desmin found in embryonic muscle (Gard et al., 1979; O'Connor et al., 1979) all label intensely. The faint spots appearing in the autoradiogram below and to the acidic side of desmin are believed to be degradation products of desmin. Their greater mobility on SDS gels precludes the possibility that they were contaminants of the immunizing antigen. The same pattern of peptides on two-dimensional gels is generated by partial digestion of purified desmin with a protease present in commercial preparations of collagenase (B. Hubbard and E. Lazarides, unpublished observations) or with a purified calcium-activated protease from muscle (D. Gard and E. Lazarides, unpublished observations). These peptides co-purify with desmin from chicken gizzard and bind to anti-desmin affinity columns (our unpublished observations). The acidic satellite of α–desmin is highly homologous to α– and β–desmin by one-dimensional peptide mapping (Gard et al., 1979) and is phosphorylated along with α–desmin (O'Connor et al., 1979). It thus appears that this antiserum reacts specifically with desmin and has no significant activity against any other antigen in this system. This antiserum has been characterized by other means (Lazarides and Hubbard, 1976; Lazarides, 1978) and also shown to be specific for desmin.

Figure 4a shows a gel treated identically to those in Figures 2 and 3, except that the vimentin preimmune serum was used. The autoradiogram in Figure 4b was exposed for the same period of time as the other two (21 hr). Clearly, none of the proteins were labeled.

Immunofluorescence of Myofibrils

Indirect immunofluorescence of skeletal myofibrils reveals that vimentin is localized along the Z line. Figures 5–7 show phase-contrast and fluorescence images of three types of muscle fiber stained with rabbit anti-vimentin followed by fluorescein-conjugated goat anti-rabbit IgG: pectoralis major, a glycolytic twitch muscle; anterior latissimus dorsi (ALD), a slow muscle; and posterior latissimus dorsi (PLD), a twitch muscle with its transverse tubular system near the level of each A-I junction (Page, 1969). In each case, the fluorescence lies along the Z line. The same pattern is obtained using peroneus longus, an oxidative twitch muscle (not shown). Figure 8 demonstrates that vimentin in PLD myofibrils remains insoluble and in

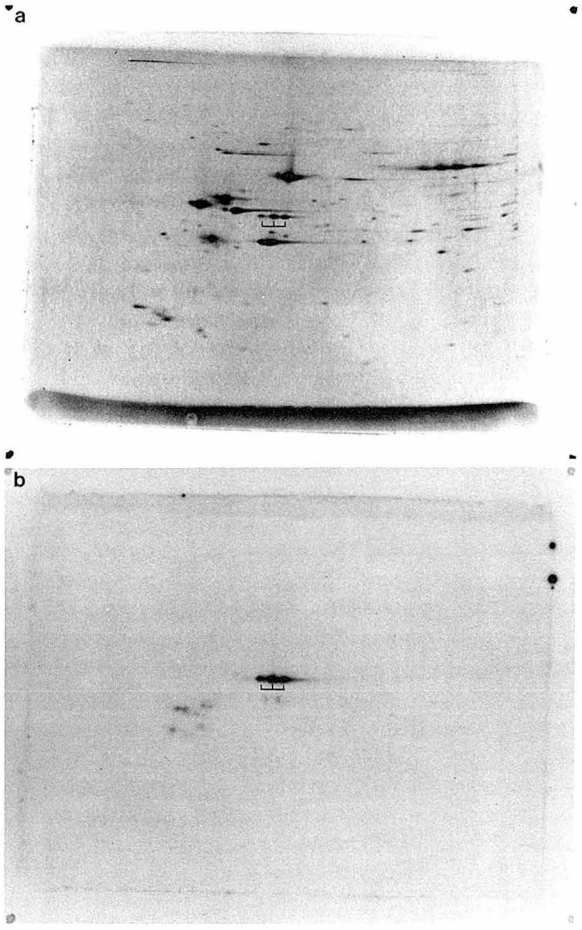


Figure 3. Two-Dimensional Immunoautoradiography Using Anti-desmin Antiserum

(a) Dried two-dimensional gel as in Figure 2a. Brackets mark (from left to right) the acidic variant of α–desmin, α–desmin and β–desmin. (b) Corresponding autoradiogram showing specific labeling of desmin and its degradation products with this antiserum.

place when actomyosin is extracted with 0.6 M KI before immunofluorescence. This is also true for ALD, pectoralis and peroneus longus (not shown).

Staining of each of these muscle types with anti-desmin gives the same pattern as with anti-vimentin: a fluorescent Z line. Figures 9 and 10 show staining of PLD and ALD muscles with anti-desmin; pectoralis and peroneus longus give the same result, and all exhibit Z line staining after extraction with KI (not shown; see Granger and Lazarides, 1978; Lazarides and Balzer, 1978). All of the myofibrils shown are glycerinated, but the same results are obtained using myofibrils isolated from fresh muscle.

Immunofluorescence of Z Disc Sheets

Z disc sheets were prepared as described (Granger and Lazarides, 1978). Strips of glycerinated muscle were extracted in a solution containing 0.6 M KI and then sheared briefly in a blender. Cleavage of the extracted fibers occurs preferentially between planes of Z discs; the Z discs of each Z plane remain laterally connected to one another in such a way that sheets of interlinked Z discs are produced. These sheets present a face-on view of the Z discs, and can provide information on the spatial distribution of antigens within the Z plane which myofibrils alone cannot give.

Figure 11c shows that vimentin is localized at the periphery of each Z disc, forming a network within the Z plane. For comparison, Figures 11a and 11b show the distributions of desmin and α–actinin in Z disc sheets; desmin is at the periphery of each disc and α–actinin is within the discs themselves. Thus vimentin exhibits the same distribution as desmin, which is complementary to that of α–actinin.

If Z disc sheets are doubly labeled (indirectly with anti-desmin or anti-vimentin and fluorescein-conjugated goat anti-rabbit, followed by direct labeling with rhodamine-conjugated anti-α–actinin; Gard and Lazarides, 1980) the patterns of Figures 12 and 14 emerge, clearly demonstrating the complementarity and specificity of the staining (shown for desmin but the same for vimentin). Within the same Z disc sheet, desmin and vimentin are present at the periphery of each Z disc while α–actinin is confined to the interior of each disc.

Z disc sheets can be stretched manually to many times their usual size by smearing the Z disc scaffold homogenate across the surface of a coverslip with several strokes of the side of a pipette tip. Figures 13 and 14 show the stretched configurations that result. Figure 13 is printed at the same magnification as Figures 11 and 12; the Z discs have been stretched to several times their usual diameter of about 1 μm. The general integrity of the sheets is maintained, implying a high degree of plasticity in the system. Z discs do not separate, but remain firmly linked to one another at their peripheries. Residual α–actinin maintains a fairly even distribution within the stretched disc (Figure 14b), while desmin and vimentin retain their continuous net-like distribution throughout the Z plane (shown for desmin in Figures 13 and 14). The maintenance of the complementary relationship between desmin (or vimentin) and α–actinin in a stretched Z disc sheet is illustrated in Figure 14. These patterns are strongly suggestive of a structure based on a malleable network of filaments (desmin and vimentin) within the Z plane.

Figure 11a shows several good examples of the proposed phenomenon of myofibril proliferation by longitudinal splitting as discussed previously (Granger and Lazarides, 1978). Z discs stained for desmin or vimentin occasionally show localization at a spot near the center of the disc, or a radial arm extending from the center to the periphery. Double labeled Z disc sheets suggest that α–actinin is absent from these locations (Figure 12). These patterns correlate well with the fissures down the lengths of larger myofibrils

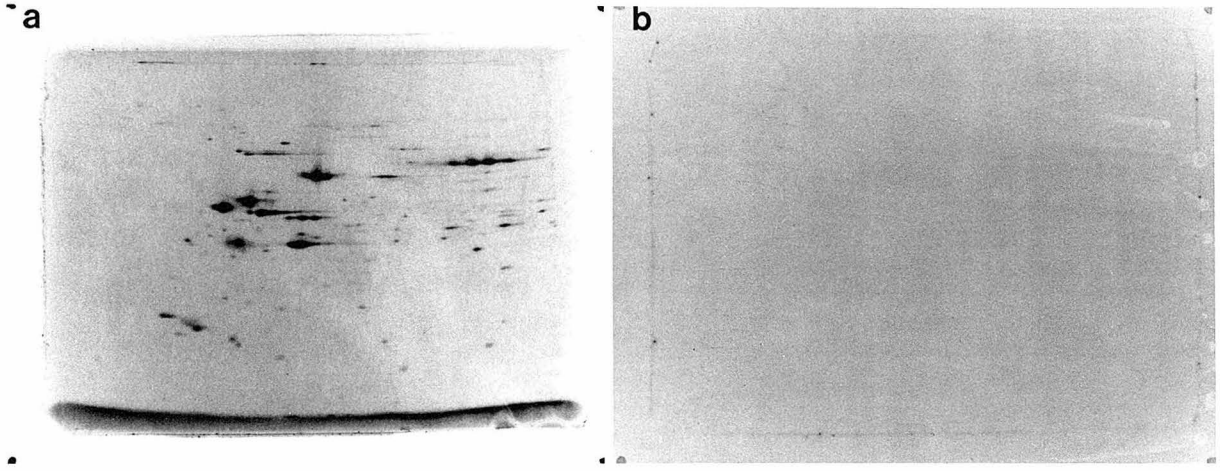


Figure 4. Two-Dimensional Immunoautoradiography Using Vimentin Preimmune Serum
(a) Dried two-dimensional gel as in Figure 2a. (b) Corresponding autoradiogram.

(as observed by electron microscopy), which are presumably manifestations of subdivision (Goldspink, 1970, 1971; Shear, 1978).

Discussion

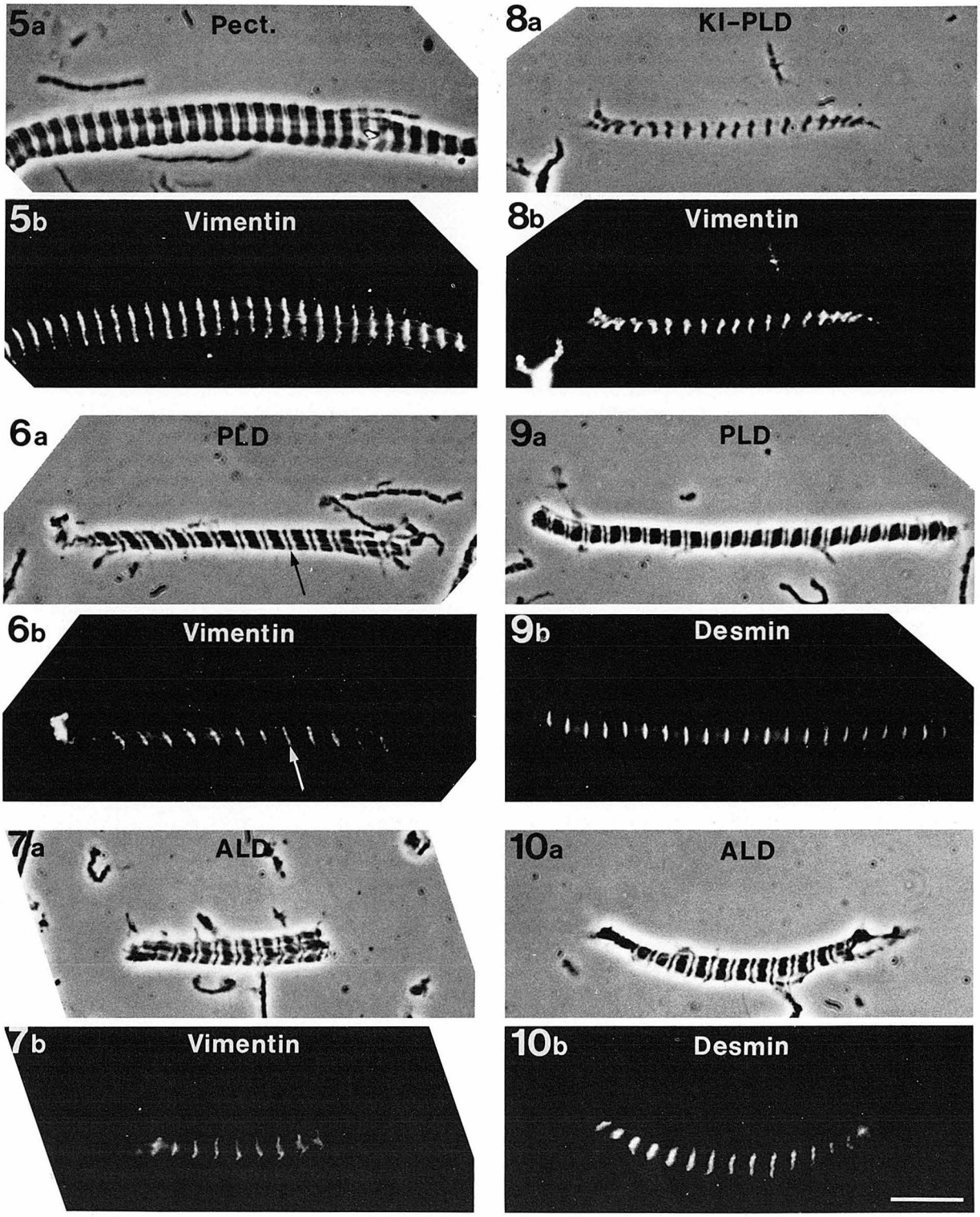
Existence of Intermediate Filaments in Striated Muscle

The recognition of a third class of filaments distinct from the thin (actin) and thick (myosin) filaments prevalent in the cytoplasm of developing muscle (Ishikawa, Bischoff and Holtzer, 1968; Kelly, 1969; Rash, Biesele and Gey, 1970a; Rash, Shay and Biesele, 1970b) has led to a number of papers on the morphological characterization and distribution of these filaments. Electron microscopic evidence from developing newt skeletal muscle initially suggested that these filaments do not have a direct role in myofibrillogenesis, but become deposited as transverse supportive elements at the level of the Z lines (Kelly, 1969). This view is supported by studies of mature frog lymph heart (Lindner and Schaumburg, 1968) and mammalian heart (Sandborn et al., 1967; Virágh and Challice, 1969; Ferrans and Roberts, 1973; Behrendt, 1977; Junker and Sommer, 1977; Eriksson and Thornell, 1979), which show networks of filaments surrounding and presumably interconnecting adjacent Z discs. These filaments are not as apparent in mature skeletal muscle, where convincing ultrastructural evidence of their existence in association with the peripheries of Z discs has come only from chicken ALD (Page, 1969). A similar system seems to exist in the flight muscles of the bee (Ernst and Garamvölgyi, 1956; Garamvölgyi, 1962, 1965).

It is not clear why these filaments are less apparent in skeletal muscle than in cardiac muscle, but it may be due to the much tighter packing of myofibrils and membranous organelles in skeletal muscle. If the desmin and vimentin, which we propose are the components of these filaments, are indeed in a predominantly filamentous form, they may be obscured by close association with the sarcoplasmic reticulum and T system membranes that ensheath each myofibril. Cardiac muscle lacks this regularity of cytoplasmic membranes; in addition, the myofibrils are often separated by greater distances, so that filaments spanning adjacent Z discs might be obscured less frequently by membranes.

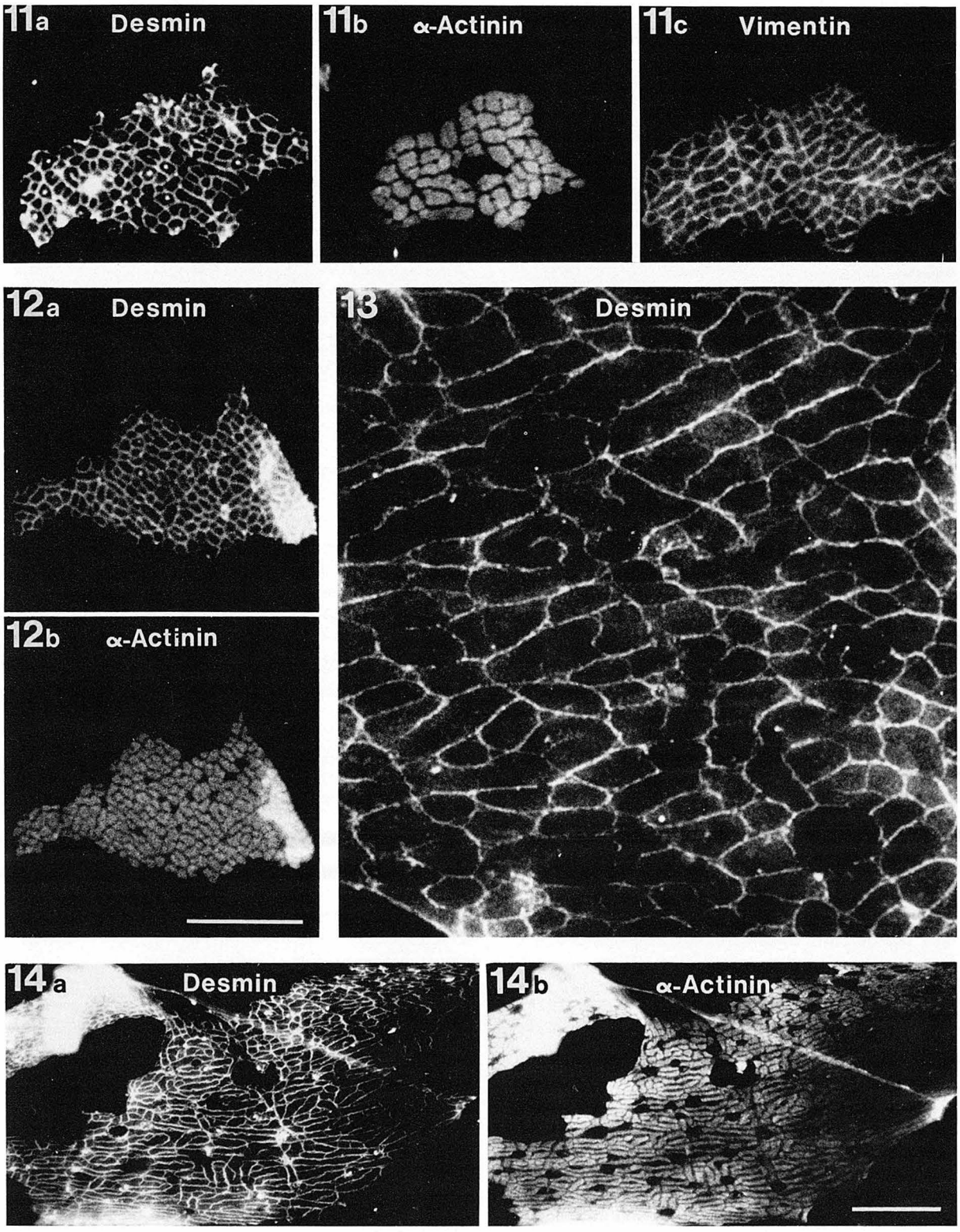
The biochemical and immunological characterization of intermediate filaments from avian muscle (Lazarides and Hubbard, 1976; Small and Sobieszek, 1977; Hubbard and Lazarides, 1979) allowed immunofluorescent localization of their major subunit (desmin) at the periphery of myofibril Z discs (Granger and Lazarides, 1978). This suggested that the transverse filaments observed near the Z lines were indeed intermediate filaments composed of desmin. It also permitted more extensive visualization of the range of intermediate filaments, which appears to be continuous throughout the Z plane.

In this paper we show that not only desmin is found surrounding the Z discs, but that vimentin, the major subunit of intermediate filaments of most cells of mesenchymal origin, is present as well. As desmin and vimentin filaments seem to be morphologically indistinguishable, the transverse filaments observed in electron micrographs must be composed of both desmin and vimentin (see below). It is known that during skeletal myogenesis, desmin and vimentin exist as seemingly random cytoplasmic filaments without obvious connections to other organelles (Ishikawa et al., 1968; Gard and Lazarides, 1980). In mature muscle, however, both molecules are found surrounding the Z discs. A molecular modification or maturation process must take place which causes desmin and vimentin to travel to the Z disc during development. Recent im-



Figures 5–10. Indirect Immunofluorescence of Myofibrils Using Anti-vimentin and Anti-desmin Antisera

(a) Phase-contrast image; (b) fluorescence image. The muscles from which the myofibrils were obtained and the antisera used are as indicated. Arrow denotes position of myofibril Z line. (Pect) Pectoralis major; (PLD) posterior latissimus dorsi; (ALD) anterior latissimus dorsi; (KI) potassium iodide extracted. Bar = 10 μm.



Figures 11–14. Immunofluorescence of Z Disc Sheets

Antisera used for staining are as indicated. Figures 12 and 14 are Z disc sheets that have been doubly labeled with antisera to desmin (a) and α–actinin (b). Figures 13 and 14 show Z disc sheets that have been stretched manually. Figures 11–13 are all at the same magnification; bar in Figure 12b = 10 μm. Bar in Figure 14 = 20 μm.

munofluorescence studies clearly show a redistribution of these antigens as myotubes differentiate into contractile fibers (Gard and Lazarides, 1980). If desmin and vimentin are chemically modified to effect this change, the alteration must be subtle: the existing and newly synthesized molecules maintain their same molecular weights and isoelectric points (as judged by IEF/SDS-PAGE) before, during and after the transition period (Gard and Lazarides, 1980).

Function of Muscle Intermediate Filaments

Proposed functions of intermediate filaments have all been based on their cytoplasmic distribution. No enzymatic activity has been assigned to them, despite the fact that they can be phosphorylated (O'Connor et al., 1979). Several lines of evidence suggest that they may have a cytoskeletal role in muscle: their deployment around Z discs in striated muscle (as discussed in this paper), their insertion into smooth muscle dense bodies (Uehara, Campbell and Burnstock, 1971; Ashton, Somlyo and Somlyo, 1975; Cooke, 1976) and their association with cardiac intercalated discs (Virágh and Challice, 1969; Ferrans and Roberts, 1973; Lazarides and Hubbard, 1976; Oliphant and Loewen, 1976; Behrendt, 1977). The seemingly random disposition of intermediate filaments in undifferentiated cells and during the early stages of myogenesis makes functional assignments more speculative.

We previously suggested that skeletal muscle intermediate filaments mechanically integrate and unify the contractile actions of each fiber by linking myofibrils laterally at their Z discs and by linking the Z discs to the plasma membrane (Lazarides and Hubbard, 1976; Granger and Lazarides, 1978). These filaments may thus be responsible for bringing about the characteristic cross-striated appearance of muscle.

The presence of desmin around the Z discs originally suggested that desmin might interact with or be closely associated with the transverse tubular (T) system (Granger and Lazarides, 1978; Lazarides and Granger, 1978). In the course of investigating additional types of muscle fiber, we have found that both desmin and vimentin always seem to be present along the Z line regardless of the position of the T system. For example, the PLD muscle of the chicken has two triads (junctional complexes of T system and sarcoplasmic reticulum) per sarcomere, one near each A-I boundary in relaxed muscle (Page, 1969); immunofluorescence reveals desmin and vimentin along the Z line, but they are not coincident with the T system. Either there is not a general one-to-one correspondence or necessary association between intermediate filaments and T tubules, or PLD is anomalous in this respect, desmin and vimentin are antigenically masked at these positions, or this material is lost in the preparation of myofibrils. Alternatively, there may be an interaction between intermediate filaments at the Z line and the sarcoplasmic reticulum that is present at that level in PLD.

Vertebrate slow muscle is characterized by poorly defined myofibrils, an irregularly developed T system and a Z line that zigzags across the fiber (see Page, 1969; Hess, 1970). We have determined that both desmin and vimentin are present along the Z line in chicken slow muscle (ALD). Even though the Z line of ALD does not follow a straight line across the fiber as it does in twitch muscle, there is still a high degree of lateral registry of adjacent sarcomeres. This level of intracellular order may well be based on a filamentous network of desmin and vimentin within each Z "plane" that is easily stretched or deformed, as suggested by Figure 13. The regular occurrence of desmin and vimentin at the Z lines, together with the irregular nature of the T system in ALD, suggests again that there is no invariant relationship between the transverse filaments and cytoplasmic membranes, although such relations may exist. For example, the coincident appearance of desmin, vimentin and membranes in the fissures of subdividing myofibrils (see Results) implies some degree of interaction between these two systems. Whatever the relationship may be between desmin and vimentin and the margin of the Z disc, the presence of these two proteins at that location seems to be a generalized feature of different muscle types.

Desmin- and vimentin-containing filaments cannot be distinguished morphologically or by their response to colcemid. Using immunofluorescence techniques, we have determined that they have coincident distributions at all stages of skeletal muscle development (Gard and Lazarides, 1980). Due to the limited resolution of immunofluorescence, it is not clear whether desmin and vimentin co-polymerize into a single set of filaments or whether they exist as distinct populations of similarly distributed filaments. If they do co-polymerize, then the stoichiometry of association may be variable; the ratio of vimentin to desmin, as observed on two-dimensional gels, declines as the fiber matures. Desmin and vimentin are present in comparable amounts during early myogenesis (Gard and Lazarides, 1980), but desmin predominates over vimentin in mature muscle (see Figure 1). The extreme susceptibility of these molecules (particularly vimentin) to proteolysis, however, precludes accurate quantitation. Indeed, this may explain why only low proportions of vimentin were observed (in retrospect) on gels of Z disc scaffolds that had undergone prolonged extraction (Granger and Lazarides, 1978). From the data presented here, we suggest that all of the functions originally proposed for desmin in skeletal muscle (Lazarides and Hubbard, 1976; Granger and Lazarides, 1978; Lazarides, 1979) be extended to include vimentin.

It is unclear why both molecules are needed at the periphery of the Z discs. If they are co-polymerized into one set of filaments, this set could perform either

a single function or different functions at different points along its length. If discrete desmin and vimentin filaments coexist in the inter-Z disc region, they could perform either subunit-specific functions or the same generalized functions. To reiterate, we propose that they form a transverse network within each Z plane and are responsible for the lateral linkage and registration of myofibrils; such a system would effect mechanical integration of the muscle fiber and provide a molecular basis for its striated phenotype. The frequent close association of this network with cytoplasmic membranes suggests a possible role in the positioning of these systems along the myofibril, and maintenance of positional relationships during contraction.

While this paper was being written, it was reported that vimentin disappears during early skeletal myogenesis in vitro (Bennett et al., 1979). These data were based solely on immunofluorescence, and are in conflict with our findings. It is unclear why this discrepancy exists, but we feel that the data presented in this paper and studies on the synthesis and deployment of vimentin and desmin during myogenesis (Gard and Lazarides, 1980) clearly demonstrate the presence of vimentin in mature muscle fibers.

Experimental Procedures

Sample Preparation

Strips of muscle were tied to wooden applicator sticks, immersed in a solution of 50% glycerol, 50% PBSA [163 mM Na^+, 140 mM Cl^-, 4 mM K^+, 10 mM PO_4^{--}, 10 mM N_3^- (pH 7.4)], 1 mM EGTA, 0.5 mM 0-phenanthroline, and stored for 1–6 months at −15°C. Myofibrils were prepared by blending in the glycerol solution on ice for 5 min in a Lourdes MM-1B homogenizer.

Glass coverslips spread with a drop of myofibril suspension and immediately rinsed had large numbers of adherent myofibrils on which immunofluorescence could be performed. Extraction was performed by immersion of these coverslips in a 0.6 M KI solution (see below) for 15 min.

Z disc sheets were prepared by first extracting small tied strips of glycerinated muscle (peroneus longus or iliotibialis) with 0.6 M KI, 10 mM $Na_2S_2O_3$, 20 mM Tris-Cl (pH 7.5), 0.1 mM EGTA, 0.1 mM 0-phenanthroline for about 1 week at 4°C or 1 day at room temperature. Subsequent blending in the KI solution in a Virtis "45" microhomogenizer produced Z disc sheets that would adhere tenaciously to glass coverslips. A drop of homogenate would normally be placed on a coverslip and then immediately rinsed off. Alternatively, the drop could be smeared onto the coverslip with the side of the tip of a Pasteur pipette; this would result in a large proportion of the sheets being stretched to several times their original size.

For two-dimensional gels, myofibrils were washed three times (by pelleting and resuspension) in 20 mM sodium phosphate (pH 7.2), 0.1 mM EGTA, 0.1 mM 0-phenanthroline, 10 mM NaN_3, 1% NP40. The final pellets were resuspended in the same solution containing 55% (w/w) sucrose, layered on top of the same solution containing 57% (w/w) sucrose and centrifuged for 2 hr at 240,000 × g. The interface material was collected, washed, extracted for 1 hr in the 0.6 M KI solution, washed with water and solubilized in 8 M urea, 2% NP40, 0.5% 2-mercaptoethanol for application to the isoelectric focusing gel.

Immunofluorescence

All immunofluorescence was performed on myofibrils and Z disc sheets adhering to glass coverslips. Incubations and washes were performed in either PBSA or in PBSA containing 0.5% Triton X-100 and 0.1% SDS, which gave the same results but generally resulted in cleaner backgrounds. Incubations with primary antisera were typically for 1 hr at 37°C; incubations with fluorescein-conjugated goat anti-rabbit IgG (Miles Laboratories) were for ½ hr at 37°C. Washes were for ½–2 hr at room temperature. Coverslips were mounted with Elvanol or 50% glycerol and examined with a Leitz phase/fluorescence microscope using a 100X oil immersion lens and filter module H or K (or N2 for rhodamine fluorescence). Photographs were taken on Kodak Tri-X film and developed in Diafine. Rhodamine B (Sigma)-conjugated anti-α-actinin IgG was prepared essentially by the method of Cebra and Goldstein (1965), as described (Gard and Lazarides, 1980).

Immunodiffusion

Meloy precast 1% agarose Ouchterlony plates were equilibrated in 120 mM NaCl, 20 mM Tris-Cl (pH 8.0), 0.1 mM EGTA, 0.1 mM 0-phenanthroline, 10 mM NaN_3, 0.5% Triton X-100, 0.1% SDS. For antigen, leg and breast muscle from a 12 day chick embryo was homogenized in either the above buffer (except 5 mM EGTA, 1 mM 0-phenanthroline and 1 mM PMSF) or this buffer with 4 M urea, and clarified by centrifugation. Diffusion was carried out at 4°C for 3 days; the gel was washed in the above buffer for 1.5 days, then stained for 1 hr with Coomassie blue and destained as described below for polyacrylamide gels.

Polyacrylamide Gel Electrophoresis

One-dimensional SDS-PAGE for antigen preparation was based on the method of Laemmli (1970), and two-dimensional IEF/SDS-PAGE was performed according to the method of O'Farrell (1975), each as modified and described by Hubbard and Lazarides (1979). Analytical gels contained 12.5% acrylamide and 0.1% N,N′-methylene bisacrylamide, and were 1.6 mm thick, 14 cm wide and 10 cm long.

Immunization

The production of rabbit antiserum against chicken smooth muscle desmin has been described (Lazarides and Hubbard, 1976). A similar procedure was used for vimentin. Antigen was prepared from Triton-KCl cytoskeletons of embryonic chicken muscle: leg and breast muscle from 14–16 day old embryos was dissected out and homogenized in a Dounce homogenizer in 100 mM KCl, 50 mM Tris-Cl (pH 8.0), 5 mM EGTA, 5 mM 2-mercaptoethanol, 1 mM 0-phenanthroline, 0.5 mM PMSF. The muscle homogenate was washed (by pelleting and resuspension) twice in this buffer, filtered through a 60 mesh screen, and then extracted three times in 0.6 M KCl, 2 mM $Na_4P_2O_7$, 0.5% Triton X-100, 20 mM Tris-Cl (pH 7.5), 1 mM EGTA, 10 mM 2-mercaptoethanol, 0.5 mM 0-phenanthroline, 0.1 mM PMSF. The final insoluble residue was washed with water, dissolved in hot solubilization buffer [2% SDS, 50 mM Tris-Cl (pH 6.8), 0.5% 2-ME, 10% glycerol, bromophenol blue] and loaded onto preparative slab gels.

Bands were visualized by staining for 15 min in 0.25% Coomassie brilliant blue R-250, 47.5% ethanol, 10% acetic acid and destaining for 1–2 hr in 12.5% ethanol, 5% acetic acid. The vimentin band was carefully excised with a razor blade, cut into small cubes and neutralized for 5–10 min in 0.15 M sodium phosphate (pH 7.4). The gel was homogenized in a motor-driven Teflon/glass homogenizer; after adding a few drops of 10% $AlCl_3$ and 0.5–1 vol of adjuvant (Freund; Difco), the mixture was emulsified by repeated passage between two syringes. Subcutaneous dorsal injections in a female New Zealand white rabbit were made with complete adjuvant on days 0 and 14, and with incomplete adjuvant on days 28, 47, 65 and 106. 200–300 μg of protein were injected at two sites each time, in a volume of 5–10 ml. Blood was collected from the marginal ear vein on days 34, 40, 53, 72, 82, 94 and 112. After clot formation and contraction, the serum was clarified by centrifugation; gamma globulins were partially purified by precipitation at 0°C with ammonium sulfate at 50% saturation, dialyzed against 130 mM NaCl, 5 mM KCl, 10 mM Tris-Cl (pH 8.0), 10 mM NaN_3, 1 mM ε-amino-n-caproic acid (to inhibit plasmin activity), and stored in liquid nitrogen. Sera from the fourth and fifth bleeds were used in this study; both gave single precipitin lines in double immunodiffusion (performed as described above) and identical

results in immunoautoradiography.

Antiserum to α-actinin was raised against purified porcine skeletal muscle α-actinin as described (Lazarides and Burridge, 1975; Lazarides, 1976).

Immunoautoradiography

Leg and breast muscle of one 14 day chick embryo was disrupted in a Dounce homogenizer in 4 ml of 20 mM Tris-Cl (pH 8.0), 5 mM EGTA, 1 mM 0-phenanthroline, 0.5 mM PMSF, and spun for 1 hr at 150,000 × g. The supernatant was made 10 M in urea, 2% in NP40 and 0.5% in 2-ME, and 75 μl were loaded on each of the two-dimensional gels.

After electrophoresis the gels were fixed for 6 hr in 50% ethanol, 10% acetic acid (all incubations were carried out in Pyrex baking dishes rocking at room temperature), and then neutralized for 1 day in several changes of 50 mM Tris-Cl (pH 7.5), 100 mM NaCl, 10 mM NaN_3. After another day of washing in buffer I [10 mM Tris-Cl (pH 7.5), 140 mM NaCl, 10 mM NaN_3, 0.1% gelatin (Difco; dissolved by heating)], the gels were incubated for 1 day in 100 ml of buffer I to which 100 μl of antiserum had been added (1:1000 dilution). This was followed by three days of washing in several changes of buffer I and then a 1 day incubation in 100 ml of buffer I containing ^{125}I-protein A (see below). 2 days of washing in buffer I and 1 day in buffer I without the gelatin were followed by staining in 0.1% Coomassie blue, 47.5% ethanol, 10% acetic acid and destaining in 12.5% ethanol, 5% acetic acid. The gels were dried, photographed and then autoradiographed for 15-25 hr at −70°C on Kodak X-Omat R XR5 film with a DuPont Cronex Lightning-Plus intensifying screen; autoradiograms were developed in Kodak X-ray developer.

Protein A (Pharmacia) was iodinated essentially by the chloramine T method of Greenwood, Hunter and Glover (1963), except that the reaction was terminated by adding an excess of tyrosine. 100 μl of 0.5 M potassium phosphate (pH 7.5) were added to 1 mCi of Na^{125}I [ICN; in 2 μl of NaOH (pH 8.8)]; 20 μl of protein A (5 mg/ml) and 20 μl of chloramine T (2.5 mg/ml) were added next. After 2 min, 150 μl of tyrosine (0.4 mg/ml) were added. The mixture was passed centrifugally through a 3 ml bed of Sephadex G-25 and the void fraction was used for labeling. Specific activities on the order of 0.1 μCi/μg protein were obtained, and each gel was incubated with approximately 1 μCi.;1;;;1;

Acknowledgments

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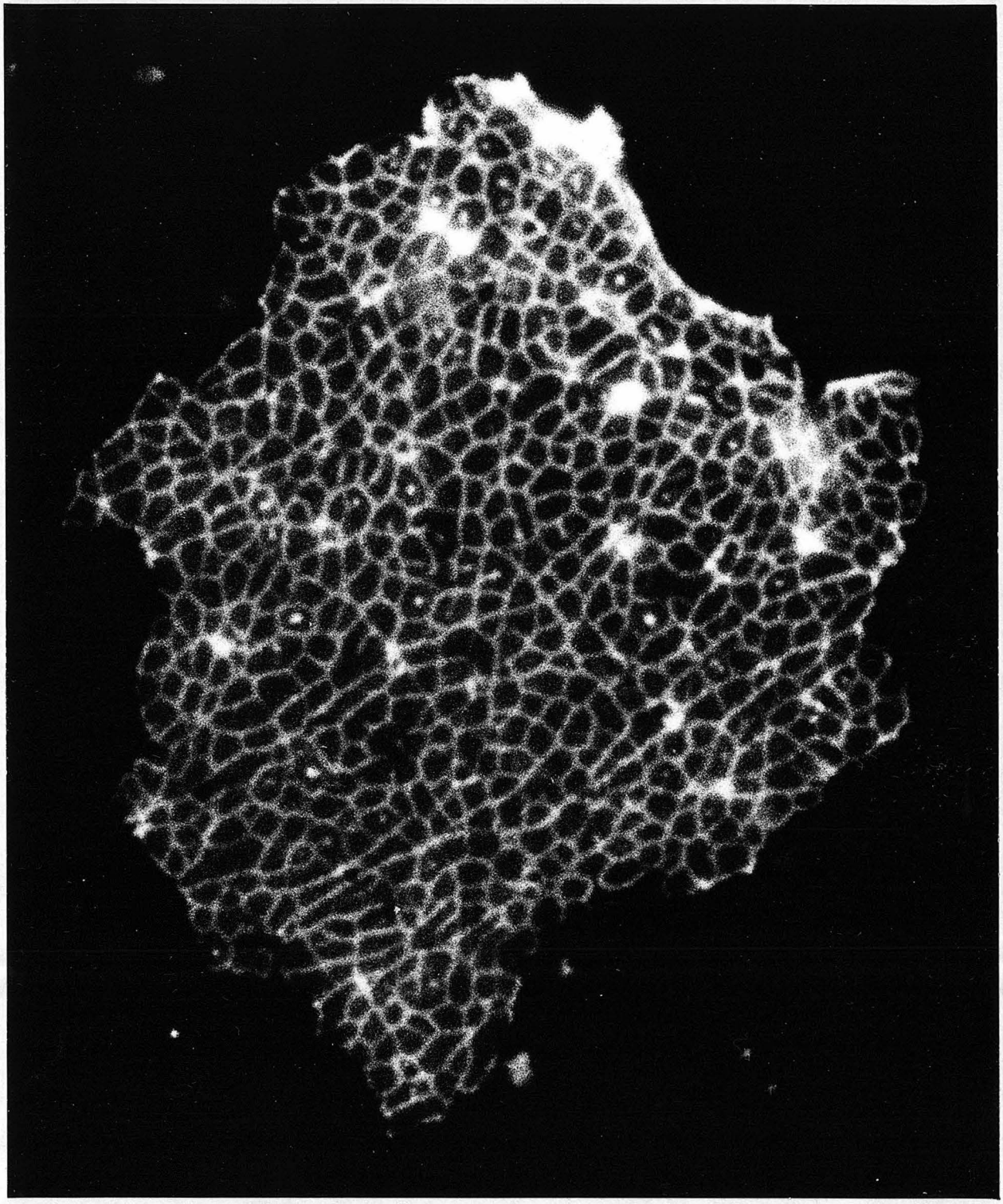

Most spectacular example of desmin immunofluorescence of a Z-disc sheet observed in these studies. Contains over 500 Z-discs. Photographed 3 July 1979. Appeared in Nature 17 January 1980 (Vol. 283, No. 5744). Magnification 4350X.

Chapter 4

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Synemin: a New High Molecular Weight Protein Associated with Desmin and Vimentin Filaments in Muscle

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Summary

A 230,000 dalton polypeptide co-purifies through cycles of depolymerization and polymerization with the intermediate filament subunits, desmin and vimentin, from avian smooth muscle. This protein is also present in skeletal muscle and is distinct from myosin and filamin. Double immunofluorescence microscopy of cultured cells, using antisera shown to be specific by immunoautoradiography, has revealed that this protein has the same spatial distribution as desmin and vimentin. During skeletal myogenesis, all three antigens exist initially in multinucleate myotubes as wavy filaments throughout the cytoplasm. Within a week after myoblast fusion, they begin to coalesce at the peripheries of the myofibril Z discs, thereby attaining the distribution observed in mature muscle, a network of interlinked rings within the Z plane. Treatment of cultured myotubes with colcemid causes the filamentous forms of these three proteins to co-aggregate into cytoplasmic bundles, but has little effect on them when they are associated with the Z discs. Extraction of cells with nonionic detergent and high salt leaves cytoskeletons containing desmin, vimentin and the 230,000 dalton polypeptide with immunofluorescent patterns that are indistinguishable from one another. These data suggest that this high molecular weight protein is closely associated with desmin and vimentin filaments in muscle cells; to indicate this, we have named the protein synemin, from the Greek σύν (with) and νήμα (filament).

Introduction

Cytoplasmic filaments with diameters of 7–12 nm are widespread in higher eucaryotes and are collectively termed intermediate filaments. Although similar in morphology, they are quite heterogeneous by chemical and immunological criteria (see review by Lazarides, 1980). In muscle cells, these filaments are intermediate in diameter between the contractile thin (actin) and thick (myosin) filaments (Ishikawa, Bischoff and Holtzer, 1968; Kelly, 1969; Rash, Biesele and Gey, 1970; Campbell et al., 1971). Initial biochemical investigations showed that desmin, the major subunit of intermediate filaments in muscle, has a molecular weight of approximately 50,000 (Cooke, 1976; Lazarides and Hubbard, 1976; Small and Sobieszek, 1977). Immunological characterization of desmin led to the demonstration of its presence along the Z lines of skeletal myofibrils, where it appeared to function in linking adjacent myofibrils to each other and to the sarcolemma (Lazarides and Hubbard, 1976). This proposal was substantiated by the observation that sheets of interconnected Z discs could be isolated mechanically from skeletal muscle fibers from which actomyosin had been extracted with high salt (Granger and Lazarides, 1978). Each sheet was derived from a plane of laterally registered myofibril Z discs; immunofluorescence showed that desmin was present at the periphery of each Z disc and thus formed a network of interlinked collars within each Z plane.

A protein with a molecular weight of 52,000 and an isoelectric point slightly more acidic than that of desmin was characterized as the major subunit of intermediate filaments from a variety of fibroblast-like cells (Brown, Levinson and Spudich, 1976; Hynes and Destree, 1978a; Gard, Bell and Lazarides, 1979). This protein, named vimentin (Franke et al., 1978), is similar to desmin in that both remain insoluble in solutions containing nonionic detergent and high salt; similarities in peptide maps suggest that desmin and vimentin may be evolutionarily related (Gard et al., 1979) even though they are antigenically distinct (Lazarides and Hubbard, 1976; Lazarides, 1978; Bennett et al., 1978a; Granger and Lazarides, 1979).

It has recently been demonstrated that both desmin and vimentin are present in both muscle and nonmuscle cells (Gard et al., 1979), and that both molecules co-purify through cycles of solubilization and precipitation from smooth muscle (this paper). During early skeletal myogenesis, desmin and vimentin exist as random cytoplasmic filaments, but move to the periphery of the myofibril Z disc as maturation proceeds (Granger and Lazarides, 1979; Gard and Lazarides, 1980). Throughout myogenesis, the distributions of desmin and vimentin are indistinguishable by immunofluorescence microscopy. It has not been determined however, whether the two molecules co-polymerize into the same filaments or whether they form separate filaments with identical cytoplasmic localizations.

In this paper we report the existence in avian muscle of a third intermediate filament protein. A 230,000 dalton polypeptide, named synemin, co-purifies through cycles of depolymerization and polymerization with desmin and vimentin from smooth muscle. Double immunofluorescence microscopy reveals that the spatial distribution of synemin throughout the course of skeletal myogenesis and in mature myofibrils is indistinguishable from that of desmin and vimentin. This holds true even when the distribution of intermediate filaments is altered with the drug colcemid, or when cells are treated with nonionic detergent and high salt.

Results

Purification of Desmin and Associated Polypeptides

Purification of desmin from chicken gizzard smooth muscle was based on the procedure of Hubbard and Lazarides (1979). The final step involves extraction of protein from muscle cytoskeletons with acetic acid, followed by precipitation of the protein upon neutralization. This cycle of solubilization and precipitation, or depolymerization and polymerization, can be repeated several times. As assayed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), the result is a preparation consisting primarily of desmin with small amounts of actin. Overloading of SDS gels, however, reveals the presence of a large number of minor components. Figure 1 shows an overloaded two-dimensional isoelectric focusing (IEF) SDS-polyacrylamide gel of such a preparation. In addition to desmin (50,000 daltons) and actin (43,000 daltons), a minor component more acidic and slightly larger (52,000 daltons) than desmin has been identified as vimentin (Gard et al., 1979).

A high molecular weight polypeptide (230,000 daltons), which we have named synemin, is also present (see also Figure 2, lanes 2). Synemin is invariably present with desmin and vimentin after cycles of polymerization and depolymerization of gizzard intermediate filament proteins. Densitometric scans of one-dimensional gels stained with Coomassie blue suggest a molar ratio of synemin to desmin of 1–2%; visual inspection of published gels (Hubbard and Lazarides, 1979) suggests that this ratio does not change appreciably through four cycles of depolymerization and polymerization. Synemin is distinct from myosin (200,000 daltons) and filamin (250,000 daltons) and migrates midway between the two variants of human erythrocyte spectrin (220,000 and 240,000 daltons) on SDS gels (not shown).

On isoelectric focusing gels, synemin does not focus with a discrete isoelectric point, but focuses as a streak of variants primarily in the pH range of 5.6–6.1 (see Figure 3). On overloaded two-dimensional gels, as in Figure 1, synemin focuses discretely with several variants of desmin more basic than β-desmin. This pattern is quite reproducible, but is believed to be an artifact of loading excessive amounts of protein on the IEF gel, and is anomalous for both desmin and synemin. The proteins appearing as vertical arrays of spots in the second dimension SDS gel are present as very sharp bands in the IEF gel; if these are cut from an IEF gel and rerun individually on a new set of two-dimensional gels, each band electrophoreses as α– and β–desmin with synemin directly above. This rules out the possibility that desmin and synemin are focusing as complexes with isoelectric points more basic than that of β–desmin. The presence or absence of disulfide reducing agents, either in the original sample or prior to running the second dimension, has no effect on the final pattern, showing that disulfide-linked aggregates are not forming in the nonreducing matrix of the IEF gel.

Immunoautoradiographic Characterization of Anti-synemin

The co-purification of synemin with desmin and vimentin suggested that synemin may be associated in some way with intermediate filaments. For this reason, antiserum specific for synemin was produced to investigate the distribution of synemin in muscle by immunofluorescence. Antigen was obtained in pure form for immunization by excision from preparative SDS-polyacrylamide slab gels of the 230,000 dalton polypeptide present in preparations of desmin that had been through two or three cycles of solubilization and precipitation. The antiserum obtained was assayed for specificity by immunoautoradiography (Burridge, 1976, 1978), as described previously (Granger and Lazarides, 1979).

A comparison of the Coomassie blue-stained gel (Figure 2a) with its corresponding autoradiogram (Figure 2b) shows that anti-synemin reacts with a 230,000 dalton protein in gizzard smooth muscle (lanes 2 and 4), adult skeletal myofibrils (lanes 3 and 5) and whole extracts of cultured embryonic myotubes (lanes 6). Neither desmin nor vimentin reacts with this antiserum in any of these samples. Lanes 1 of Figure 2 show that synemin is not detectable in preparations of, and is not crossreactive with, chick tendon collagen. This latter sample was included in the assay to ensure that synemin was not a form of collagen, since the gizzard acetone powders used for synemin isolations contain large amounts of collagen which may also be solubilized by acetic acid. Processing of an identical set of samples with the anti-synemin preimmune serum reveals no labeling of bands in the separating gel, although material at the top of the stacking gel in lanes 2 and 6 does label significantly (Figure 2c).

In preparations of acetic acid-cycled desmin (lanes 2), a number of lower molecular weight bands are labeled in addition to the 230,000 dalton synemin band. Many, if not all, of these bands are also present in a low ionic strength EGTA extract of smooth muscle (lanes 4). Most of them are present in concentrations too low to be detected readily by Coomassie blue staining, and they are most prevalent in preparations enriched in synemin. These bands are believed to be degradation products of synemin that are generated during the process of purification. These bands do not react with antibodies in the preimmune serum (Figure 2c), and could not have been contaminants of the material used for immunization, since the synemin used as antigen was purified twice by preparative SDS-PAGE. Furthermore, when special precautions are taken to minimize potential proteolysis of samples during processing for electrophoresis, labeling of

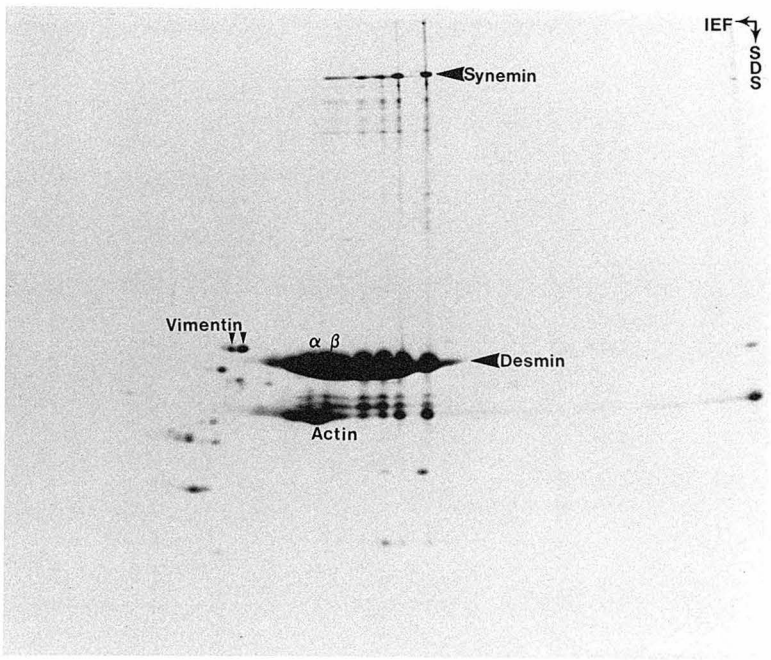

Figure 1. Two-Dimensional Electrophoresis of Smooth Muscle Intermediate Filaments

A preparation of chicken gizzard desmin was partially purified by two cycles of polymerization and depolymerization, and assayed by overloading a two-dimensional gel. Isoelectric focusing (IEF) was performed from right to left (basic to acidic), and SDS-polyacrylamide gel electrophoresis (SDS) was from top to bottom.

lower molecular weight bands is greatly reduced. This is evident in lanes 6, which contains cultured myotubes that were processed in such a manner (see Experimental Procedures); in this case, labeling of the lower molecular weight proteins is not detectable. Figure 3 shows a two-dimensional gel of a similar preparation of cultured skeletal myotubes; again, a band at 230,000 daltons is the predominant labeled protein. Desmin and vimentin do not label. Even in these samples, however, overexposed autoradiograms as in Figure 3 reveal the presence of an arc of slightly labeled peptides below and to the acidic side of synemin; this arc is more prevalent on immunoautoradiograms of two-dimensional gels as in Figure 1, and is evident by Coomassie blue staining of two-dimensional gels of electrophoretically pure synemin that has been partially proteolyzed with trypsin (not shown). It is noteworthy that this pattern is similar to the "diagonal protein" fragment pattern seen for both vimentin and desmin (Granger and Lazarides, 1979; Gard et al., 1979).

Whole skeletal myofibrils show no synemin by Coomassie blue staining; however, synemin exists and is detectable with anti-synemin immunoautoradiography (Figure 2, lanes 3). In myofibrils that have been extracted with 0.6 M KI to remove much of the actomyosin, synemin is enriched with respect to total protein and can be visualized with Coomassie blue staining as well as immunoautoradiography (Figure 2, lanes 5).

In none of these samples is either desmin or vimentin labeled with the anti-synemin antiserum. Conversely, using two-dimensional immunoautoradiography of an extract of embryonic chicken skeletal muscle, we have shown that anti-desmin and anti-vimentin do not label synemin (Granger and Lazarides, 1979). Gels of similar extracts show a 230,000 dalton streak as in Figure 3 when labeled with anti-synemin (not

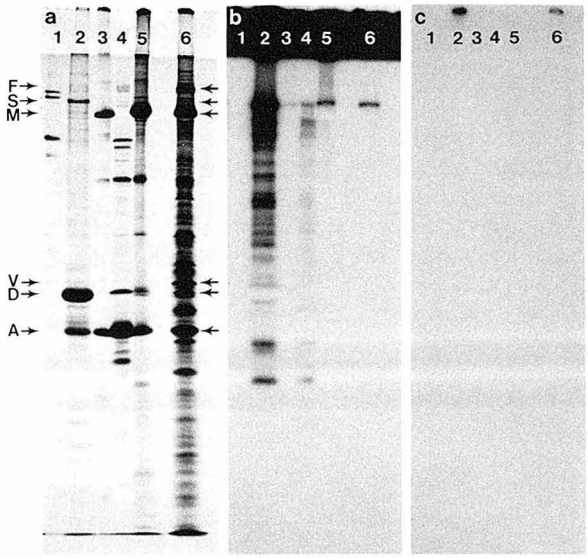

Figure 2. Immunoautoradiography Using Anti-synemin Antiserum

(a) Coomassie blue-stained, dried gel processed with anti-synemin and ^{125}I protein A; (b) corresponding autoradiogram; (c) autoradiogram of a similar gel processed with preimmune serum. (Lanes 1) Chicken tendon collagen; (lanes 2) preparation of chicken gizzard desmin after two cycles of polymerization and depolymerization; (lanes 3) skeletal myofibrils; (lanes 4) low ionic strength Tris/EGTA extract of chicken gizzard smooth muscle; (lanes 5) skeletal myofibrils after brief extraction with high salt; (lanes 6) whole SDS extract of cultured chicken embryonic skeletal myotubes. Identifiable proteins include filamin (F), synemin (S), myosin (M), vimentin (V), desmin (D) and actin (A).

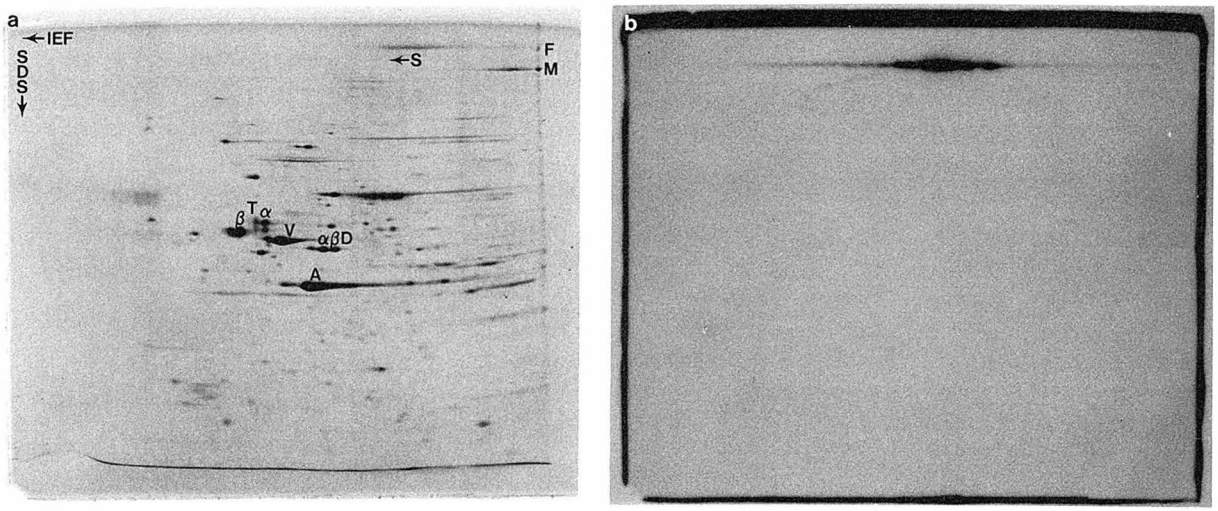

Figure 3. Two-Dimensional Immunoautoradiography Using Anti-synemin Antiserum

(a) Two-dimensional gel of whole SDS extract of cultured chicken embryonic myotubes, processed for immunoautoradiography; identified proteins are filamin (F), synemin (S), myosin (M), α- and β-tubulin (T), vimentin (V), α- and β-desmin (D) and actin (A). Synemin is barely visible on this gel. (b) Corresponding autoradiogram, showing specific labeling of synemin.

shown). It therefore appears that desmin, vimentin and synemin are antigenically distinct, and that the antisera used in the immunofluorescence study presented below reliably reflect the distributions of the corresponding proteins.

Synemin in Mature Skeletal Muscle

The spatial distribution of synemin in skeletal muscle was investigated by indirect immunofluorescence microscopy with the anti-synemin antiserum. Figure 4 shows that synemin is present along the Z lines of isolated myofibrils; this fluorescence pattern is the same as that observed for several other muscle antigens, including α-actinin (Lazarides and Burridge, 1975), desmin (Lazarides and Hubbard, 1976), vimentin (Granger and Lazarides, 1979) and filamin (Bechtel, 1979).

Synemin exhibits a Z line distribution in myofibrils from a variety of chicken skeletal muscle types, including anterior latissimus dorsi [a slow muscle with a zigzag Z line and irregular transverse tubular (T) system], posterior latissimus dorsi (a fast twitch muscle with T systems on either side of the Z line) and peroneus longus and pectoralis major muscles (oxidative and glycolytic twitch muscles with T systems at the Z lines).

The Z disc can be divided into two domains, as determined by the immunofluorescent localization of the various proteins in isolated Z disc sheets. The central domain contains actin and α-actinin, and the peripheral domain contains actin, desmin, vimentin and filamin (Granger and Lazarides, 1978, 1979; R. Gomer and E. Lazarides, manuscript submitted). The distribution of synemin with respect to these domains was also determined by immunofluorescence on isolated Z disc sheets (Granger and Lazarides, 1978). Figure 4c shows that synemin exists at the periphery of each Z disc and thereby forms a network of interlinked rings within the Z plane. This distribution is identical to the distributions of desmin and vimentin and complementary to that of α-actinin within a given Z plane (Granger and Lazarides, 1978, 1979).

Synemin retains its peripheral localization in Z discs that have been manually stretched prior to immunofluorescence (Figure 4d). The continuity of the fluorescence, also seen for desmin and vimentin (Granger and Lazarides, 1979), suggests that these molecules exist as components of a highly elastic system within the muscle fiber.

The specimens in Figure 4 were treated with nonionic detergent (a, b) or detergent and high salt (c, d); the fact that synemin remains associated with the Z discs after these treatments shows that it has solubility properties similar to those of desmin and vimentin, which exhibit the same distribution after similar treatments (Granger and Lazarides, 1979).

Synemin is occasionally present in foci or short segments in the interiors of Z discs, as shown in Figure 4c. Similar patterns are seen for both desmin and vimentin (Granger and Lazarides, 1979). These Z discs presumably correspond to those myofibrils undergoing longitudinal subdivision as a means of proliferation (Goldspink, 1970, 1971; Shear, 1978).

Synemin during Myogenesis in Vitro

Primary cultures of embryonic chicken skeletal muscle were used to investigate the role of synemin during myogenesis. In these cultures, mononucleate myo-

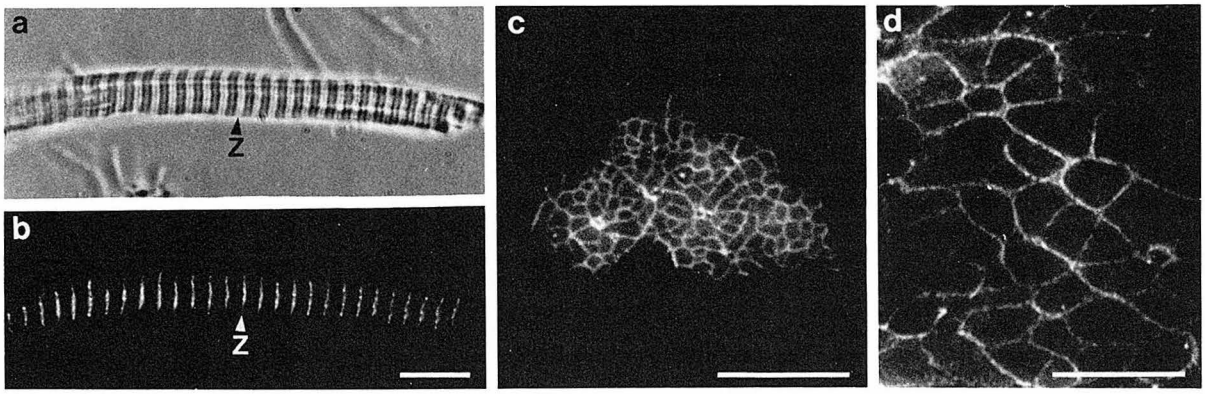

Figure 4. Immunofluorescent Localization of Synemin in Myofibrils and Z Disc Sheets

(a) Phase-contrast micrograph of a bundle of skeletal myofibrils; (b) indirect immunofluorescent image of this bundle labeled with anti-synemin. Only the Z lines (Z) are stained. (c) Fluorescence micrograph of an isolated Z disc sheet labeled with anti-synemin. The periphery of each Z disc is labeled. (d) Stretched Z disc sheet labeled with anti-synemin and printed at the same magnification as (c); again, the peripheries of the Z discs are labeled. Synemin is also present occasionally in foci or short segments in the interiors of Z discs. Bars represent 10 μm.

blasts fuse to form multinucleate myotubes within the first few days of plating. Myotubes assemble myofibrils and within a week are capable of twitching. α-Actinin begins to organize into Z lines by day 3; desmin and vimentin exist initially as seemingly random cytoplasmic filaments, but begin to coalesce at the Z lines by day 7, thereby establishing their final distribution at the peripheries of the Z discs in mature muscle (Granger and Lazarides, 1979; Gard and Lazarides, 1980).

Double immunofluorescence microscopy was performed at various times after plating to follow the distribution of synemin during myogenesis and to compare it with that of desmin and vimentin. Figure 5 is a set of low-power micrographs of a primary myogenic culture five days after plating. In the top row, the cells were doubly labeled with anti-synemin and anti-vimentin. Vimentin can be seen in all the cells in the culture (fibroblasts, myoblasts and myotubes); synemin is confined primarily to the myotubes, but is also present in an occasional myoblast. The lower row shows a different field stained with both anti-synemin and anti-desmin. In these photographs, synemin and desmin exhibit coincident distributions in the myotubes. Fibroblasts remain nonfluorescent, but mononucleate myoblasts occasionally show a positive stain for these two antigens.

It has previously been reported that desmin can be detected in post-mitotic myoblasts, but not presumptive myoblasts, by immunofluorescence (Bennett, Fellini and Holtzer, 1978b); only a small proportion of myoblasts stain positively for desmin and synemin, suggesting that these cells fuse into multinucleate myotubes soon after initiation of synthesis of these proteins. Whether or not synthesis of desmin and synemin is temporally coordinated has not been determined conclusively, due partially to the nonquantitative nature of immunofluorescence. At higher magnifications it is evident that synemin is present in early myotubes in the form of cytoplasmic filaments; this pattern is also characteristic of desmin and vimentin at this stage of development (Gard and Lazarides, 1980). By double immunofluorescence it is apparent that these three antigens exist in the same structures (see below).

If synemin is present in early muscle as wavy filaments throughout the cytoplasm, and in mature muscle at the periphery of the myofibril Z disc, then it must alter its distribution at some point during myogenesis, as do desmin and vimentin. Figure 6 shows a segment of a myotube after 35 days in culture. Well developed sarcomeres can be discerned within the cytoplasm by phase-contrast microscopy; indirect immunofluorescence shows that synemin is distributed predominantly along the Z lines. Examination of a series of earlier time points shows that synemin begins its transition to the Z line within a week of myoblast fusion, and that this coincides both spatially and temporally with the transition of desmin and vimentin to this structure (see Figures 7–9).

To compare the post-transition distributions of synemin, desmin and vimentin, and to determine whether synemin shares the solubility properties of desmin and vimentin in cultured cells, double immunofluorescence was performed on myotube cytoskeletons. Myotubes were extracted in a buffer containing 0.5% Triton X-100 and 0.6 M KCl to leave cytoskeletons (Brown et al., 1976; Gard et al., 1979), which were then fixed and incubated with the appropriate antisera. Figure 7 shows that synemin, along with desmin and vimentin, remains an insoluble cytoskeletal component. Both cytoplasmic filamentous and Z line-associated forms resist extraction with detergent and high salt. Comparison of the exact localizations of synemin with desmin and vimentin reveals no salient differences. At this level of resolution there appears to be an invariant correspondence in the distributions of these three antigens.

Intermediate filaments can be rearranged in the

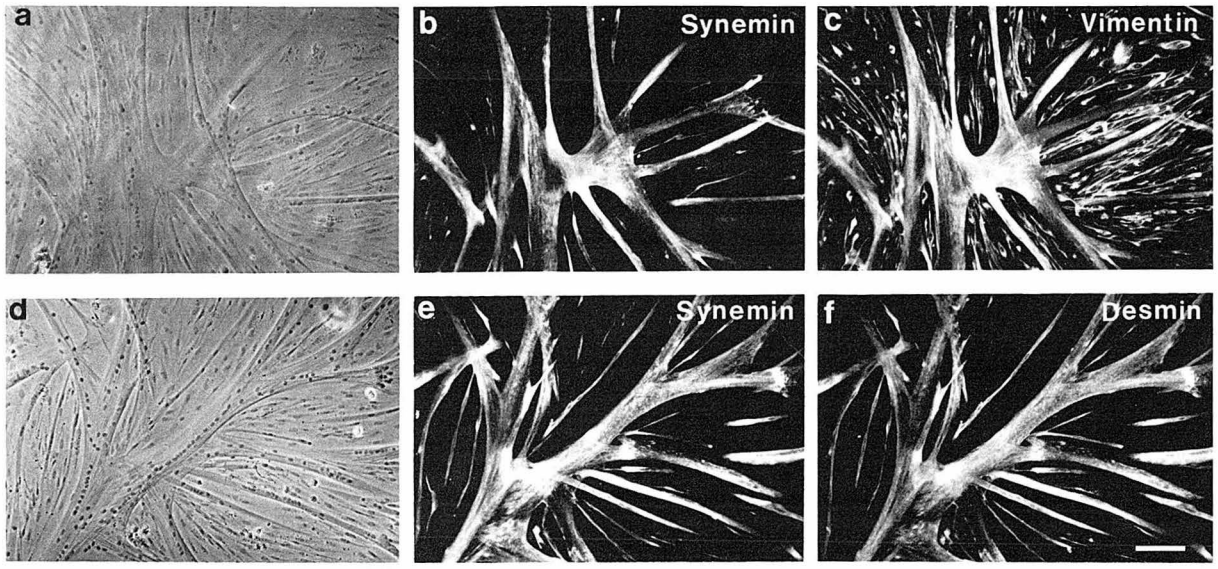

Figure 5. Double Immunofluorescence of 5 Day Myotubes

(a, d) Phase-contrast micrographs; (b, c, e, f) fluorescence micrographs of cells labeled with anti-synemin (b, e), anti-vimentin (c) or anti-desmin (f). The top row shows a field of cells doubly labeled to show the distributions of both synemin (b) and vimentin (c). The lower row compares synemin (e) and desmin (f). Desmin and synemin are found primarily in multinucleate myotubes, while vimentin is also found in fibroblasts. Bar represents 100 μm.

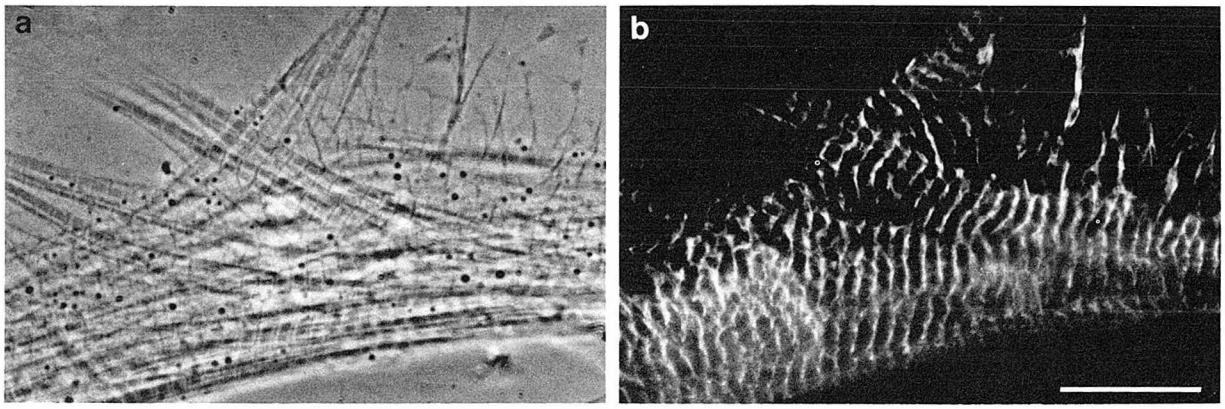

Figure 6. Localization of Synemin in a Fully Differentiated Myotube

(a) Phase-contrast micrograph of 37 day myotube, showing well developed sarcomeres; (b) fluorescence micrograph showing that synemin is present predominantly at the Z lines. Bar represents 20 μm.

cytoplasm of many cell types by treatment with the drug colcemid (Ishikawa et al., 1968). Exposure to 5 μM colcemid for 18 hr causes the filaments to aggregate into sinuous cytoplasmic bundles. In muscle, it has been shown that the cytoplasmic intermediate filament subunits desmin and vimentin co-aggregate after exposure to colcemid. Before transition to the Z line, they form thick cable-like accumulations in the sarcoplasm; after transition, the Z line-associated forms of desmin and vimentin resist rearrangement (Gard and Lazarides, 1980).

To determine whether the distribution of synemin could be affected by colcemid, we performed double immunofluorescence on cells treated with colcemid both before and after the Z line transition period. Figure 8a shows the result on 3 day myotubes; synemin co-aggregates with desmin and vimentin (shown only for desmin) into sarcoplasmic bundles. Figure 8b shows that in 37 day myotubes, the Z line-associated form of synemin resists this induced rearrangement, and its distribution again appears identical to that of both desmin and vimentin (shown only for desmin).

To ensure that each of the antisera used in these studies was reacting with its respective antigen and not crossreacting with either of the other two antigens of interest, the immunofluorescence and immunoau-

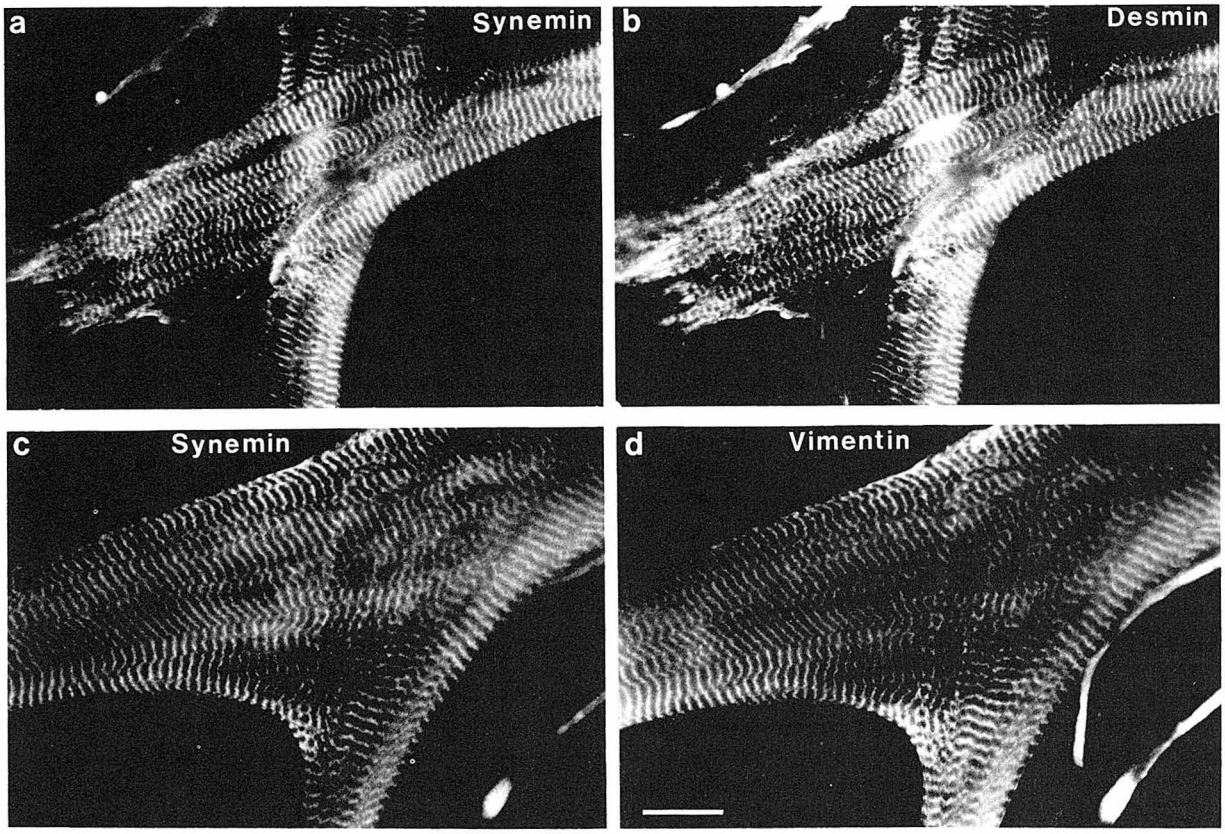

Figure 7. Double Immunofluorescence of Myotube Cytoskeletons

Nine day myotubes were extracted with 0.6 M KCl and 0.5% Triton X-100 prior to processing for immunofluorescence. Cells were doubly labeled for synemin and desmin (a and b) or synemin and vimentin (c and d). Bar represents 20 μm.

toradiographic results were supplemented with appropriate adsorption controls. Synemin, desmin and vimentin were purified by preparative SDS-PAGE; proteins were eluted from gel slices, dialyzed against distilled water and lyophilized. Antisera were mixed with portions of lyophilized pure protein and incubated at room temperature for 1 hr; the mixtures were centrifuged to remove large aggregates, and the supernatants were used on myotubes fixed for immunofluorescence. Figure 9 shows representative results. Adsorption of anti-synemin with purified synemin blocks fluorescence, but adsorption with purified desmin has no effect on the immunofluorescence observed. Conversely, anti-desmin is blocked by desmin but not by synemin. Identical results were obtained using vimentin instead of desmin (not shown): purified vimentin blocks anti-vimentin but not anti-synemin fluorescence, and purified synemin has no effect on anti-vimentin fluorescence. It thus seems highly unlikely that the synemin fluorescence observed is due to contamination of the synemin antiserum with anti-desmin or anti-vimentin antibodies, or due to antigenic crossreactivity between synemin, desmin and vimentin.

Preliminary data (not shown) suggest that rabbit anti-chicken synemin crossreacts little if at all with mammalian muscle. In this respect it is similar to several anti-desmins produced in this laboratory that are fairly specific for the avian antigen (Lazarides and Balzer, 1978; our unpublished observations). Isolation of a mammalian synemin will be useful for corroboration of its importance as an intermediate filament-associated protein.

Discussion

Characterization of Synemin

A 230,000 dalton polypeptide co-purifies through cycles of depolymerization and polymerization with desmin from chicken gizzard smooth muscle. Although present in small amounts relative to desmin, this protein seems to be an invariant component of such preparations; it has been visible but not studied in previous investigations of desmin (Hubbard and Lazarides, 1979). By electrophoretic and immunological criteria we have determined that the 230,000 dalton protein from chicken smooth muscle is antigenically crossreactive with, and has the same molecular weight as, a protein from skeletal muscle, and that this protein has a cytoplasmic distribution identical to that of the

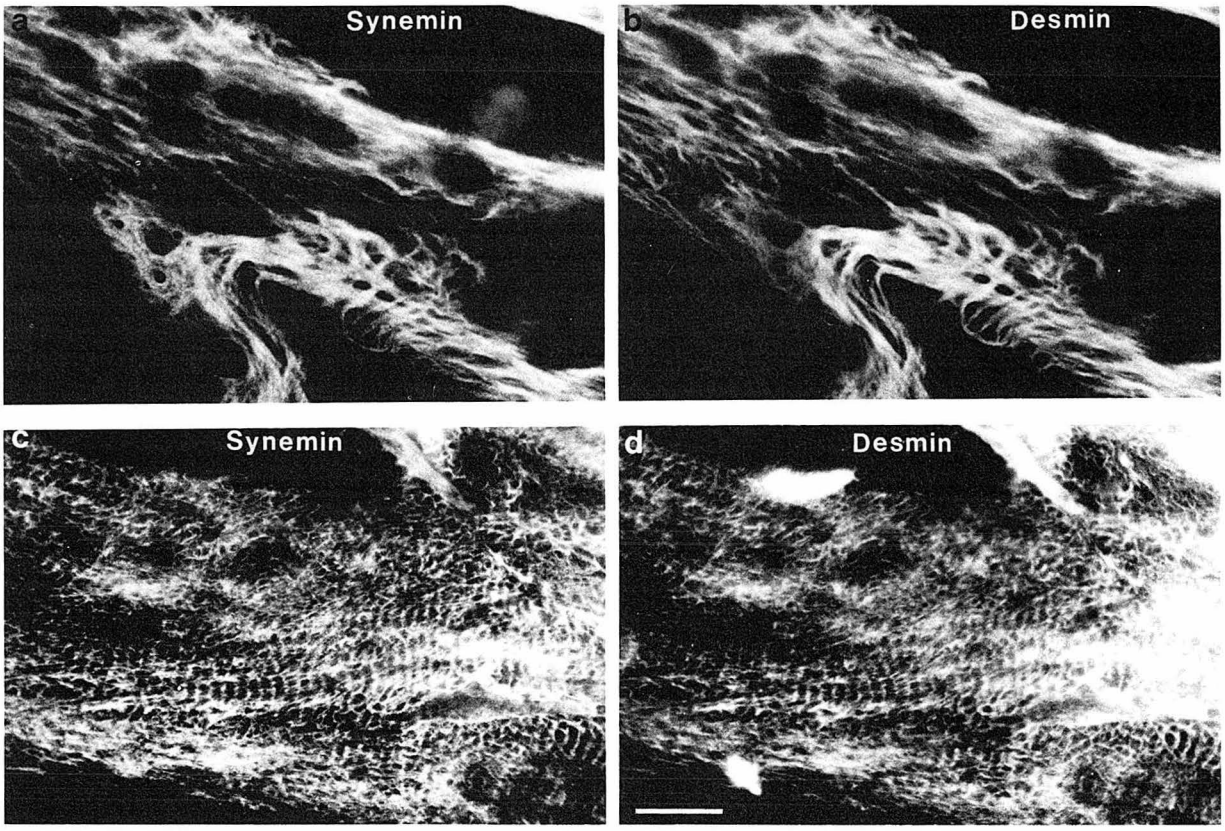

Figure 8. Double Immunofluorescence of Colcemid-Treated Myotubes

Three day (a, b) or 37 day (c, d) myotubes were treated with colcemid and doubly labeled for synemin (a, c) and desmin (b, d). Double labeling for synemin and vimentin similarly produced patterns that were indistinguishable from each other. Bar represents 20 μm.

intermediate filament subunits desmin and vimentin. For these reasons we have chosen to name it synemin (sin-NEM-in), from the Greek syn (with) and nema (filament).

The coincident localization of synemin, desmin and vimentin, as assayed by double immunofluorescence microscopy, has been demonstrated under a variety of experimental conditions; this suggests strongly that these molecules coexist in the same filamentous structures within the cell. All are present in the form of long, wavy filaments in the cytoplasm of early myotubes in culture, and begin to move to the myofibril Z lines within a week of plating. In mature skeletal muscle, all are present at the periphery of the myofibril Z disc, where they form a network of interlinked collars within the Z plane. At these locales, all resist solubilization by nonionic detergents and high salt concentrations. When present as cytoplasmic filaments, all are rearranged indistinguishably by colcemid, but resist this rearrangement when associated with the Z discs. Differential localization of synemin, desmin and vimentin in skeletal muscle has not yet been detected experimentally at the level of resolution afforded by immunofluorescence microscopy; elucidation of the exact relationships of these three molecules in intermediate filaments therefore awaits new experimental approaches.

The interpretation of the immunofluorescence data presented in this paper is entirely dependent on the lack of crossreactivity of the antisera used. Since desmin, vimentin and synemin exhibit indistinguishable fluorescence patterns, it was essential to demonstrate that the antigens being visualized were indeed distinct. This was accomplished by preadsorption of antisera with purified antigens to show that any two of the three antigens did not affect the specific fluorescence observed with the third antibody. This result was supported by the technique of immunoautoradiography, an extremely sensitive assay of antibody-binding activity, which showed that each antiserum was reacting only with its respective antigen.

The Existence of Vimentin in Smooth Muscle

The detection of vimentin in preparations of acetic acid-cycled smooth muscle desmin is another new finding. The ratio of vimentin to desmin is much lower in smooth muscle than in skeletal muscle, and may explain why it has not been seen previously (Izant and Lazarides, 1977; Hubbard and Lazarides, 1979). This vimentin could conceivably be derived solely from the

nonmuscle cells present in smooth muscle tissue, as it has been shown that fibroblastic connective tissue cells and vascular endothelial cells are rich in vimentin (Brown et al., 1976; Hynes and Destree, 1978a; Franke et al., 1978, 1979b; Blose, 1979; Gard et al., 1979). Alternatively, vimentin could coexist with desmin in smooth muscle cells only in very low proportions relative to desmin. The latter possibility is favored, since immunofluorescence of primary cultures of embryonic gizzard tissue reveals that all cells label with anti-vimentin, whereas only a subpopulation of cells labels with anti-desmin and anti-synemin (data not shown). It therefore seems probable that the vimentin present in these preparations comes from both smooth muscle and nonmuscle cells. This is the first direct demonstration that vimentin, like desmin, can be enriched with respect to total protein by cycles of solubilization and precipitation using acetic acid. The solubility of vimentin in acetic acid is further substantiated by its detection in preparations of collagen extracted from chick tendons (see Figure 2, lanes 1), where it is presumably derived from the collagen-secreting tendon fibroblasts.

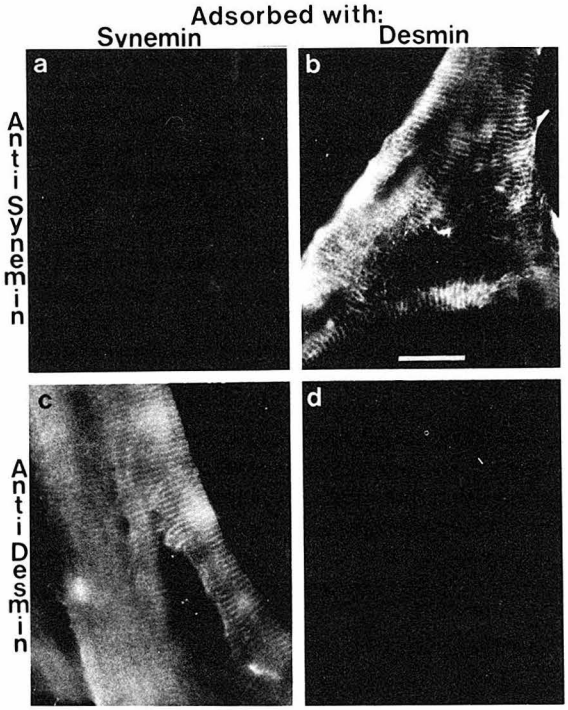

Figure 9. Immunofluorescence Using Antisera Preadsorbed with Purified Antigens

Seven day (a, c) or nine day (b, d) myotubes were labeled with anti-synemin that had been preadsorbed with purified synemin (a) or desmin (b), or anti-desmin that had been preadsorbed with purified synemin (c) or desmin (d). Similar controls using vimentin and anti-vimentin in place of desmin and anti-desmin gave the same result. Bar represents 20 μm.

Function of Synemin

Since little is known about the properties and dynamics of intermediate filaments, speculation about their functions must be derived primarily from their observed distributions. In skeletal muscle, synemin coincides spatially with desmin and vimentin, so that possible functions of synemin are postulated to be similar to those already proposed for desmin and vimentin (Granger and Lazarides, 1978, 1979). As components of the peripheral domain of the skeletal myofibril Z disc, these molecules form an interlinked network of collars that may serve to integrate the muscle fiber mechanically. They may promote or maintain the lateral registry of adjacent sarcomeres, giving skeletal muscle its characteristic striated appearance, and may thus be involved in unifying the contractile actions of its constituent myofibrils. By extrapolation of this hypothesized function back to early, premyofibril muscle cells, in which long undulating intermediate filaments permeate the cytoplasm, it may be inferred that here, too, these filaments are involved in the maintenance of cell structure (see Lazarides, 1980).

The coincident localization of synemin, desmin and vimentin raises the possibility that these three polypeptides co-polymerize into single filaments. Since the ratio of vimentin to desmin decreases during early myogenesis (Gard and Lazarides, 1980), co-polymerization in vivo might require the permissiveness of a nonuniform intermediate filament structure. The low molar ratios of both synemin and vimentin to desmin in smooth muscle suggests that co-polymerization of these molecules is not obligatory for filament formation. Synemin may mediate some specialized function by either co-polymerizing as an integral part of the filament or by binding to the filament in a specific manner as an extrinsic associated polypeptide. The apparent uniform distribution of synemin along all the filaments, and the lack of apparent concentration around or association with any other cytoplasmic structure, suggests that synemin does not function to link intermediate filaments specifically to major cellular organelles. Studies are currently under way to investigate the possible presence of synemin in nonmuscle cells, and to determine whether it is invariably associated with desmin or can exist with other intermediate filament subunits as well.

Definition of Intermediate Filaments

Intermediate filaments are defined morphologically as cytoplasmic filaments with diameters intermediate between those of the thin actin filaments and the thick myosin filaments and microtubules. This classification is useful in that all are believed to have some sort of structural or cytoskeletal function, but does not take into account their biochemical diversity. Differences in antigenicity, solubility, and number and size of component subunits make it a very heterogeneous group (see Lazarides, 1980). Subclasses of interme-

diate filaments can generally be associated with particular cell types or origins, but the discovery that many cell types have more than one type of filament subunit suggests that each type of subunit may perform unique functions. Distributions of filaments coexisting in the same cell are sometimes distinguishable, such as those of keratin and vimentin filaments in ectodermal cells (Franke et al., 1978, 1979a); in other cases, as with desmin and vimentin in skeletal muscle cells, separate distributions have not been observed to date (Granger and Lazarides, 1979; Gard and Lazarides, 1980). This raises the question of whether the different intermediate filament subunits are capable of co-polymerizing into mixed filaments, and to what extent the roles of the different subunits are distinct. It is also unclear whether all polypeptides that co-purify with intermediate filaments can be considered true subunits of the filaments, or whether they are specifically or adventitiously bound to the filaments during isolation.

In this study we characterize a new polypeptide as a component of intermediate filaments in muscle. It is present in a low proportion relative to the major filament subunit with which it co-purifies, yet it may still deserve classification as a filament subunit. Evidence is mounting that filament subunit ratios can be quite variable in different cell types; for example, the ratio of vimentin to desmin is high in fibroblasts, low in smooth muscle, and changes from high to low during skeletal myogenesis. Functional correlates of these ratios have not yet been discerned. Synemin may thus also be variably present in different cell types; differential synthesis of subunits might result in heterogeneous mixed filaments with a wide range of properties tailored to the activities or functions of the cell.

It is anticipated that as work in the field progresses, the morphological term "intermediate filament" will be superseded by classifications based more on the biochemical properties and functions of these structures.

Experimental Procedures

Polyacrylamide Gel Electrophoresis

One-dimensional SDS-polyacrylamide gel electrophoresis was based on the method of Laemmli (1970), and two-dimensional isoelectric focusing/SDS-PAGE was performed according to the method of O'Farrell (1975), each as modified and described by Hubbard and Lazarides (1979). Separating SDS gels contained 12.5% acrylamide and 0.11% N,N′-methylene-bisacrylamide, and were 1.6 mm thick, 14 cm wide and 11 cm long.

Myogenic Cultures

Primary cultures of embryonic chicken leg muscle were prepared according to the method of Konigsberg (1979) from 10 day old embryos. Cells were plated at a density of 2.3×10^4 cells per cm^2 on glass coverslips coated with collagen from either rat tail tendons or chick foot tendons. Collagen was prepared as described by Ehrmann and Gey (1956). Initial plating was in growth medium (Eagle's minimal essential medium, nonessential amino acids, 15% horse serum, 5% chick embryo extract, 0.01% streptomycin and 100 U penicillin/ml); on day 3 or 4, cells were given complete medium (growth medium containing only 10% horse serum and 2% embryo extract) with 10 μM cytosine arabinofuranoside (araC) to prevent fibroblast overgrowth (except for cells in Figure 5, which received no araC). Cells were fed with complete medium at 7–10 day intervals thereafter.

Sample Preparation

Several modifications of published procedures for the isolation of desmin (Small and Sobieszek, 1977; Hubbard and Lazarides, 1979) were used in this study. A protocol useful for yielding both low salt-soluble and high salt-insoluble desmin fractions will be described.

Fresh gizzard muscle (~400 g) was homogenized and washed by pelleting and resuspension four times in 2 liters each of cold 140 mM KCl, 2 mM EGTA, 10 mM Tris–Cl (pH 7.5). Two washes in 2 liters each of 1 mM EGTA, 10 mM Tris–Cl (pH 7.5) were followed by two 15–20 hr extractions with 2 liters each of 10 mM EGTA, 10 mM Tris–Cl (pH 7.5). These extracts contained considerable amounts of desmin (see Hubbard and Lazarides, 1979; Lazarides, Hubbard and Granger, 1979) and were used for the isolation of desmin used as antigen in this study (see next section). The resulting pellets were further extracted four times with 1 liter each of 0.6 M KI, 10 mM $Na_2S_2O_3$, 1 mM EGTA, 10 mM 2-mercaptoethanol, 10 mM Tris–Cl (pH 7.5) over a period of 30 hr. The residue was washed five times with 0.5 liter each of distilled water, then five times with cold acetone. The final pellet was dried under nitrogen at room temperature, then vacuum-dried and stored at −70°C; the weight ratio of acetone powder to wet tissue was 1.5–2%. Desmin was extracted directly from acetone powders by extraction with 1 M acetic acid on ice, and precipitated from clarified extracts by raising the pH to around 5 with NaOH. The precipitate was pelleted and redissolved in 1 M acetic acid; this cycle was repeated 2–4 times. Final cycles often involved polymerization of the extract by dialysis against distilled water. The end product was usually lyophilized for storage.

Glycerinated skeletal myofibrils were prepared as described (Granger and Lazarides, 1979). A suspension of glycerinated anterior latissimus dorsi myofibrils was mixed with 50 vol of 0.6 M KI, 20 mM $Na_2S_2O_3$, 20 mM Tris–Cl (pH 7.5), 0.1 mM EGTA at room temperature, and immediately spun down. The pellet was washed once with water and then boiled in SDS sample buffer (Figure 2, lanes 5).

For electrophoretic analysis, primary myogenic cultures were grown on collagenized 100 mm plates; araC was administered on day 5. After 9 days, plates were rinsed 3 times with PBS at 37°C and 3–5 times with absolute ethanol at −20°C. Cells were scraped off with a rubber policeman into ethanol, transferred to a centrifuge tube and spun down. The pellet was lyophilized and stored at −70°C. Material from one plate was boiled in 30 μl of 1% SDS; 300 μl of 10 M urea, 2% NP40, 0.5% 2-mercaptoethanol were then added, and the solubilized protein was sufficient for four two-dimensional gels (as in Figure 3). Alternatively, one plate of lyophilized material was boiled in 600 μl of 2% SDS, 50 mM Tris–Cl (pH 6.8), 10% glycerol, 0.1 mM EDTA, 0.005% bromophenol blue; after centrifugation 25 μl of supernatant were loaded in lanes of one-dimensional gels (Figure 2, lanes 6).

Collagen was obtained by extraction of chick foot tendons with 1% acetic acid at 4°C for several days. Extracts were dialyzed against distilled water and lyophilized or run directly on gels (Figure 2, lanes 1).

Immunization

Synemin used in the production of antibodies was obtained from preparations of chicken gizzard desmin that had been cycled three times by polymerization and depolymerization as described (above; Hubbard and Lazarides, 1979). After electrophoresis of this material on preparative SDS-polyacrylamide slab gels, the synemin band was excised and reelectrophoresed on another set of gels (7.5% acrylamide, 0.19% N,N′-methylene-bisacrylamide). Visualization of bands was accomplished by brief staining with Coomassie Brilliant Blue R-250 followed by overnight destaining, as described (Granger and Lazarides, 1979). The synemin band was equilibrated in phosphate-buffered saline [PBS: 145 mM Na^+, 4 mM K^+, 10 mM PO_4^{2-}, 130 mM Cl^- (pH 7.4)] and homogenized in a motor-driven Teflon/

glass homogenizer. 2–3 ml of packed gel homogenate, containing approximately 0.5 mg of protein, were suspended in 6 ml PBS and divided into four aliquots; these were administered to a female New Zealand white rabbit by multiple subcutaneous dorsal injections on days 0, 41, 66 and 92. The first injection consisted of an aliquot emulsified with an equal volume of Freund's complete adjuvant, while subsequent injections contained no additives. Some of the smallest gel particles (those sedimentable between 15,000 and 25,000 × g for 10 min) were injected intravenously (marginal ear vein) on day 0. Serum used in this study was collected on day 104 and processed as described (Granger and Lazarides, 1979).

Rabbit anti-desmin was prepared using desmin present in 10 mM EGTA, 10 mM Tris–Cl (pH 7.5) extracts of chicken gizzard smooth muscle. The second extract (see above and Figure 2, lanes 4) was precipitated with ammonium sulfate (10% w/v), dialyzed against water and lyophilized. This material was electrophoresed on SDS slab gels (12.5% acrylamide, 0.11% N,N′–methylene–bisacrylamide) and processed subsequently as an antigen, as described (Granger and Lazarides, 1979). Desmin band homogenate was injected without adjuvant on days 0, 17, 38, 75 and 100; each injection contained approximately 2 mg of protein. Bleeds for this study were made on days 108 and 111. Characterization of this antiserum by two-dimensional immunoautoradiography of high-speed supernatants of embryonic chicken skeletal muscle gave results identical to those shown for another chicken gizzard desmin antiserum (Granger and Lazarides, 1979).

Rabbit anti-vimentin was prepared from embryonic chicken skeletal muscle tissue as described (Granger and Lazarides, 1979).

Immunofluorescence

Cells grown on coverslips were fixed for 10 min at 37°C in 2% formaldehyde, 150 mM KCl, 5 mM NaCl, 1 mM NaN_3, 10 mM $NaPO_4$ (pH 7.5) and then washed in buffer T [0.5% Triton X-100, 130 mM NaCl, 5 mM KCl, 5 mM NaN_3, 1 mM EGTA, 1 mM ϵ–amino–n–caproic acid, 10 mM Tris–Cl (pH 7.5)]. Subsequent incubations and washes for immunofluorescence were in buffer T, or in buffer T containing 0.1% SDS (Figures 7 and 8a). Some cells (Figures 6, 8b and 9) were prefixed for 5 min at 37°C in the same fixative as above, except with 0.5% Triton X-100 and only 0.1% formaldehyde; this was followed by normal fixation and washing as outlined above.

Z disc sheets were prepared from glycerinated chicken iliotibialis muscle; extraction with the 0.6 M KI solution was typically for 0.5–1 day at room temperature (Granger and Lazarides, 1979). Myofibrils were prepared from glycerinated peroneus longus muscle as described (Granger and Lazarides, 1979). For immunofluorescence, incubations and washes of myofibrils and Z disc sheets were in buffer T or in Tris-buffered saline [140 mM NaCl, 5 mM KCl, 10 mM NaN_3, 10 mM Tris–Cl (pH 7.5)].

Antibodies were used at 1/30 serum concentration for immunofluorescence.

Directly labeled antibodies were prepared according to the methods of Cebra and Goldstein (1965) and Brandtzaeg (1973). Antisera were partially purified by ammonium sulfate fractionation and DEAE-cellulose chromatography, and concentrated by ultrafiltration. Anti-vimentin (from day 82 bleed) was conjugated with rhodamine B isothiocyanate (Sigma); anti-desmin (from day 108 bleed) was conjugated with tetramethyl rhodamine isothiocyanate, isomer R (BBL). Conjugations were carried out for 9–10 hr in the cold in 0.15 M sodium carbonate (pH 9.5) with 40 μg dye per mg protein and 7–10 mg protein per ml. Unbound dye was removed by passage through a column of Sephadex G-25 or G-50, and the conjugated antibodies were fractionated further on DEAE-cellulose; those eluting with 40 mM NaCl, 10 mM $NaPO_4$ (pH 7.5) (with a molar dye/protein ratio of approximately 2) were used in this study.

Fluorescein-conjugated goat anti-rabbit IgG was purchased from Miles-Yeda and used at a dilution of 1:150.

Double immunofluorescence was performed according to the indirect/direct method of Hynes and Destree (1978b). Sequential incubations of fixed cells were with rabbit anti-synemin, fluorescein-conjugated goat anti-rabbit IgG, normal rabbit serum (preferably the preimmune serum of the following, directly labeled antibody) to block free anti-IgG sites, and finally with rhodamine-conjugated rabbit anti-desmin or anti-vimentin.

Coverslips were mounted in 90% glycerol in Tris-buffered saline, examined with a Leitz phase/fluorescence microscope (filter modules K or N2) and photographed immediately on Kodak Tri-X film.

Immunoautoradiography

Immunoautoradiography was performed essentially as described (Granger and Lazarides, 1979), with the following modifications. Buffer I contained 140 mM NaCl, 10 mM Tris–Cl (pH 7.5), 5 mM NaN_3, 0.1 mM EGTA, 0.1% gelatin. Fixation was followed by washes in water and then buffer I without gelatin; gels were equilibrated in buffer I as usual before application of antisera. Antisera were used at a dilution of 1:1000. Protein A was found to be useful at least a month after iodination when diluted 10 fold with buffer I and stored at 4°C.

In the context of the methods of antibody production and characterization used in this study, two experimental observations are worth describing. First, we found that gels could be stained first with Coomassie blue, destained and then reacted with antibodies for immunoautoradiography. The success of this approach demonstrates that antibodies will still bind specifically to proteins that have been stained with Coomassie blue. It makes it possible to examine a gel for defects before the long process of immunoautoradiography. However, dye slowly leaches from the protein spots into the buffer I, so that the final gel is stained poorly. Leached dye in buffer I does not seem to affect specific antibody binding, but the desirability of destaining the gel extensively before antibody application would preclude making this method more expedient than the one described originally. In addition, highly overexposed autoradiograms of such gels revealed very faint labeling of most other proteins on the gel; such labeling was not detectable on gels that were not prestained. This may be a result of nonspecific binding of antibody or protein A to Coomassie blue-stained proteins, or it may be caused by specific binding of antibodies to the dye: since the antigen had been stained lightly with Coomassie blue, the dye may have acted as a weakly immunogenic hapten to which a low titer of specific antibodies was elicited.

A second significant finding was that the anti-synemin rabbit began producing antibodies against polyacrylamide (or a contaminant thereof). This was first suspected when the initial attempt at immunoautoradiography with antiserum from an early bleed resulted in a uniformly labeled gel. The same result with a later bleed confirmed this finding. This necessitated preadsorption of the antiserum with polyacrylamide before use in subsequent immunoautoradiographic assays. A blank area of a stained and destained gel was homogenized in a Teflon/glass homogenizer and equilibrated in buffer I. Antiserum was added to a 5–15 fold excess (v/v) of packed gel in enough buffer I to make a thick slurry. After rocking for several hours at room temperature, the slurry was centrifuged and the supernatant was applied to the slab gels.

Alternatively, the problem of anti-polyacrylamide could be overcome by transfer of protein from the polyacrylamide gel to diazotized paper for immunoautoradiography, as described by Renart, Reiser and Stark (1979). This approach is much less time-consuming than labeling in gels, due to reduced incubation and washing periods, but suffers from a slight loss in resolution and the difficulty of visualizing the exact positions of transferred proteins.

Autoradiograms accompanying this paper were exposed for 40 hr at −70°C with intensifying screens; better resolution, however, can be obtained by prolonged exposure at room temperature without screens.

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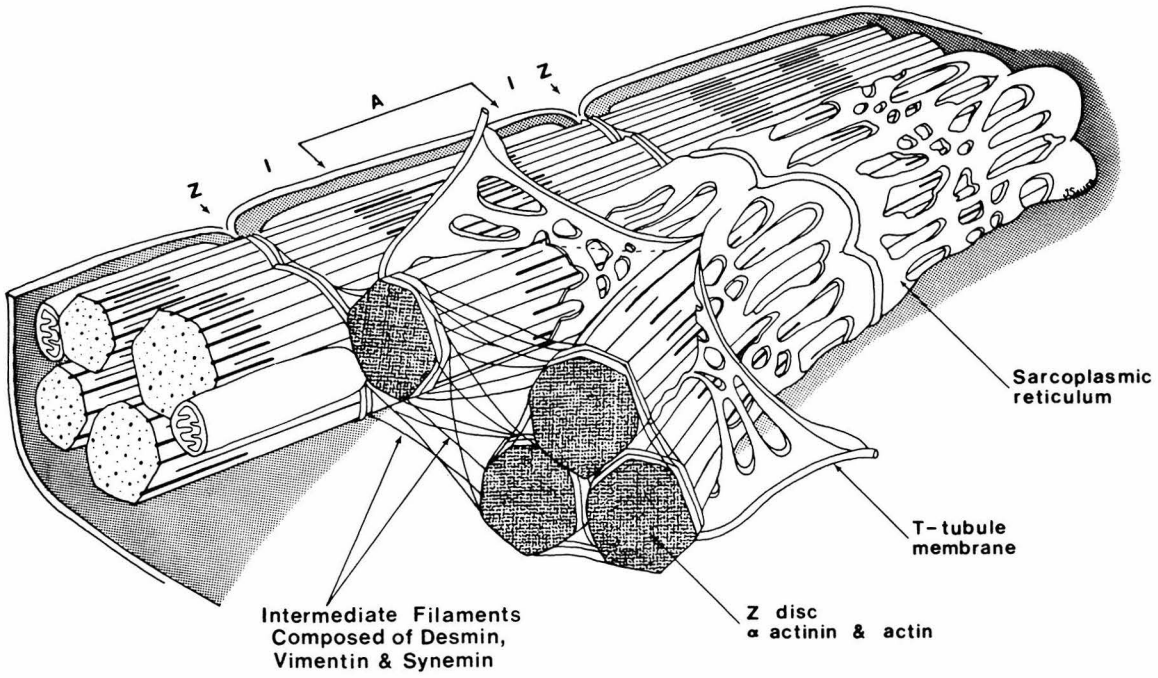

Schematic representation of chicken skeletal muscle showing the distribution of intermediate filaments. Modified slightly from Lazarides, E. (1980), Nature **283:** 249-256.

Chapter 5

Synemin and Vimentin are Components of Intermediate Filaments in Avian Erythrocytes

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ABSTRACT Synemin, a high-molecular-weight protein associated with intermediate filaments in muscle, and vimentin, an intermediate-filament subunit found in many different cell types, have been identified by immunologic and electrophoretic criteria as components of intermediate filaments in mature avian erythrocytes. Desmin, the predominant subunit of intermediate filaments in muscle, has not been detected in these cells. Two-dimensional immunoautoradiography of proteolytic fragments of synemin and vimentin demonstrates that the erythrocyte proteins are highly homologous, if not identical, to their muscle counterparts. Double immunofluorescence reveals that erythrocyte synemin and vimentin co-localize in a cytoplasmic network of sinuous filaments that extends from the nucleus to the plasma membrane and resists aggregation by Colcemid. Erythrocytes that are attached to glass cover slips can be sonicated to remove nuclei and nonadherent regions of the plasma membrane; this leaves elliptical patches of adherent membrane that retain mats of vimentin- and synemin-containing intermediate filaments, as seen by immunofluorescence and rotary shadowing. Similarly, mechanical enucleation of erythrocyte ghosts in suspension allows isolation of plasma membranes that retain a significant fraction of the synemin and vimentin, as assayed by electrophoresis, and intermediate filaments, as seen in thin sections. Both synemin and vimentin remain insoluble, along with spectrin and actin, in solutions containing nonionic detergent and high salt. However, brief exposure of isolated membranes to distilled water releases the synemin and vimentin together in nearly pure form, before the release of significant amounts of spectrin and actin. These data suggest that avian erythrocyte intermediate filaments are somehow anchored to the plasma membrane; erythrocytes may thus provide a simple system for the study of intermediate filaments and their mode of interaction with membranes. In addition, these data, in conjunction with previous data from muscle, indicate that synemin is capable of associating with either desmin or vimentin and may thus perform a special role in the structure or function of intermediate filaments in erythrocytes as well as in muscle.

Mature avian erythrocytes are nucleated, biconvex, elliptical discs that contain relatively few cytoplasmic organelles. Removal of hemoglobin from these cells by hypotonic lysis (15) reveals an equatorial bundle of microtubules known as the marginal band (5), a submembranous spectrin-actin shell (9), as well as a residual network of cytoplasmic filaments that surrounds the mitochondria and extends from the nucleus to the plasma membrane (27). This latter network of filaments is probably a component of the "trans marginal band material" noted in many nonmammalian vertebrate erythrocytes (13). These filaments appear to be of the type known as intermediate filaments (33, 39), due to their characteristic ultrastructural morphology and insolubility in nonionic detergents (59, 63). The close association of these filaments with the plasma membrane and nucleus, as shown by electron microscopy, suggests that they might function to maintain the shape of the cell or position the nucleus within the cell (13, 27, 59, 63).

We have examined these filaments biochemically, immunologically, and ultrastructurally and have determined that they are composed predominantly of vimentin, an intermediate filament subunit common to many different cell types (18, 39). The other major component of these filaments is synemin, a high molecular weight protein originally isolated from avian smooth muscle in association with desmin and subsequently shown to co-localize with desmin and vimentin in skeletal muscle (25). Double immunofluorescence shows that erythro-

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cyte synemin and vimentin also coexist in a network of cytoplasmic filaments. The electrophoretic and immunologic criteria used to identify vimentin and synemin in these cells fail to detect desmin, the major intermediate filament subunit of muscle (40, 53) that is also found in some nonmuscle cells (19, 55, 58).

Various cell fractionation procedures based on differential centrifugation have indicated that synemin and vimentin sediment with both nuclear and membrane fractions. In this study we have concentrated on those intermediate filaments that remain associated with the erythrocyte plasma membrane after mechanical enucleation of the cells. These filaments resist dissociation from the membranes by sonication and treatment with high salt and nonionic detergent, suggesting that they are in some way anchored to the membrane cytoskeleton, perhaps to the spectrin-actin network. However, we have found that the filaments can be selectively removed from the membranes by treatment with low ionic strength solutions and that the predominant proteins thus released are vimentin and synemin.

The evidence presented here that synemin associates with vimentin in erythrocytes, in conjunction with the evidence presented previously that synemin associates with desmin in smooth muscle and desmin and vimentin in skeletal muscle (25), suggests that synemin may be capable of associating with different intermediate filament subunits in different cell types. Because synemin is not detectable in all cell types, it may play a special role in the structure or function of certain types of intermediate filaments or filaments in certain cell types.

MATERIALS AND METHODS

Preparation of Erythrocyte Membranes

White leghorn chickens were given intravenous injections of 3–4 mg (500–700 USP units) of heparin (17) and then ~50 mg of sodium pentobarbital. Blood was collected from the neck vein into a solution of 0.01% heparin, 5 mM HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid), 155 mM choline chloride (pH 7.1 at room temperature). Phosphate-buffered saline was simultaneously injected into a wing vein, and the perfusate was also collected until it became relatively clear. Alternatively, blood for some experiments was drawn from wing veins or neck veins of uninjected chickens and collected in 1–2 vol of the above heparin-containing solution. Blood cells were pelleted by centrifugation for 5 min at 1,000 *g*, and the top white layer of cells (buffy coat) and supernatant were removed by aspiration. The erythrocyte pellet, exclusive of a dark-red layer adhering to the bottom of the centrifuge tube, was resuspended in 155 mM choline chloride, 5 mM HEPES (pH 7.1 at room temperature) and recentrifuged. Again, the supernatant, buffy coat, and dark-red layer were discarded. This cycle was repeated for a total of 4–8 washes, and was performed either at room temperature or at 4°C.

The final pellet of erythrocytes was rapidly resuspended in at least 10 vol of ice-cold hypotonic lysis buffer (Buffer H) [5 mM Tris-Cl (pH 7.5), 5 mM NaN_3, 5 mM $MgCl_2$, 1 mM EGTA (ethyleneglycol-bis(β-amino-ethyl ether) N,N′-tetra-acetic acid), 1 mM dithioerythritol (DTE) or dithiothreitol (DTT), 0.5 mM phenylmethyl sulfonyl fluoride (PMSF)]. $MgCl_2$ was included to keep the nuclei intact, EGTA and PMSF were included as protease inhibitors, and DTE (or DTT) was found to increase both the yield and size of the plasma membrane fragments (see below). The resulting nucleated erythrocyte ghosts were pelleted by centrifugation for 5 min at approximately 10,000 *g*, then resuspended in at least 10 vol of the same solution. This cycle was repeated for a total of three or four washes in Buffer H.

For the preparation of plasma membranes, the final pellet was resuspended in 2–4 vol of Buffer H, loaded into a syringe, and forced rapidly through a 23 gauge hypodermic needle bent into the shape of a Z (with two 30° angles). Centrifugation for 10–20 min at 1,000 *g* in a swinging bucket rotor resulted in three layers: a firm, white pellet of free nuclei on the bottom, a loose, pink layer of undisrupted cells in the middle, and a supernatant containing soluble proteins and membrane fragments. The middle layer was resuspended in Buffer H and again forced through the needle and centrifuged to give the three layers. This was usually repeated three times; the yield of membrane fragments increased with each cycle. The supernatants from each centrifugation were combined and recentrifuged for 30 min at 100–200 *g* to remove any remaining nuclei. The supernatant of this low speed centrifugation was respun for 10 min at 20,000 *g* to pellet the membranes which were subsequently resuspended in 10–20 vol of Buffer H and stored on ice. Contaminating nuclei visible by phase contrast microscopy could be removed by another low speed centrifugation. After storage for a month on ice, little change was seen in the electrophoretic protein profile as judged by one dimensional SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (see below).

Selective Extraction of Synemin and Vimentin From Erythrocyte Membranes

The suspension of erythrocyte membranes was mixed with 10–30 vol of 2 mM EDTA 10 mM Tris (pH adjusted with HCl to 7.4 at 0°C); after 10 min on ice the membranes were pelleted by centrifugation at 20,000 *g* for 10 min. The pellet was resuspended (with a Pasteur pipette) in distilled water at 0°C and centrifuged as above. The supernatant was then lyophilized directly for electrophoretic analysis, or first recentrifuged for 1–5 h in a Beckman SW 50.1 swinging bucket rotor at 50,000 RPM (Beckman Instruments, Inc., Fullerton, Calif.).

Polyacrylamide Gel Electrophoresis

One-dimensional SDS-PAGE was based on the discontinuous, Tris-glycine system of Laemmli (37), as modified and described previously (29). Separating gels were 11 × 14 × 0.16 cm and contained 12.5% acrylamide and 0.11% N,N′-methylene-bisacrylamide. Samples were solubilized with 1% SDS, 125 mM Tris-Cl (pH 6.8), 10% glycerol, 1% 2-mercaptoethanol, 1 mM EDTA, 0.004% bromophenol blue ("1% SDS sample buffer"), and immediately placed in a boiling water bath for ~1 min.

Two-dimensional isoelectric focusing (IEF)/SDS-PAGE was performed according to the method of O'Farrell (48), as modified and described previously (29), except that nonidet P-40 (NP-40) was omitted from all gels and samples (this enhanced the resolution of the isoelectric variants of several proteins of interest in this system). Second-dimension SDS slab gels were as described above.

Phosphorylation

Incorporation of [^{32}P]-phosphate into erythrocyte proteins was performed essentially as described by Beam et al. (4) and Alper et al. (2). Blood was collected from the wing vein of an adult hen turkey in cold heparinized choline chloride buffer and washed as described above. Erythrocytes were then washed once at room temperature with 40 vol of 157.5 mM NaCl, 2.5 mM KCl, 11.1 mM D-glucose, 10 mM HEPES (brought to pH 7.65 at room temperature with NaOH). 1 ml of packed cells was resuspended in 9 ml of this solution; 2 mCi of ^{32}P-phosphoric acid (New England Nuclear, Boston, MA; 100 μl of carrier-free in 20 mM HCl) were then added and the suspension was incubated in an orbital shaker bath at 39°C. After 3.5 h the suspension was divided in half; to one-half was added 50 μl of 0.1 mM DL-isoproterenol-HCl (Sigma Chemical Co., St. Louis, MO) in the above solution (final concentration 1 μM). Both aliquots were incubated for another 20 min at 39°C and then processed in parallel for gel electrophoresis. The erythrocytes were spun down and washed once with 40 vol of the above solution at 39°C, then lysed with 80 vol of 10 mM Tris-Cl (pH 8.0), 5 mM $MgCl_2$, 1 mM EGTA, 1 mM o-phenanthroline, 0.5 mM PMSF, 1 mM NaF at 0°C. The nucleated ghosts were spun down and washed once with this solution, then disrupted by one passage through a bent hypodermic needle as described above. Intact cells and nuclei were removed by low-speed centrifugation, and free membrane fragments were collected by high-speed centrifugation. The membrane pellet was boiled in 1% SDS sample buffer and analyzed by SDS-PAGE. After staining and destaining, the gel was dried on filter paper and exposed to Kodak X-Omat R XR5 film at room temperature for 10 d without an intensifying screen.

In a separate experiment, erythrocytes were collected, washed and labeled as described above, except that the labeling period was 20 h and no isoproterenol was added. Cells were lysed with 5 mM sodium phosphate, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM PMSF (pH 7.4 at 4°C) and disrupted as described above. The membrane fraction was dissolved in 10 M urea containing 1% 2-mercaptoethanol, analyzed by IEF/SDS-PAGE, and autoradiographed.

Immunoautoradiography

Immunoautoradiography was performed as described previously (25). Gels were incubated with antisera diluted 1,000-fold, followed by radioiodinated protein A (4–7 μCi ^{125}I/μg protein A; each gel was incubated with 20–30 μCi in 100 ml of solution). The dried gels were exposed to x-ray film for the following times with (+) or without (−) an intensifying screen: Fig. 3*b*, 27 h (−); Fig. 3*c*, 15 h (+); Fig. 4*b*, 36 h (−); Fig. 4*c*; 55 h (+); Fig. 5*b*, 405 h (−); Fig. 5*d*, 42 h (+).

Samples for electrophoresis were prepared as follows. Fig. 3: The white cell layer (buffy coat) was collected from the first centrifugation of heparinized chicken blood and recentrifuged twice in HEPES-buffered saline containing 1 mM EDTA to give an erythrocyte-free preparation. The cells were mixed with 40 vol of ethanol at 0°C, pelleted, and boiled in 1% SDS sample buffer. Whole adult chicken-gizzard smooth-muscle tissue was frozen and pulverized in liquid nitrogen, thawed in ethanol, pelleted, and boiled in 1% SDS sample buffer. Turkey erythrocyte membranes were extracted at 0°C for 20 min with 10 mM Tris, 1 mM EDTA, 1 M NaCl, 1% 2-mercaptoethanol, 1% Triton X-100, and the residue was pelleted and boiled in 1% SDS sample buffer. Chicken erythrocyte membranes were extracted at 0°C for 70 min with 10 mM Tris, 2 mM EDTA, 0.5 M KCl, 1% Triton X-100 (pH 7.4), and the residue was pelleted and boiled in 1% SDS sample buffer. Another aliquot of chicken erythrocyte membranes was washed at 0°C with 10 mM Tris-2 mM EDTA (pH 7.4) and then treated for 4 h at 0°C with 10 mM Tris-1 mM EGTA (pH 7.4); the extract was dialyzed against water, lyophilized, and boiled in 1% SDS sample buffer.

Fig. 4: Chicken erythrocyte membranes were treated with 60 vol of ethanol to permeabilize the vesicles, pelleted, and dissolved at room temperature in a saturated urea solution containing 1% 2-mercaptoethanol.

Fig. 5: Chicken erythrocytes were extracted twice at 0°C with 1% Triton X-100, 150 mM NaCl, 2 mM EGTA, 5 mM $MgCl_2$, 1 mM sodium tetrathionate, 1 mM ϵ-amino-n-caproic acid, 1 mM o-phenanthroline, 1 mM PMSF, 10 mM Tris-Cl (pH 7.2) and rinsed twice with this solution without the Triton X-100. The resulting cytoskeletons were then extracted with this latter solution containing 6 M urea for 1.5 h at 0°C. This extract was dialyzed against water, and the resulting precipitate was collected and dissolved in a saturated urea solution containing 0.5% 2-mercaptoethanol. Whole adult chicken-gizzard smooth-muscle tissue was frozen and pulverized in liquid nitrogen, thawed in ethanol, pelleted, boiled for 30 s in 10 μl of 1% SDS, and then dissolved in 90 μl of saturated urea containing 1% 2-mercaptoethanol.

Immunofluorescence

Glass cover slips were pretreated with Alcian Blue to promote erythrocyte adhesion (54). Cover slips were cleaned, simmered for 5 min in 0.1% Alcian Blue 8GX (Sigma Chemical Co., St. Louis, Mo.), rinsed with distilled water, and air dried. Washed erythrocytes in the choline chloride/HEPES buffer were allowed to settle on the cover slips for 5–10 min at room temperature; nonadherent cells were removed by rinsing with the same solution.

For the sonication experiments, cover slips with attached erythrocytes were hypotonically lysed at room temperature in Buffer H and then placed in Buffer F [130 mM KCl, 5 mM NaCl, 1 mM NaN_3, 5 mM $MgCl_2$, 1 mM EGTA, 20 mM potassium phosphate (pH 7.5)]. Cover slips were laid face-up in a beaker of buffer F and sonicated for 20 s at 20 watts using a Braunsonic 1510 sonicator (B. Braun Instruments, San Francisco, Calif.) with a 4-mm titanium probe tip positioned 3–4 cm above the cells. A glass rod was used to hold the cover slips in position on the bottom of the beaker during sonication. Incubations with antisera and subsequent washes were all performed at room temperature in buffer TM (0.5% Triton X-100, 130 mM NaCl, 5 mM KCl, 5 mM NaN_3, 5 mM $MgCl_2$, 1 mM EGTA, 10 mM Tris-Cl (pH 7.5)].

Alternatively, sonication was performed in Buffer H and antibody incubations and washes in Buffer F without the $MgCl_2$ and EGTA (Fig. 8 *a,b,c,h*).

To investigate the Colcemid sensitivity of the filaments, turkey erythrocytes attached to cover slips were incubated for 16 h at 37°C in growth medium (Eagle's minimal essential medium, nonessential amino acids, 15% horse serum, 5% chick embryo extract, 0.01% streptomycin and 100 U penicillin/ml) containing 5 μM Colcemid (demecolcine; Calbiochem-Behring Corp., La Jolla, Calif.). Control cells were treated identically, except that they were not exposed to Colcemid. Cover slips were placed in Buffer TM containing 0.5 mM PMSF for 1 min at room temperature to make cytoskeletons, then fixed for 10 min at room temperature in Buffer F containing 2% formaldehyde. Subsequent incubations and washes were done in Buffer TM containing a 0.5 mM PMSF. Similar results were obtained with unfixed cells, with cells incubated in suspension rather than attached to cover slips, and with cells incubated with 100 μM Colcemid.

Rabbit anti-vimentin was prepared using antigen from embryonic chicken skeletal muscle cytoskeletons (24). Rabbit anti-desmin was prepared using desmin present in a low-salt extract of chicken-gizzard smooth muscle (25). Rabbit anti-synemin was prepared using antigen obtained from chicken-gizzard intermediate-filament proteins that had been solubilized and precipitated three times in acetic acid (25). All antigens were ultimately purified by preparative SDS-PAGE before injection.

Conjugation of anti-vimentin with rhodamine B was performed as described (25). Double immunofluorescence was performed by the indirect/direct method (32). Fluorescein-conjugated goat anti-rabbit IgG was purchased from Miles-Yeda, Ltd. (Rehovot, Israel) and diluted 150-fold for use. Primary antisera were partially purified by precipitation with ammonium sulfate at 50% saturation and used at ~1/30 serum concentration.

Cover slips were mounted in 90% glycerol in Tris-buffered saline and photographed with Kodak Tri-X film using a Leitz phase/epifluorescence microscope and filter modules K and N2. The patches of plasma membrane in Fig. 8 *s* are visible by phase-contrast microscopy because an air bubble was present under that portion of the cover slip.

Electron Microscopy

For thin sectioning, chicken erythrocyte membranes were pelleted and fixed with 1% glutaraldehyde, 5 mM $MgCl_2$, 10 mM sodium phosphate (pH 7.5), postfixed in 1% OsO_4, 0.1 M sodium cacodylate (pH 7.4), stained at 60°C with 1% uranyl acetate, 0.1 M sodium maleate (pH 5.15), dehydrated in ethanol and propylene oxide, and embedded in a 9/16/1 mixture of 1,2,7,8-diepoxyoctane (Aldrich; Milwaukee, Wis.)/nonenyl succinic anhydride (ICN K/K Laboratories, Inc., Plainview, N. Y.)/DMP-30. The resin was cured for 2 d at 60°C and sectioned with glass knives on a Reichert OmU2 ultramicrotome. Sections were stained for 3 min with 0.2% lead citrate, examined with a Philips EM201 at 80 kV, and photographed on 35-mm film.

Shadowed replicas were made as follows: Chicken erythrocytes adhering to Alcian Blue-coated cover slips were lysed and sonicated as described under Immunofluorescence. The cover slips were immersed in buffered 1% glutaraldehyde followed by 1% OsO_4, dehydrated with ethanol, dried in a carbon dioxide critical-point drier, rotary shadowed with platinum/palladium (80/20) at an angle of 6°, and then carbon coated. Replicas were separated from cover slips with 5% HF, washed with water, mounted on 300-mesh copper grids, and viewed by transmission electron microscopy.

RESULTS

Membrane Fractionation and Ultrastructure

The study of minor protein components of avian erythrocytes is hampered by the relative abundance of hemoglobin and chromatin in these cells. Removal of these two components makes biochemical, immunological and ultrastructural characterization of the remaining structures easier. Hypotonic lysis (to remove hemoglobin) and subsequent mechanical enucleation (to remove chromatin) gives a preparation of membranes that can be studied in a manner analogous to the study of the simpler mammalian erythrocyte ghosts. To this end, chicken and turkey erythrocytes were isolated from fresh blood by differential centrifugation and lysed in a low osmolarity buffer containing magnesium ions to keep the nuclei intact (27). These nucleated ghosts were then disrupted by passage through a bent hypodermic needle, and a membrane fraction was separated from the nuclei and unbroken cells by differential centrifugation. This membrane fraction is the main object of this study and will hereafter be referred to as "erythrocyte membranes."

Representative thin sections of the erythrocyte membranes are shown in Fig. 1. The preparation is composed primarily of plasma membranes, both complete and in pieces (compare with sections of whole cells in references 4, 5, 63, 66). Close examination reveals that in a given thin section many of the membranes have filaments associated with them. These filaments are ~9 nm in diameter and are therefore classified as intermediate filaments. They are present on the cytoplasmic side of the plasma membrane fragments and often appear to be in close apposition to the protein network (analogous to the spectrin network in mammalian erythrocytes) just inside the lipid bilayer. This is especially apparent in grazing sections of the membrane in these relatively thick sections. The filaments are usually curved and randomly distributed and do not exhibit any obvious association with specific cell structures.

Thin sections of the free nuclei (not shown) also reveal intermediate filaments associated with these structures, as previously shown by Woodcock (63). It has not been determined what proportion of the filaments remains with the membranes and what proportion with the nuclei, due to the difficulty of

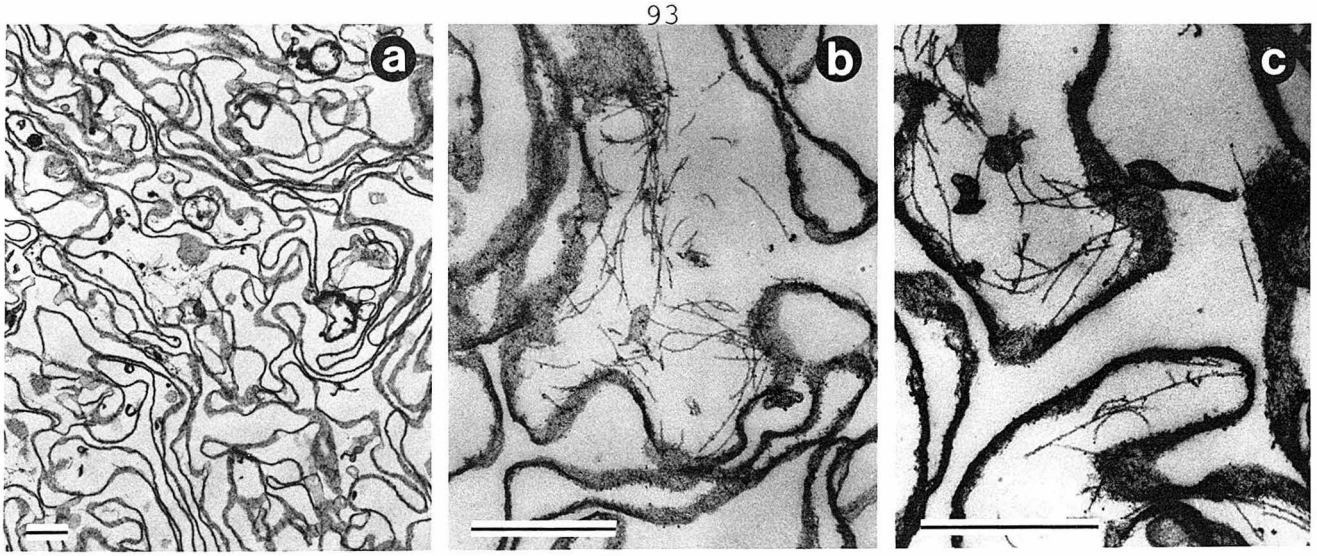

FIGURE 1 Thin sections of chicken erythrocyte membranes. (*a*) Medium-power view of pelleted membranes showing that filaments can be seen associated with many, but not all, membranes in a given section (× 11,000). (*b*, *c*) High-power views. Note cytoplasmic filaments and frequent close associations between filaments and membranes (*b*: × 38,000; *c*: × 47,000). Bars, 500 nm.

enucleating the ghosts quantitatively and completely separating the resulting fractions. Such a quantitation is also complicated by the fact that the proportion of the filaments associated with the nucleus before cell disruption that remains associated with the nuclei after fractionation is probably a function of the severity of the disruption procedure and the extent of loss of the outer nuclear membrane. However, based on various biochemical and ultrastructural data (see below), it appears that on the average less than half of the cell's intermediate filaments end up in the membrane fraction.

It is apparent from these thin sections that the erythrocyte membrane fraction contains low levels of contamination by fragments of structures other than the plasma membrane. Even though this enucleation procedure results in free nuclei that appear to be intact by phase-contrast microscopy, the relatively fragile outer nuclear membrane may become partially fragmented and fractionate with the plasma membranes (27, 65). Fragments of mitochondrial membranes may also be present in this fraction. However, because our studies were concerned primarily with intermediate filaments rather than specific membrane proteins, further purification of the membrane fraction was not deemed necessary for subsequent biochemical studies. The purpose of the fractionation was to remove chromatin that would have physically interfered with the membrane extraction experiments, and this was accomplished. Negligible amounts of histone could be seen when the membrane fraction was analyzed by SDS-PAGE, and nuclear membrane lamins (21, 52) could not be detected by IEF/SDS-PAGE, showing that the level of contaminating material was low.

Electrophoretic Analysis of Membrane Fraction

Analysis of the protein composition of avian erythrocyte membranes was performed with regard to the voluminous work on mammalian erythrocyte ghosts. Similarities between the two systems include two major high-molecular-weight proteins in avian membranes that correspond to the mammalian erythrocyte spectrins (see Figs. 2 and 6). Avian α-spectrin comigrates by SDS-PAGE with mammalian α-spectrin, but the β variant has a higher mobility and can be resolved into a closely spaced doublet on underloaded gels (not shown). Both systems contain actin at 42,000 daltons as well as a broad band of membrane proteins around 100,000 daltons (Band 3; reference 16). Among the characteristic differences are the presences in avian membranes of goblin, a hormonally-regulated phosphoprotein (4), and of the intermediate filament proteins, vimentin and synemin. The presence of these two intermediate filament components in association with avian erythrocyte membranes was demonstrated by two-dimensional gel electrophoresis (IEF/SDS-PAGE; see Fig. 4*a*). Erythrocyte vimentin coelectrophoreses in this gel system with vimentin identified in other avian cell types (19, 24); the identification of synemin was tentative at this stage and required immunological and biochemical confirmation, as described below. Desmin was not detected on these electrophoretograms.

Initial biochemical studies of erythrocyte membranes began with attempts to remove peripherally bound proteins from the membrane lipid bilayer. We found that the solubilization or release of any protein components from the erythrocyte membranes, without the use of detergents or strongly chaotropic agents, required the removal of divalent cations. Therefore, before most biochemical experiments, the magnesium ions present in the membrane suspension (in the hypotonic lysis buffer) were removed from the membranes by washing with a low-salt buffer containing EDTA.

Treatment of membranes with solutions of very low ionic strength was expected to release spectrin, by analogy to the mammalian erythrocyte system (42, 43). However, it was observed that if such an extraction was performed briefly at 0°C, then the primary protein released was vimentin. Fig. 2*a* shows a two-dimensional gel of the extract obtained by treatment of chicken erythrocyte membranes with distilled water for 30 min on ice. In addition to the four or five isoelectric variants of vimentin at 52,000 daltons, are synemin at 230,000 daltons and actin at 42,000 daltons. Identification of the 230,000 dalton polypeptide as synemin is based on its immunological cross-reactivity with smooth muscle synemin and its immunoautoradiographic peptide map, both as detailed below, as well as its copurification with vimentin. No desmin can be detected on this gel. Distilled water was found to be the optimal solvent for extraction of relatively pure vimentin and synemin, but other low ionic strength solutions (eg., 1–2 mM EDTA or EGTA

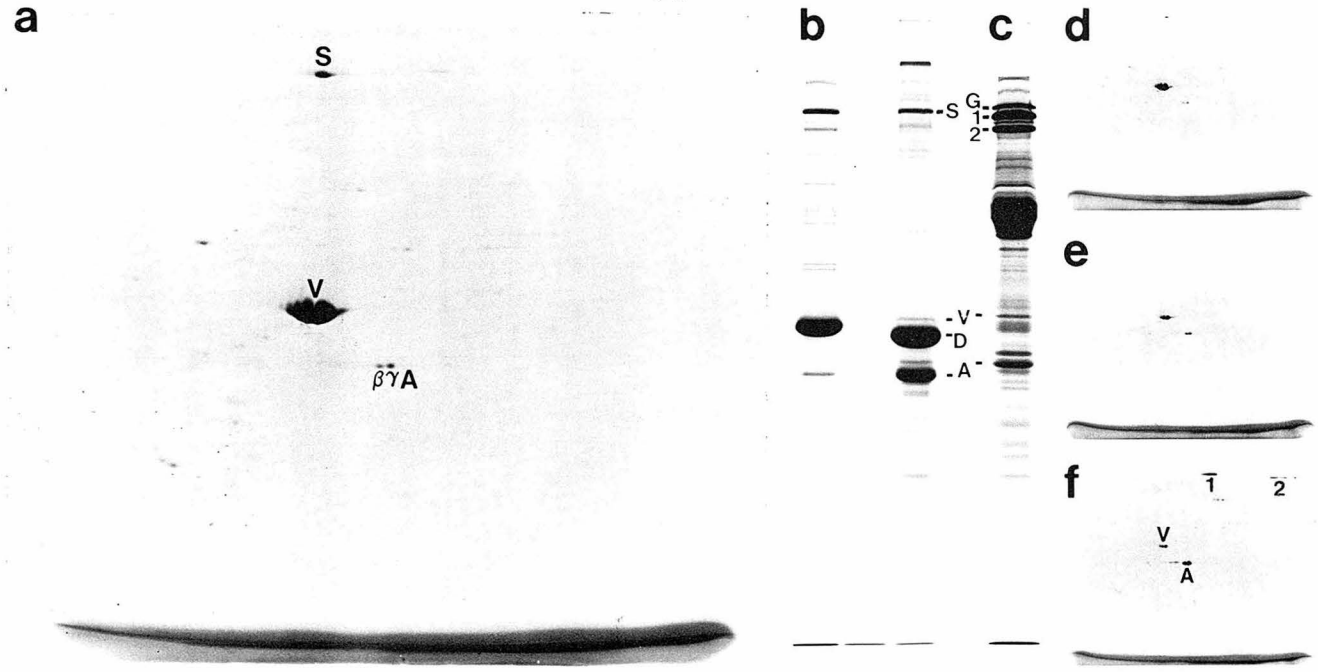

FIGURE 2 Water extracts of chicken erythrocyte membranes. (*a*) Two-dimensional gel of a distilled water extract of erythrocyte membranes showing predominance of vimentin (*V*) and synemin (*S*). The β and γ variants of actin (*A*) are present. (*b*) One-dimensional gel; extract as in (*a*) was centrifuged for 1 h at 240,000 *g* (av.); lane 1 contains the supernatant and lane 2 the pellet. For comparison, lane 3 shows a preparation of gizzard desmin (*D*) and synemin that was cycled twice by solubilization and precipitation using acetic acid. (*c*) Erythrocyte membrane residue after two sequential extractions with distilled water over 2-h period. *G*, goblin (see Fig. 6 for documentation of this identification); 1 and 2, α and β spectrin, *A*, actin. (*d*, *e*, *f*) Sequential water extracts of a single aliquot of erythrocyte membranes; extraction periods were for 1 min, 0.5 h and 9 h, respectively, and each gel represents all of the protein extracted at each step. All membrane extractions in this figure were performed at 0°C. Panel *a* represents the protein extracted from 35–40 μl of packed erythrocyte membranes, and lane 1 from 20–25 μl of membranes.

brought to pH 7.4 with Tris) will produce extracts of comparable purity but lower yield (approximately one-half). If the pH of the extracting solution is raised, release of several components in addition to vimentin is favored, resulting in a less pure preparation of vimentin and synemin. At pH 11, the membranes are nearly quantitatively stripped of non-integral proteins (not shown; see reference 56). Similarly, many proteins can be released from the membranes by treatment with acetic acid (cf. reference 41), but this results in a low-yield, very impure preparation of vimentin and synemin that exhibits extensive proteolytic degradation. Acetic acid thus appears to be undesirable for use in the extraction and purification of intermediate filaments from erythrocytes as it has been used previously for smooth muscle (29, 53).

This technique of obtaining highly enriched preparations of vimentin and synemin takes advantage of the fact that spectrin and actin are released very slowly from the membranes in distilled water or low salt buffers at 0°C. Fig. 2*d–f* show sequential extracts of a membrane aliquot made with distilled water at 0°C for 1 min, 30 min and 9 h. Most of the vimentin and synemin are released from the membranes within 1 min. The ratio of actin to vimentin increases with each extraction, and spectrin becomes a major component of the extract after a few hours. After prolonged extraction, though, even in the presence of EDTA and reducing agents, most of the spectrin (and much of the actin) is still associated with the membrane (not shown). If the extractions are performed at 37°C instead of 0°C, all of these proteins are released more rapidly; spectrin is solubilized rapidly enough to become the major component of short-interval extracts, making vimentin a minor component (not shown).

After repeated and prolonged extractions with distilled water at 0°C, the membranes tend to break up into smaller fragments and vesicles. Partial loss of the spectrin network may account for this.

Lane 1 of Fig. 2*b* is the one-dimensional gel profile of a distilled water extract identical to that in Fig. 2*a*, except that it was centrifuged for 1 h at 170,00–310,000 *g* (240,000 *g* av.); the supernatant was lyophilized and run in Lane *1*, and the pellet was run in Lane *2*. It is apparent that under these conditions little of the protein is sedimented. A similar result was obtained with a 5-h centrifugation. Minor polypeptides that do not focus discretely in the two-dimensional gel system can be visualized here. Electrophoresis of this material on less porous polyacrylamide gels shows that most of the material migrating with the dye front in Lane *1* is residual hemoglobin. Lane *3* is a preparation of chicken-gizzard smooth-muscle intermediate filaments that shows synemin, vimentin, desmin, and actin for molecular weight comparison. Synemin and α spectrin are difficult to resolve from one another on normally loaded one-dimensional gels but are clearly resolved on underloaded gels and two-dimensional gels.

Not all of the vimentin is extracted from erythrocyte membranes with a single distilled water treatment. Fig. 2*c* shows a sample of membranes that was extracted twice with water over a 2-h period; the amount of vimentin is less, but some still remains. Residual vimentin is evident even after four extractions over a 5-d period (not shown). However, in thin sections of membranes treated with distilled water for 5 min, cytoplasmic intermediate filaments cannot be found. This suggests that the residual vimentin may not be in the form of free cytoplasmic filaments (see Discussion).

Immunological Characterization of Erythrocyte Intermediate Filaments

The technique of immunoautoradiography (10), which uses antibodies to detect protein antigens in polyacrylamide gels, was used in this study for three purposes: (*a*) to determine whether the erythrocyte intermediate-filament subunits were antigenically crossreactive with their muscle counterparts; (*b*) as a form of peptide map analysis to determine whether the subunits in erythrocytes were homologous or identical to their muscle counterparts; and (*c*) to detect these antigens in gels with a sensitivity much greater than that afforded by Coomassie Blue staining. Antisera used in this study were all elicited against chicken muscle proteins, purified by SDS-PAGE, and each appears to be specific for its respective antigen as assayed by two-dimensional immunoautoradiography (24, 25).

Fig. 3 shows the presence of immunoreactive forms of both vimentin and synemin in various fractions of chicken and turkey erythrocyte membranes. Fig. 3*a* shows a Coomassie Blue-stained SDS-polyacrylamide gel of a variety of samples; this gel was labeled with anti-synemin followed by radioiodinated protein A, and the corresponding autoradiogram is shown in Fig. 3*c*. A duplicate gel was processed with anti-vimentin, and its autoradiogram is shown in Fig. 3*b*.

Lane *1* represents whole white cells from chicken blood, examined to ensure that the vimentin and synemin being studied in the erythrocyte preparation were not originating from the extremely low level of contamination by white cells. Little if any synemin is detectable, and the quantity of vimentin is low relative to the amount of actin present in these cells.

Lane *2* is whole chicken-gizzard smooth-muscle tissue. It is the tissue from which synemin was originally purified and was the source of the synemin used for immunization (25). Vimentin is also present in this tissue (25; Fig. 3*b*). The two most prominent bands near the top of the lane are filamin and myosin; the autoradiogram of Fig. 3*c* shows that synemin migrates between these two proteins (see also reference 25). α-Actinin is visible at 100,000 daltons, and the two major bands in the middle are desmin and actin (50,000 and 42,000 daltons).

The remaining lanes demonstrate the presence of vimentin and synemin in erythrocyte membranes, membrane cytoskeletons, and low salt extracts. Lanes *3* and *8* display chicken erythrocyte membranes, and lane *4* displays turkey erythrocyte membranes. Both samples contain polypeptides that have molecular weights and antigenicities similar to those of muscle vimentin and synemin. Lanes 5 and 6 represent turkey and chicken erythrocyte membranes that have been extracted with high salt and Triton X-100. Both vimentin and synemin remain insoluble in the cytoskeletal residue, as do the spectrin and actin. Goblin, the 100,000 dalton cluster, a 44,000 dalton polypeptide and many minor components are partially or completely solubilized (compare lanes *5* and *6* with lanes *3* and *4*). Lane *7* contains a low-salt extract of chicken erythrocyte membranes that is highly enriched in vimentin and synemin; the greater amount of handling and processing of these samples relative to the other samples on the gel probably accounts for the increased quantity of proteolytic fragments of vimentin and synemin evident in these lanes of the autoradiogram.

Anti-desmin does not label any of the proteins in these samples, except for the desmin present in the gizzard tissue (not shown).

Fig. 4*a* depicts chicken erythrocyte membrane proteins resolved by IEF/SDS-PAGE. This gel was processed in immunoautoradiography with anti-vimentin, and the resulting autoradiogram is shown in Fig. 4*b*. An identical gel was processed with anti-synemin, and its autoradiogram is in Fig. 4*c*. Proteins readily identified by Coomassie Blue staining include goblin, α and β spectrin, synemin, the multiple isoelectric variants of

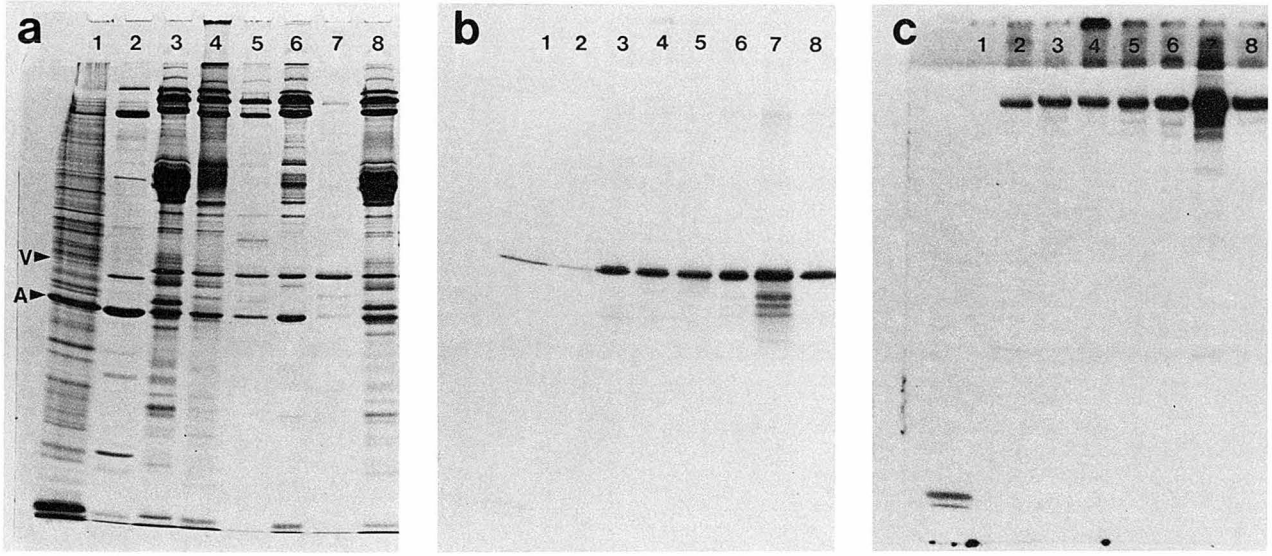

FIGURE 3 Immunoautoradiography using antivimentin and antisynemin. (*a*) Polyacrylamide gel stained with Coomassie Blue after labeling with antisynemin and radioiodinated protein A; (*b*) Autoradiogram of duplicate gel labeled ith anti-vimentin; (*c*) Autoradiogram of gel in (*a*). Samples are from chickens unless otherwise noted. Lane *1*: Buffy coat. The identity of the prominant labeled polypeptide just above the dye front is not known. It labels comparably with both antisera and may therefore be an IgG receptor or protein *A* receptor that survives SDS denaturation and acetic acid/ethanol fixation. Lane *2*: Whole gizzard muscle. Lane *3*: Erythrocyte membranes. Lane *4*: Turkey erythrocyte membranes. Lane *5*: High salt plus detergent residue of turkey erythrocyte membranes. Lane *6*: High salt plus detergent residue of erythrocyte membranes. Lane *7*: Low salt extract of erythrocyte membranes. Lane *8*: Erythrocyte membranes. *A*, actin; *V*, vimentin. Synemin is the major high molecular weight protein in lane *7*; it migrates just beneath α spectrin.

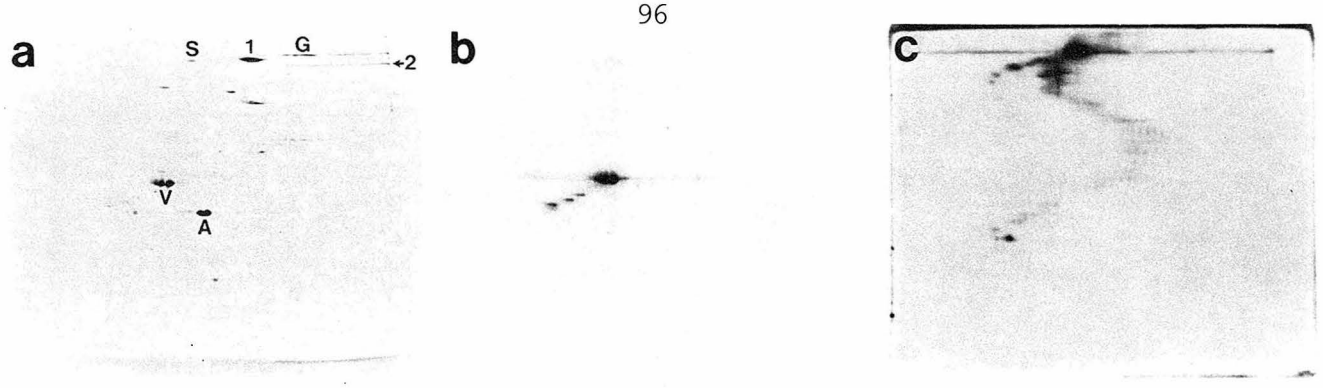

FIGURE 4 Two-dimensional immunoautordiography of chicken erythrocyte membranes using antivimentin and antisynemin. (*a*) Two-dimensional gel of chicken erythrocyte membranes stained with Coomassie Blue after labeling with antivimentin and radioiodinated protein *A*. (*b*) Autoradiogram of same gel. (*c*) Autoradiogram of duplicate gel labeled with antisynemin. *A*, actin; *V*, vimentin; *S*, synemin; *1*, α spectrin *2*, β spectrin; *G*, goblin.

vimentin, and nearly equal amounts of β and γ actin. Antivimentin and anti-synemin label only their respective proteins on the gel and do not crossreact with other proteins in this system. Desmin is not detectable by Coomassie Blue staining, nor by immunoautoradiography with anti-desmin (not shown). The diagonal string of polypeptides smaller and more acidic than vimentin, visible in Fig. 4*b*, represents breakdown products of vimentin (19, 24); the same probably holds true for the numerous polypeptides under synemin that label with antisynemin. Because the vimentin and synemin used for immunization were excised from an SDS-polyacrylamide gel, it seems unlikely that all of these minor polypeptides could be unrelated contaminants. The relative quantity of these other polypeptides increases with increased processing of the samples. They can be reduced in amount or completely eliminated if special precautions are taken to inhibit proteolytic enzymes (19, 25). Also, the same patterns are seen in samples from different tissues (references 19, 24, 25, and below).

To determine the antigenic homology of erythrocyte synemin and gizzard synemin, a fortuitous form of peptide mapping was used. Fragments of synemin generated by endogenous proteases during processing of the tissues were detected with antibodies to synemin as visualized by two-dimensional immunoautoradiography. An antiserum specific for a given protein thus allows visualization of the protein's peptide map without the necessity of prior purification of that protein. The degradation pattern of erythrocyte vimentin as seen in Fig. 4*b* is similar to that already published for muscle vimentin (24).

Fig. 5 compares the synemin present in chicken erythrocyte cytoskeletons and that in chicken gizzard smooth muscle tissue. Fig. 5*a* shows the proteins that remain after extraction of erythrocytes with 1% Triton X-100 in a physiological salt buffer; a major difference in the protein pattern, compared with the membranes in Fig. 4*a*, is the presence of the nuclear lamins. There is also more vimentin relative to the amount of actin present, as this preparation also includes the nucleus-associated intermediate filaments. The autoradiogram of this gel after processing with anti-synemin is shown in Fig. 5*b*. Similarly, a two-dimensional gel of whole gizzard tissue processed with anti-synemin, and its autoradiogram, are shown in Fig. 5*c* and *d*. The similarities in the two synemin patterns are striking (see also Fig. 4*c*); in each case, the parent molecule is most heavily labeled, and the arcs of daughter products terminate in what appears to be a particularly stable fragment at ~34,000 daltons (pI ~4.9).

We have noted no consistent differences between erythrocyte synemin and smooth muscle synemin. Minor differences in the fragment patterns may be attributable to different endogenous proteases, different processing schemes, or slight differences in electrophoresis; the latter two would explain the very minor differences between the erythrocyte synemins in Figs. 4*c* and 5*d*. There is a slight variation in the observed isoelectric points of erythrocyte and smooth-muscle synemin, but there is variation even among different samples of erythrocyte synemin (compare Figs. 2*a* and 4*a*). The focusing of synemin seems to be influenced by the amount of protein loaded on the isoelectric focusing gel; the apparent isoelectric point of synemin is often the same as that of desmin or vimentin if either of the latter is present in large quantities on the gel (Fig. 2*a* and reference 25). We conclude from these immunoautoradiographic data that erythrocyte synemin and muscle synemin are similar if not identical; similarities in solubility properties and cellular distribution (below) strengthen the conclusion that the erythrocyte polypeptide may be regarded as synemin as defined previously in smooth muscle.

Phosphorylation

Goblin is a high molecular weight protein of the turkey erythrocyte plasma membrane characterized by hormone dependent phosphorylation (4). Both goblin and synemin have reported molecular weights of ~230,000 daltons; although their solubility properties and distributions appeared to differ, we thought it was necessary to conclusively determine whether goblin and synemin were indeed different proteins. We identified goblin by its characteristic properties of being a large membrane-associated protein and the most hyperphosphorylated polypeptide in turkey erythrocytes treated briefly with the β-adrenergic agonist, isoproterenol (4). Fig. 6*a* shows a Coomassie Blue-stained gel of membranes of turkey erythrocytes labeled with [^{32}P] inorganic phosphate; those on the left were also treated with isoproterenol, whereas those on the right were not. Fig. 6*b* is the corresponding autoradiogram. By the above criteria, we conclude that the band designated in the figure is goblin. In this gel system, goblin migrates more slowly than the two spectrin variants, rather than migrating between them as in the system of Beam et al. (4). Using their gel system, we found that the electrophoretic pattern of our samples was indeed different: the relative positions of some bands was different, and goblin and the spectrins were not resolved as well.

A 44,000 dalton polypeptide is also noticeably hyperphos-

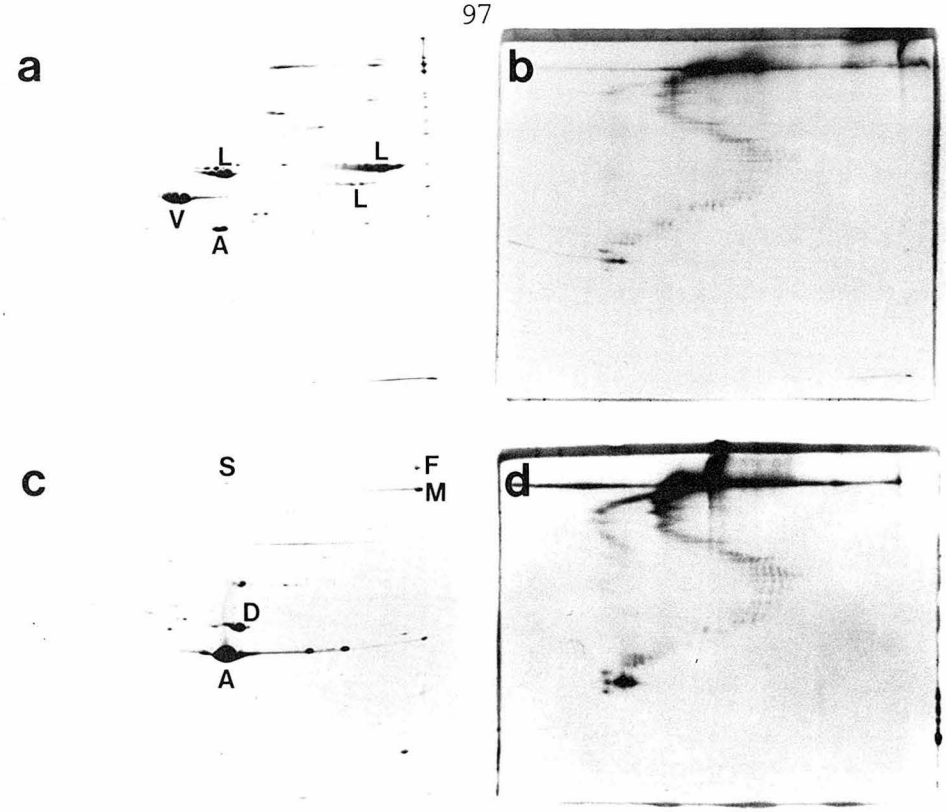

FIGURE 5 Comparison of erythrocyte and gizzard synemins by two-dimensional immunoautoradiography. (*a*, *c*) Two-dimensional gels stained with Coomassie Blue after labeling with antisynemin and radioiodinated protein *A*. (*b*, *d*) Corresponding autoradiograms. Samples are (*a*) a Triton X-100 insoluble cytoskeleton of chicken erythrocytes, and (*c*) whole gizzard smooth muscle tissue. *A*, actin; *D*, desmin; *V*, vimentin; *L*, lamins *A*, *B*, and *C* (references 21 and 52); *M*, myosin; *S*, synemin; *F*, filamin.

phorylated in the presence of isoproterenol (4), and this hormone-dependent phosphorylation seems to apply to a lesser degree to several other proteins as well. Vimentin appears to be one of these, because a slight increase in labeling of this protein with isoproterenol can be detected on the autoradiogram (Fig. 6*b*). This is consistent with the hormone-dependent phosphorylation of vimentin that has been observed in other cell types (D. L. Gard and E. Lazarides, manuscript submitted for publication). Synemin migrates too close to α spectrin on one-dimensional gels to be able to determine whether it is phosphorylated, but two-dimensional gels of erythrocytes phosphorylated to a steady state (1) suggest that synemin is indeed phosphorylated (not shown). These gels also show that all but the most basic isoelectric variant of vimentin are phosphorylated, as is the case with vimentin in other cell types (46, 47).

Localization of Synemin and Vimentin by Immunofluorescence

With antibodies specific for synemin and vimentin, it was possible to determine the spatial distributions of these antigens within the avian erythrocyte by immunofluorescence. The abundance of hemoglobin in these cells was not a problem because synemin and vimentin remained insoluble after removal of the hemoglobin by hypotonic lysis or detergent lysis. Typically, erythrocytes were allowed to attach to Alcian Blue-coated glass cover slips (54), then lysed with a physiological salt solution containing Triton X-100 and magnesium ions, and processed for examination by immunofluorescence microscopy without any fixation. The result with both anti-vimentin and anti-synemin was a cytoplasmic network of sinuous filaments extending from the nucleus to the plasma membrane. This network gave the impression of being composed of a small number of long filaments, since relatively few free ends could be seen. A high concentration of filaments was often noted near the poles of the nuclei (Fig. 7*d*). However, the small size and relatively great depth of field of the cells, coupled with the dense packing of the filaments, prevented adequate resolution of the individual filaments after photographic reproduction of the network. Nevertheless, this technique of immunofluorescence allowed us to determine the effects of Colcemid on the filaments in these cells.

Colcemid has been found to cause an aggregation and bundling of intermediate filaments in a variety of cultured cell types (8, 18, 23, 31, 33, 38). When turkey erythrocytes were incubated with Colcemid under conditions that are normally used for cultured cells, their intermediate filaments did not aggregate. Chicken embryo fibroblasts incubated in the same petri plate showed normal filament aggregation. Fig. 7 shows the distribution of the erythrocyte intermediate filaments after this incubation, as revealed by immunofluorescence using anti-vimentin. The top row depicts cells that received Colcemid, and the bottom row shows control cells that were treated identically, except that they received no Colcemid. After incubation with or without Colcemid, the cells were briefly lysed with Triton X-100 to remove hemoglobin, then fixed with formaldehyde to ensure preservation of filament distribution. There was no obvious Colcemid-induced aggregation of the filaments, and what appeared to be individual filaments could clearly be seen extending to the plasma membrane. No consistent difference between the Colcemid-treated cells and con-

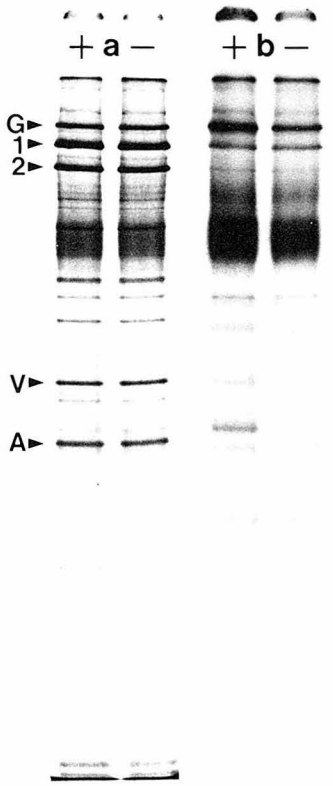

FIGURE 6 Phosphorylation of turkey erythrocyte plasma membrane proteins: identification of goblin. Erythrocytes were incubated for 4 h with ^{32}P-phosphate; half were treated with isoproterenol for the final 20 min. Plasma membranes were isolated and the proteins were resolved by SDS-PAGE. The Coomassie Blue-stained gel was dried (*a*) and autoradiographed (*b*). Goblin (*G*), the high-molecular-weight protein that is most noticeably hyperphosphorylated in the presence (+) of isoproterenol, is distinct from synemin, which runs just beneath α spectrin (*1*). Abbreviations: *G*, goblin; *1* and *2*, α and β spectrin; *V*, vimentin; *A*, actin; + and − refer to the presence and absence of isoproterenol in the incubation mixture.

trols could be detected. (This filament distribution is the same as in erythrocytes that have not been subjected to an in vitro incubation but examined soon after removal from the animal.) Similar results were obtained with anti-synemin. Unfixed cells exhibit a similar filament distribution, but the nuclei shrink somewhat and become more dense during processing. High concentrations of Colcemid (100 μM rather than 5 μM) also had no effect on the intermediate filaments, and cells treated in suspension rather than after attachment to cover slips were similarly unaffected.

To examine the association of the filaments with the membrane and allow visualization of the filament network more clearly, a technique was developed that removed the erythrocyte nuclei and most of the filaments to leave a residual mat of membrane-associated filaments in which the individual strands could be resolved and photographed. This technique is based on ideas that stemmed from a number of sources (3, 26, 35, 44, 54). It involves attaching erythrocytes to Alcian Blue-coated glass cover slips, then disrupting the cells by cavitation (with a sonicator) forcefully enough to remove nuclei and other cellular structures not firmly anchored to the cover slip. This results in a cover slip covered with residual elliptical patches of membrane, each with its most firmly associated structures. Immunofluorescence shows that these patches often have vimentin- and synemin-containing filaments attached to them. This was the first good indication that intermediate filaments might in some way be physically anchored to the erythrocyte plasma membrane. Fig. 8 is a montage of fluorescence micrographs showing the presence and distribution of vimentin and synemin on these residual patches of membrane. Tangled and wavy networks of filaments and fragments of filaments can be visualized. The uniform-diameter filaments probably represent individual intermediate filaments rather than bundles (see electron microscopic correlates in Fig. 9). Their measured diameter of 200–300 nm is close to the resolution limit of the light microscope and consistent with the immunofluorescence image of individual microtubules (49). Anti-desmin gives negligible fluorescence, which is consistent with our inability to detect it electrophoretically. Synemin and vimentin preimmune sera also give negligible fluorescence, and preadsorption of the antisera with the corresponding purified proteins has been shown previously to block fluorescence (25).

The quantity of filaments remaining on the membrane patches is probably a function of the degree of sonication. Sonication was monitored by phase-contrast microscopy and performed at a level that removed most of the nuclei. This produced a wide range of anucleate membrane patches; most patches retained no intermediate filaments (see Fig. 8 *s*), whereas the rest displayed patterns ranging from short fragments (Fig. 8 *l–p*) to complex networks of filaments (Fig. 8 *a–d*). Fig. 8 *s* is a combination phase-contrast/fluorescence micrograph showing bare membrane patches as well as filament-containing patches. It is noteworthy that the remaining filaments are not always distributed uniformly over the membrane patch.

Double immunofluorescence was performed to directly compare the distributions of vimentin and synemin on these patches. Fig. 8 *q* and *r* show that the distributions are the same. Both antigens appear along the same filaments, and nowhere at this level of resolution is one antigen present and the other absent. However, the synemin fluorescence sometimes gives the impression of being slightly punctate along filaments that show uniform vimentin fluorescence.

We have obtained comparable immunofluorescence results with cells sonicated in low ionic strength and physiologic salt buffers, by following sonication with a detergent/high-salt extraction, and with subsequent incubations and rinses in the presence or absence of Triton X-100. In none of the preparations shown in Fig. 8 were the samples fixed with protein crosslinkers or denaturants.

Ultrastructure of Sonicated Membranes

Sonicated erythrocyte membrane patches on glass cover slips, similar to those used for immunofluorescence, were fixed and rotary-shadowed for examination by transmission electron microscopy. Fig. 9 shows portions of three such membrane patches with their adherent filaments. The pattern of the filaments is similar to the pattern seen in immunofluorescence; the filaments tend to be relatively long, and can be straight, wavy or curved into loops. Occasionally, the filaments extend beyond the edge of the membrane patch (Fig. 9 *b* and *c*), presumably as a result of the sonication. Similar patterns are obtained if the erythrocytes are sonicated in a physiologic salt buffer without previous hypotonic lysis, showing that the filaments are not precipitated on the membranes as a result of the low-salt treatment. Treatment with Triton X-100 also does not

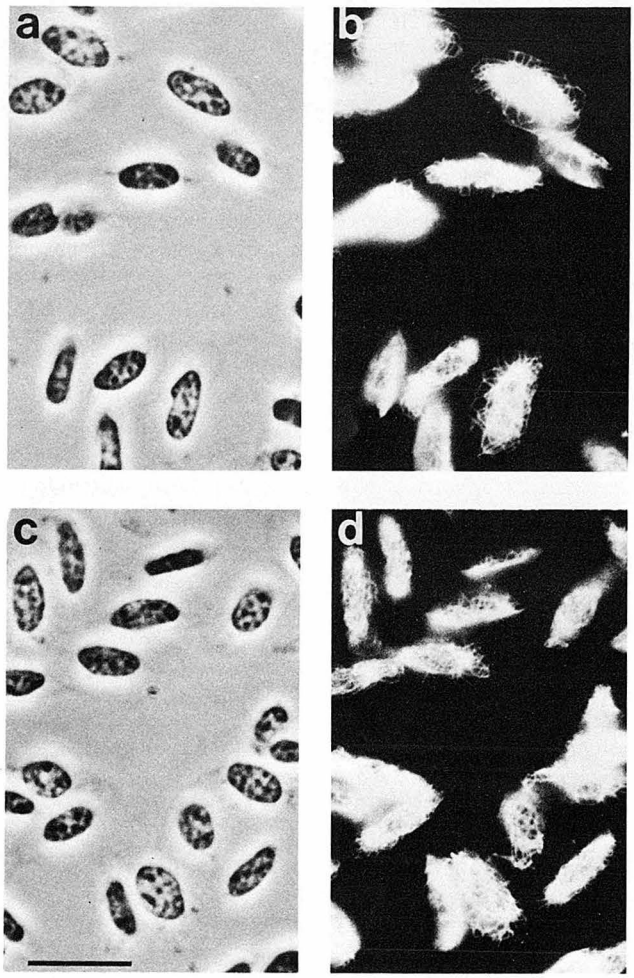

FIGURE 7 Distribution of vimentin in Colcemid-treated erythrocytes. Phase (*a*, *c*) and corresponding fluorescence micrographs (*b*, *d*) of turkey erythrocytes incubated in culture medium with (*a*, *b*) or without (*c*, *d*) Colcemid. Intermediate filaments were visualized in fixed cytoskeletons by indirect immunofluorescence using anti-vimentin. Similar patterns were obtained with antisynemin. Bar, 10 μm. × 1350.

affect this pattern. Strands of chromatin originating from the erythrocyte nuclei have a distinctive morphology and are evident in the replicas only when magnesium ions are omitted from the lysis or sonication buffers. The relatively flat, coarse, upper face of the membrane patch probably represents the spectrin network that lines the cytoplasmic surface of the plasma membrane. The intermediate filaments are of fairly uniform diameter; it is not known whether the frequent, slight bulges, constrictions, and discontinuities have a molecular basis or are just an artifact of the shadowing procedure (cf. reference 28). Detail cannot be resolved sufficiently to determine whether the filaments branch or merely associated laterally in various places. We have not been able to positively identify structures that might anchor the filaments to the membrane patch, and it is not evident from these micrographs how abundant such linkers might be. From the distribution of the filaments on the sonicated membranes, it appears likely that they are not randomly distributed over the plasma membrane.

DISCUSSION

Intermediate Filaments in Avian Erythrocytes

Avian erythrocytes provide a simple system for the study of intermediate filaments. These cells are terminally differentiated, nondividing, nonadherent, and nonmotile. They are easily obtained and purified. Their cytoplasms are relatively simple and nondynamic. Interaction of intermediate filaments with both the nucleus and plasma membrane can thus be examined without the variability and complexity inherent in most other cell types. Comparison of the avian erythrocyte with the well characterized mammalian erythrocyte provides insight into the functions and relationships between components that are not common to both, such as nuclei, microtubules and intermediate filaments.

In this study we demonstrate that vimentin and synemin are the major components of avian erythrocyte intermediate filaments. These filaments had previously been postulated to be composed of vimentin (63), and vimentin indeed appears to be their major subunit. Vimentin and synemin appear to coexist uniformly in the filaments, which form a looping, intertwined network in the cytoplasm. A portion of this filament network is associated with the plasma membrane firmly enough to resist detachment by physical disruption forces that are sufficient to remove the nucleus and fragment the plasma membrane. However, synemin and vimentin can be selectively released from the plasma membrane by treatment with divalent cation-free, low ionic strength solutions.

We have used chicken and turkey erythrocytes as representatives of avian erythrocytes in general for this work. Immunofluorescence was performed on turkey erythrocytes, because they are slightly larger than chicken erythrocytes. The protein goblin had been defined in turkey erythrocytes (4), so we used these cells for the phosphorylation experiments. Biochemical studies were based primarily on chicken erythrocytes, which were more readily available than turkey erythrocytes; electron microscopy of thin sections was performed on chicken erythrocytes to correlate with the biochemistry. However, it is evident from electrophoretograms such as in Fig. 3 *a* that chicken and turkey erythrocytes are not identical. For example, turkey erythrocyte membranes have a smaller β-spectrin and less protein in the 100,000 dalton cluster and 44,000 dalton band than chicken erythrocyte membranes. Vimentin and synemin, though, appear to be very similar, if not identical, as judged by IEF/SDS-PAGE and immunoautoradiography (not shown). We believe, therefore, that the generalizations made about vimentin and synemin in this paper are likely to be applicable to all avian erythrocytes.

Studies of Intermediate Filaments Associated with the Plasma Membrane

Our studies of the properties of erythrocyte intermediate filaments have focused on those filaments that fractionate with the plasma membrane. They have the same antigenicity and electrophoretic mobility as the filaments that fractionate with the nuclei, so we regard them as equivalent in terms of basic composition and properties. Whether a given segment of filament will fractionate with the nucleus or plasma membrane may be variable and depend as much on the homogenization conditions as on the spatial arrangement of the filaments.

We have developed an enucleation technique that gives fair yields of whole plasma membranes and large pieces of membrane (referred to here simply as "erythrocyte membranes"). Many of these membranes retain large networks of intermediate filaments; in a given thin section, though, many do not appear to possess any associated filaments. This may be a result of loss during enucleation, initial lack of attachment of the filaments to certain regions of the plasma membrane, absence

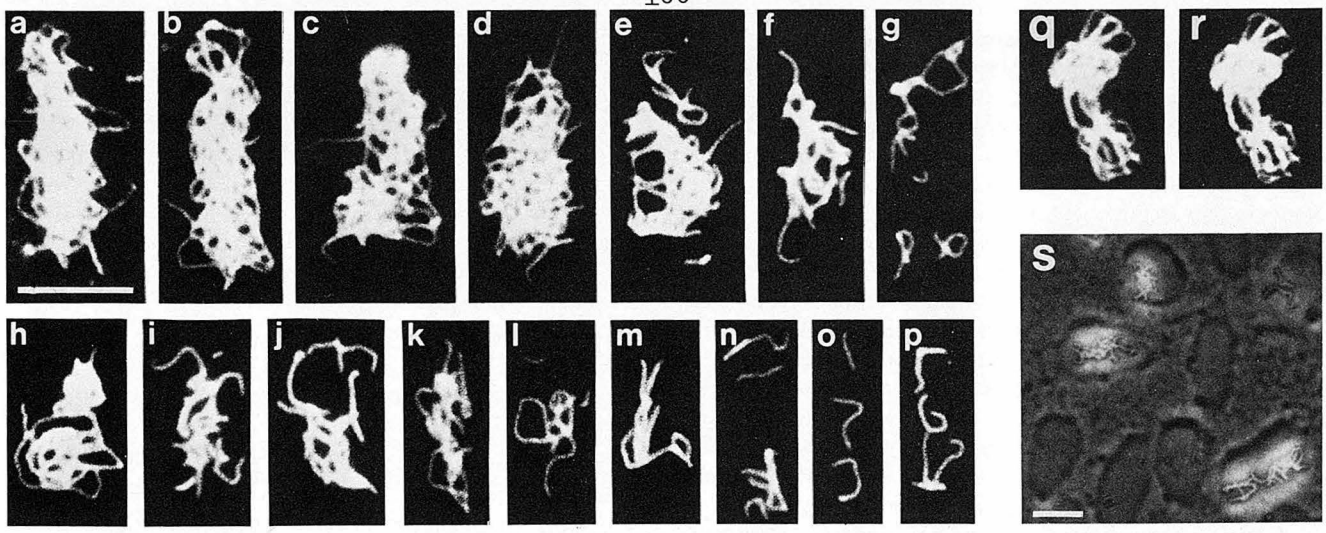

FIGURE 8 Immunofluorescence of turkey erythrocyte intermediate filaments. Erythrocytes adhering to cover slips were hypotonically lysed and sonicated to remove overlying membranes and nuclei. Intermediate filaments remaining attached to the resulting patches of plasma membrane were visualized by immunofluorescence using antibodies to synemin (*a*, *r*) or vimentin (*b*–*q*, *s*). Specimens were not fixed, and all but *a*, *b*, *c*, and *h* were treated with Triton X-100. Micrographs a–p are indirect immunofluorescence images; *q* and *r* demonstrate colocalization of vimentin (*q*) and synemin (*r*) by double immunofluorescence. Micrograph *s* is a combination phase/fluorescence image showing the distribution of vimentin on the elliptical patches of plasma membrane; note that many patches are devoid of filaments. Bars, 5 μm. *a*–*r*, × 3040; *s*, × 1330.

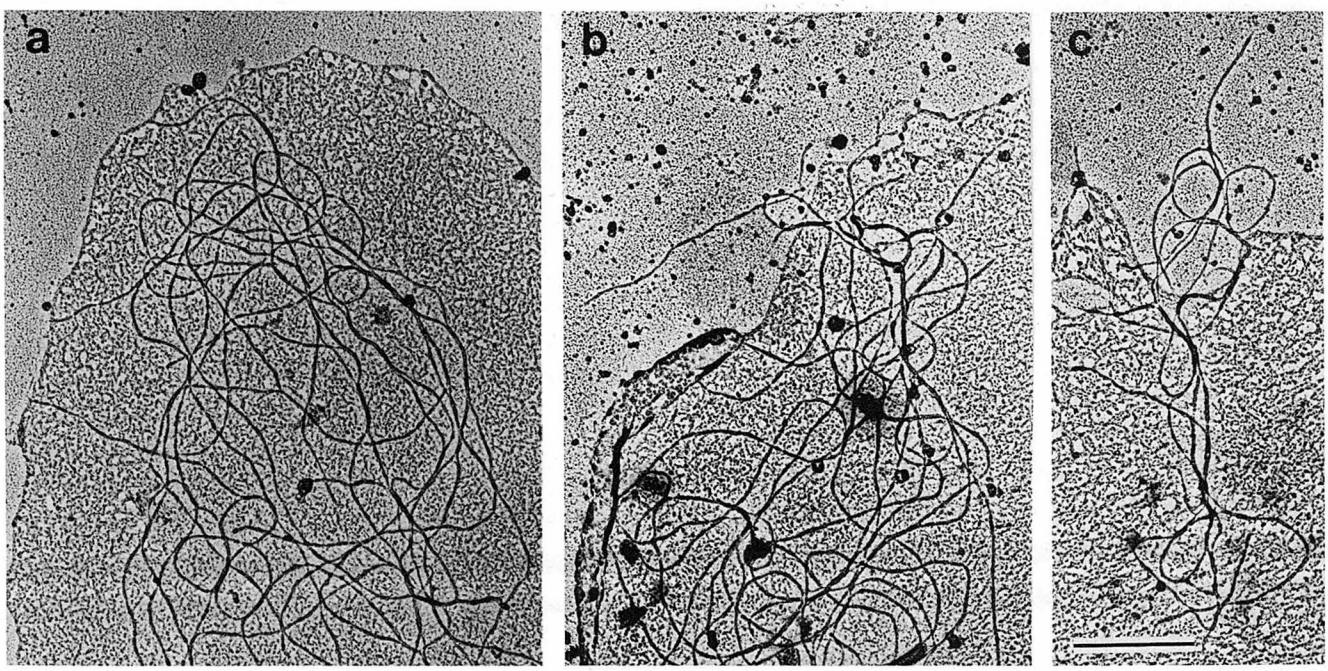

FIGURE 9 Platinum replicas of sonicated chicken erythrocyte ghosts. Samples were prepared as in Fig. 8, then fixed, dried, and rotary shadowed with platinum for examination by transmission electron microscopy. Intermediate filaments can be seen on patches of plasma membrane that remained attached to the cover slip during sonication. In *b* and *c*, a portion of the filaments have fallen beyond the edge of the membrane patch. Magnification: Bar, 1 μm. × 16,000.

from certain regions of the erythrocyte cytoplasm, or a close apposition to the plasma membrane that renders the filaments unresolvable.

Previous studies involving isolation of the avian erythrocyte plasma membrane by differential centrifugation after mechanical disruption of the cells have relied on pressure-release homogenization (7, 14, 61), sonication (4, 27), rotating blades (11, 66), or a tight-fitting Dounce (12, 22) or Potter-Elvehjem homogenizer (6). However, the presence of filaments associated with the isolated membrane fragments was noted only rarely (27), and, in comparisons to mammalian erythrocyte membranes, the presence of an extra polypeptide, similar in molecular weight to vimentin, was rarely mentioned (11). Some of these disruption techniques produce very small membrane fragments that may be largely stripped of filaments; alternatively, the filaments may assume a distribution or configuration in which they are not readily identifiable by electron microscopy. The gentler disruption techniques appear to produce membrane fragments similar to those in this study, but associated filaments have tended to escape detection. Intermediate

filaments have been most apparent in detergent-insoluble cytoskeletons of whole erythrocytes examined by thin sectioning or negative staining (59, 63).

Treatment of avian erythrocyte membranes with certain low ionic strength solutions removes the associated intermediate filaments. Filaments can no longer be found with the membranes in thin sections, and the low-salt extract contains nearly pure vimentin and synemin. This release seems to depend on low ionic strength and absence of divalent cations and be independent of reducing agents or nonionic detergents. Our highest yields have been obtained using distilled water. Roughly 60–90% of the vimentin is released after 1 min of extraction with distilled water. Selective release of vimentin and synemin, as compared to spectrin and actin, is enhanced by low temperature and brevity of treatment. Because the released vimentin and synemin cannot be sedimented by centrifugation for 5 h at 240,000 *g*, yet appear to comigrate in a gel filtration column with an exclusion limit of 15 million daltons (unpublished observations), they must exist in solution as some sort of multimeric complex or oligomer. This implies that the filaments break down or partially depolymerize during or after release from the membranes. Solubility in low salt has similarly been described for other preparations of native intermediate filaments (29, 30, 50, 51, 55, 57). These extraction conditions may thus be resulting in a dissolution of the filaments rather than a dissociation of the filaments from the membranes. It is conceivable that these extraction conditions have no disruptive effect on the anchorage points of the filaments to the membranes, which would explain why some of the vimentin remains with the membranes after extensive extraction with water. This vimentin may be a distinct population associated with anchorage points in the form of tightly bound monomers or oligomers or short segments of filament not resolvable in thin sections. These extraction data thus do not permit a conclusion about the nature of attachment of the filaments to the membranes. It can only be stated, based on the physical data of enucleation and sonication, that at least some of the intermediate filaments in avian erythrocytes are somehow anchored to the plasma membrane, and that this attachment is stable in the presence of physiologic salt, high salt, and nonionic detergent.

Comparison of Erythrocyte Proteins

An aspect of comparative biochemistry exemplified by this study is the difficulty of comparing protein profiles of a given preparation by different SDS-PAGE systems. Although useful for general comparisons, different gel systems may not be directly comparable with regard to specific polypeptides. There has classically been disagreement between different investigators about calculated molecular weights; even the relative positions of different polypeptides may not be consistent in different gel systems (for example, the high molecular weight proteins shown in this paper—see Results). This stresses caution in identifying a polypeptide solely by its mobility on an SDS-polyacrylamide gel. Our electrophoretic profiles of avian erythrocyte membrane proteins differ from those of other laboratories, which also differ among themselves (2, 4, 7, 11, 12, 34, 60, 61); some of these differences have been noted and attributed to endogenous proteases or proteases present in contaminating leukocytes (11, 34, 61). Extrapolation from one class to another (for example, mammalian (16) to avian erythrocyte membranes) may not be justified either and may lead to erroneous identification of polypeptides. Two-dimensional gel electrophoresis makes polypeptide identification less ambiguous, because another parameter (isoelectric point) is taken into account and has proved useful for several proteins in this study. Nevertheless, other (nonelectrophoretic) evidence for the identity of a protein band on a gel is essential. We have used immunologic and solubility properties, in addition to electrophoretic characteristics, to identify synemin and vimentin in avian erythrocytes, and phosphorylation characteristics to identify goblin (4). Determination of why similarly prepared samples show not only different relative mobilities but also different relative amounts using different gel systems awaits further study.

The Effects of Colcemid

One indication of a functional or interactive difference between the intermediate filaments of avian erythrocytes and most other cell types grown in vitro is the insensitivity of the former to Colcemid. Treatments with Colcemid that will cause aggregation and perinuclear bundling of intermediate filaments in most cultured cells (8, 18, 23, 31, 33, 38) appear to have no effect on the filaments of erythrocytes. Colcemid sensitivity might thus be a function of how dynamic a cell is, or perhaps its state of differentiation, as appears to be likely for skeletal muscle cells (20, 25) but not be an intrinsic property of the filaments themselves. Related to this may be the observation that chick erythrocyte marginal band microtubules are resistant to depolymerization by Colcemid (5).

Intermediate Filament Proteins

Synemin was originally found to be associated with intermediate filaments in smooth and skeletal muscle (25). Here we show that synemin is not a muscle-specific protein but is present as well in at least one nonmuscle cell, the mature avian erythrocyte. The original study also raised the possibility that synemin was a desmin-associated polypeptide; here we show that synemin can also exist and associate with vimentin. In both muscle cells and erythrocytes, synemin appears to be a component of the same filaments that contain desmin and vimentin, as determined by double immunofluorescence. Densitometric scans of Coomassie Blue-stained polyacrylamide gels of preparations of vimentin and synemin from chicken erythrocytes give a vimentin-to-synemin ratio of ~50:1. This is similar to the ratio obtained for desmin and synemin in smooth muscle and suggests a constant stoichiometry between synemin and intermediate filaments of different subunit composition. This ratio is a very rough estimate, not taking into account differential proteolysis of the proteins during processing and possible nonlinearity in dye binding and densitometry, and should therefore not be regarded as the true ratio. It is useful, however, for rough comparisons of different systems.

We have taken advantage of a novel form of two-dimensional peptide mapping to compare synemins from different tissues. This combination of partial hydrolysis of tissue proteins by endogenous proteases and two-dimensional immunoautoradiography has demonstrated a high degree of homology between synemins from avian smooth muscle and erythrocytes. Both molecules exhibit an S-shaped string of fragments that terminates in a proteolytically stable peptide of 34,000 daltons. This technique is extremely sensitive, detecting peptides much too scarce to be seen by Coomassie Blue staining, but does not resolve the high molecular weight peptides sufficiently to allow detailed comparisons. Also, slight variations from gel to gel do

not allow us to conclude that the synemins we are examining are identical. Minor differences in the maps may be artifactual or may reflect functional differences in the molecule, perhaps related to whether synemin is found in association with desmin or with vimentin.

These data do not address the question of whether synemin is an integral or an associated filament protein, or what its properties are independent of desmin and vimentin. The large size and paucity of synemin relative to desmin and vimentin tend to favor a role for synemin as an associated polypeptide. Perhaps it is analogous to the high molecular weight polypeptide of neurofilaments, which appears to be wrapped helically around the core filament (62), where it may function to stabilize the filament, promote assembly (45), or mediate interactions with other molecules or organelles.

The presence of nonmicrotubular filaments in nucleated erythrocytes has been known for some time (27, 36), but only recently were they identified as intermediate filaments (59, 63). These filaments were usually noted and studied in relation to the nucleus or nuclear membrane. In this paper we show that they also exhibit a close association with and apparent anchorage to the plasma membrane, and that they contain the intermediate-filament subunits vimentin and synemin. Nucleated erythrocytes may thus be an ideal model system for the study of filament-membrane interactions and for examining intermediate filament nucleation, assembly, and deployment during differentiation.

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Chapter 6

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Structural Associations of Synemin and Vimentin Filaments in Avian Erythrocytes Revealed by Immunoelectron Microscopy

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Running title: Erythrocyte Intermediate Filaments

Summary

Intermediate filament structure and distribution were studied by antibody decoration and low angle shadowing of sonicated chicken erythrocytes and embryonic erythroid cells. Intermediate filaments containing vimentin and synemin form a three-dimensional network in these cells, interlinking the nucleus and plasma membrane. This filament network is spatially segregated from the marginal band of microtubules, indicating that these two systems do not interact directly in the development or maintenance of cell shape. Incubation of sonicated cells with an antiserum specific for vimentin results in uniform decoration of the intermediate filaments; incubation with anti-synemin results in decoration of periodically-spaced foci. Measurement of the synemin periodicity under a specified set of sample preparation conditions gives average values of 180 nm for adult erythrocytes and 230 nm for 10-day embryonic erythroid cells, suggesting some fundamental change in the structure of the filaments during erythropoiesis. Registration of these foci in laterally associated filaments, and decoration of bridges between slightly separated filaments, suggest that synemin mediates crosslinking of intermediate filaments through self-interaction.

Introduction

The cytoskeleton of mammalian erythrocytes has been extensively characterized, while that of avian erythrocytes has received little attention. Mammalian erythrocytes are anucleate and virtually devoid of cytoplasmic organelles; a network based on the protein spectrin lines the inner surface of their plasma membrane and serves as their cytoskeleton (Branton, Cohen and Tyler, 1981). Avian erythrocytes possess nuclei and occasional organelles such as mitochondria; in addition to a spectrin based plasma membrane cytoskeleton, these cells also possess a circumferential bundle of microtubules known as the marginal band (Fawcett and Witebsky, 1964; Behnke, 1970a), and a system of cytoplasmic filaments that spans from the nucleus

to the plasma membrane (Harris and Brown, 1971). These latter filaments were recently identified as intermediate filaments (for a review of intermediate filaments, see Lazarides, 1980), based on their solubility properties and ultrastructural morphology (Virtanen, Kurkinen and Lehto, 1979; Woodcock, 1980).

A new means of visualizing the intermediate filaments of avian erythrocytes was recently developed (Granger, Repasky and Lazarides, 1982). Cells adhering to a flat glass substrate were disrupted by sonication, and their exposed, insoluble cytoskeletons were shadowed with platinum. Examination of the resulting replicas by transmission electron microscopy revealed a fraction of the intermediate filament network stably associated with the plasma membrane. Similarly, mechanical enucleation of erythrocyte ghosts in suspension resulted in the isolation of a plasma membrane fraction rich in intermediate filaments. Biochemical and immunological characterization of these filaments demonstrated that their major component is vimentin (Granger et al., 1982), a 52,000 dalton polypeptide characteristic of filaments in mesenchymally-derived cells (Franke et al., 1978). A minor component of the filaments was shown to be synemin, a 230,000 dalton polypeptide found in association with desmin filaments in smooth muscle, and with desmin and vimentin filaments in skeletal muscle (Granger and Lazarides, 1980). Immunofluorescence of erythrocyte cytoskeletons showed that vimentin and synemin coexist in the same filaments, and form a cytoplasmic network linking the nucleus and plasma membrane.

These immunological and electron microscopic techniques have been combined in this study to investigate the structural relationships of synemin and vimentin in chicken erythrocyte intermediate filaments. Decoration of the filaments with specific antibodies indicates that vimentin forms the core polymer, and synemin exists as an associated polypeptide spaced at regular intervals along the filament. The anti-synemin decoration further demonstrates a registry of laterally associated

filaments and reveals a possible role for synemin as a mediator of intermediate filament crossbridging. A difference in the synemin spacing between adult erythrocytes and embryonic erythroid cells suggests a change in the structure of the filaments during differentiation. In addition, these studies show that the intermediate filament network and marginal band are spatially segregated, and that the spectrin-based plasma membrane cytoskeleton is anisotropic in relation to these two systems.

Results

Antibody Decoration

Chicken erythrocytes were allowed to settle onto cationized (Alcian blue coated) glass coverslips. Nonadherent cells were rinsed off, the adherent cells were hypotonically lysed, and the monolayer of nucleated erythrocyte ghosts was disrupted by sonication. This results in the removal of nuclei and areas of overlying (nonadherent) plasma membrane, leaving elliptical patches of plasma membrane firmly attached to the coverslip (cf. Jacobson and Branton, 1977). These patches will hereafter be referred to as membrane patches, even though they contain, in addition to a lipid bilayer and its intrinsic proteins, a spectrin-based cytoskeleton on the exposed surface (see Branton et al., 1981; Kalish et al., 1978; Repasky, Granger and Lazarides, 1982). The minimal degree of sonication necessary for this cell disruption often leaves networks of intermediate filaments anchored to the adherent patches of membrane (Granger et al., 1982), which can be visualized in the transmission electron microscope (TEM) after dehydration and low angle shadowing with platinum (Figure 1a).

A previous study using biochemical and immunofluorescence techniques identified vimentin and synemin as the major components of these filaments (Granger et al., 1982). To investigate the spatial relationships of vimentin and synemin within the filaments at the ultrastructural level, sonicated chicken erythrocytes were incubated with appropriate antibodies prior to shadowing and viewing by

TEM in order to "decorate" the corresponding antigens. Decoration manifests itself as an increase in the dimensions of the antigenic structure, due to the binding of specific antibodies. Nonspecific decoration under the conditions employed in this study was ruled out by incubation of preparations similar to that depicted in Figure 1a with several samples of normal rabbit serum; none of these sera resulted in detectable decoration of the filaments or any other cellular structures (shown for synemin preimmune serum in Figure 1b).

Incubation with anti-vimentin results in uniform decoration of all of the intermediate filaments (Figure 1c). Using the low-angle shadowing technique, this antiserum results in an approximate doubling (to 40-50 nm) of the diameters of the intermediate (9-10 nm) filaments, which is consistent with the dimensions of immunoglobulins (10-12 nm; Valentine and Green, 1967) and the thickness of the deposited layer of platinum (5-6 nm). No consistent axial periodicity or helicity in the decoration was observed with this antiserum, which was an ammonium sulfate fractionated, polyclonal rabbit antiserum directed against chicken vimentin. Decoration of the filaments with different fractions of IgG obtained by DEAE-cellulose chromatography of this antiserum also did not reveal periodicity (see Figure 2a), nor did very high dilutions of the antiserum or different bleeds with (presumably) different micro-specificities. Also, formaldehyde fixation of the filaments before decoration, and slightly chaotropic conditions (such as high pH or high ionic strength) after decoration, both failed to reveal any consistent periodicity in the decoration. This is in contrast to a 31 nm periodicity in vimentin filaments in gerbil fibroma cells that has been visualized using a monoclonal IgM antibody (Blose, Matsumura and Lin, 1982), but similar to the decoration pattern of vimentin filaments in cultured mouse cells seen with another polyclonal anti-vimentin (Heuser and Kirschner, 1980). No decoration was observed using antibodies specific for desmin (not shown; see Granger et al., 1982).

Incubation of the sonicated erythrocytes with anti-synemin results in a pattern of decoration completely different from that observed with anti-vimentin; namely, discrete, periodically spaced foci along the lengths of all of the filaments (Figure 1d). Even though undecorated filaments appear uniform in diameter, with no visible appendages, the anti-synemin binds to sites that are present primarily on the side of the filament, rather than distributed with radial symmetry about the axis of the filament. This asymmetry is more evident in the high magnification images of Figure 2.

No decoration of the internodal segments of filament (i.e., between decorated foci) is evident with this antiserum. The diameter of these segments is the same as that of filaments exposed to no antiserum or incubated with preimmune serum. Amplification of the decoration with a secondary antibody (incubation of the filaments with goat anti-rabbit IgG after the primary incubation with rabbit anti-chicken synemin) also fails to reveal any antigenic sites in the internodal segments (Figure 2c). Further amplification with a ferritin conjugated secondary antibody similarly results in bare internodal segments (Figure 2d). The only effect of these secondary antibodies is to increase the size of the decorated foci. Conversely, incubation with very high dilutions of the anti-synemin alone (serum diluted 300-fold instead of the usual 30-fold) produces very small decorated foci, appearing as buttons rather than knobs on the filaments (not shown). The decoration pattern is not altered by prefixation of the filaments with formaldehyde, or use of immunoglobulins fractionated with DEAE-cellulose (not shown).

Although these replicas of intermediate filaments often do not clearly distinguish individual filaments from laterally-associated filaments or bundles of filaments, it is evident that many of the linear segments seen in these dense networks are indeed composed of more than one filament. The similarity between the axial spacing of the anti-synemin decoration in filament bundles and in presumed individual

filaments (e.g., as in Figure 2d) indicates an axial registry of laterally-associated filaments. The unit of registry must have the same length as the periodicity of the synemin nodes, or else bundles of two or more filaments would exhibit a shorter average node spacing than individual filaments.

This view is strengthened by the micrographs of embryonic filaments in Figure 2e and f, which show more clearly the registry of laterally associated filaments. Figure 2f shows that the membrane-associated filaments present in embryonic erythroid cells are much less numerous than in the adult (see also below); alignment of the decorated knobs on individual filaments is evident. Where the filaments are slightly separated, these knobs appear to join end-to-end to form a bridge between the filaments (arrowheads in e and f). The simplest interpretation of these micrographs is that synemins of adjacent filaments can self-associate and thus mediate lateral registry and crosslinking of the filaments. Figure 2e suggests that such bridges may contain more than two synemins, since more than two filaments appear to be connected. Additional support for a filament-linking role of synemin is provided by the observation that filament intersection points usually coincide with decorated synemin nodes (Figure 2b-f). This suggests that internodal segments of filaments are not crosslinked, and that the only crosslinking occurs at points corresponding to synemin decoration. Similar patterns are obtained if the filaments are fixed with formaldehyde before incubation with anti-synemin (not shown).

No consistent angular orientation of individual sidearms with respect to the filament axis has been noted; therefore, polarity of the filaments, if it indeed exists, is not discernible under these conditions. Also, stereo micrographs of individual filaments suspended between the nucleus and plasma membrane of partially-disrupted erythrocytes (as in Figure 4a) have failed to reveal any consistent helicity in the orientation of successive decorated synemin nodes (not shown).

Quantitation of the spacing in adult erythrocytes prepared as outlined above

gives a synemin node spacing of 180 nm ± 40 nm (mean ± S.D.; n = 1220; from 12 cells). However, erythroid cells from chicken embryos at 10 days of incubation, prepared in the same manner, show a synemin node spacing of 230 nm ± 50 nm (mean ± S.D.; n = 811; from 9 cells). In both of these cases, different preparations photographed at different magnifications give similar distributions; different dilutions of the anti-synemin, and application of a secondary antibody, also have no marked effect on the measured spacing. However, if adult erythrocytes are sonicated in a physiological salt solution instead of in a hypotonic solution, the average synemin node spacing is decreased by about 20 nm. Fixation of the filaments before anti-synemin application also seems to decrease the spacing slightly. This indicates that the spacing of the nodes is, to a certain extent, experimentally manipulable, but that the values obtained are reproducible in different samples prepared under the same conditions. The relative broadness of the frequency distributions (relatively high standard deviation) is probably a reflection of differential shrinkage artifacts caused by fixation and dehydration; it seems reasonable to assume that the standard deviation of the synemin spacing in vivo is considerably less than 40 nm. The greater deviation of the embryonic erythroid cell values is probably not a result of these cells being at different stages of maturation, since measurements from individual cells gave the same statistical values. However, the magnitude of the difference between the average values obtained for embryonic erythroid cells and adult erythrocytes (prepared under the same conditions) argues against this particular difference being experimentally induced.

Fortuitous support for the reality of this spacing difference comes from immunofluorescence: Adult erythrocyte intermediate filaments appear fairly uniformly labeled by anti-synemin and fluorescein-conjugated anti-IgG when viewed in the fluroescence microscope, with only a hint of periodicity (Granger et al., 1982). Embryonic erythroid cell filaments, however, appear distinctly punctate

when viewed under the same conditions (E. A. Repasky, unpublished observations). Since the limit of resolution for two point-sources of light with this wavelength (emission maximum at 525 nm; Chen, 1969) is approximately 230 nm (objective N.A. = 1.4; $R = 0.61\lambda/N.A.$), it is clear that the difference in synemin spacing between adult and embryonic filaments (180 nm vs. 230 nm in electron micrographs) is indeed potentially observable by light microscopy.

Topology of Membrane Associated Filaments and Microtubules

When a concentrated suspension of erythrocytes settles onto an Alcian blue coated coverslip, the cells adhere in different positions. Most cells adhere with the plane of the marginal band parallel to the coverslip, so that nearly half of the cell's plasma membrane is in contact with the coverslip; sonication then leaves an elliptical patch of membrane corresponding to one face of the originally-biconvex disc (as in Figure 1). The rest of the cells adhere with the plane of their marginal band at an angle (up to 90°) to the coverslip, so that regions of membrane from the edge of the disc (adjacent to the marginal band) are left after sonication. If hypotonic lysis and sonication are performed under conditions in which microtubules are stabilized, marginal band microtubules often remain on these membrane patches (Figure 3a). This attests to the adhesion of the marginal band to the plasma membrane or its spectrin-actin lining (cf. Wolfe, 1965, 1967). However, even under depolymerization-inhibiting conditions, many of the microtubules are lost during sonication. In this case, physical evidence of the marginal band microtubules remains on the membrane patches: low, thin, membrane patches: low, thin, parallel ridges ("tracks") are visible on the otherwise flat inner surface of the patch. Each ridge corresponds to a microtubule, as is evident when both structures are visible on the same patch (Figure 3b). Under the usual lysis and sonication conditions, microtubules are very rarely observed, yet the tracks are very common (Figure 3c; see also Figure 1). They are found along

the long axis of highly elliptical (disc edge) patches and near the edges of the less elliptical (disc face) patches. Even though the marginal band is continuous, the tracks seem to be absent from the membrane at the poles of the cell; abrupt terminations of the tracks that do not coincide with the edge of a membrane patch are evident in Figures 3a, 3c, 4b and 4d.

Knowledge of the position of the marginal band in these preparations allows determination of its spatial relationship to the intermediate filament network. Membrane patches on which both intermediate filaments and microtubules (or tracks) are visible always show spatial segregation of these two systems. The intermediate filaments do not occupy the area of each erythrocyte disc face within 1-2 μm of the marginal band; this defines an annular zone of plasma membrane to which the filaments are not firmly attached. Those cases in which intermediate filaments do approach or contact the microtubule tracks may be artifacts of sonication, since the filaments also occasionally extend beyond the margin of the membrane patch and onto the substrate (see Figures 3b, 4b and 4c).

Spatial Arrangement of Cytoplasmic Intermediate Filaments

In the range of sonication intensities employed in this study, most of the erythrocytes are ultimately visualized at either an initial (intact) stage or a final (bare patch of membrane) stage. Whereas the above examples of intermediate stages elucidate the topology of the filaments with respect to the plasma membrane, the three-dimensional arrangement of the filaments and their relationship to the cytoplasm and nucleus can be discerned by examination of cells that have been only slightly disrupted.

Figures 4 and 5 show several examples of partially disrupted chicken erythrocytes. Figures 4a and b show cells in which the nuclei and part of the overlying plasma membranes are still present. The replica in Figure 4a was freed of organic

material with bleach, while that in 4b was not; the inherent electron density of the nucleus obscures surface features that become visible upon removal from the replica. Intermediate filaments are prominent on the lower plasma membranes, and can be seen to attach to the nucleus and upper membrane in Figure 4a. Figure 4c is an enucleated erythrocyte with part of the upper membrane folded back. The tensile strength of the membrane is evidently greater than that of the filaments that anchor the nucleus to it. Elliptical mats of filaments on the elliptical patches of adherent membrane are occasionally devoid of filaments at their centers (Figure 4d); of all the membrane-associated filaments, those in the center are closest to the nucleus, and may therefore be most susceptible to detachment from the plasma membrane during mechanical enucleation.

Figure 5a and b demonstrate that intermediate filaments directly link areas of plasma membrane on opposite halves of the erythrocyte disc; filaments can be seen to span from the lower, adherent region of membrane to the torn, overlying region of membrane.

Embryonic Erythroid Cells

Erythroid cells from chicken embryos at 10 days of incubation were prepared for electron microscopy in the same manner as the adult erythrocytes. At this stage of embryogenesis, approximately 25% of the circulating erythroid cells are primitive series (an initial, transient population of erythroid cells) and 75% are definitive series (the sustained population that appears after about 5 days of incubation). The primitive cells exhibit different sizes but a similar stage of maturation; the definitive cells are at various stages of maturation, with about half erythroblasts and half mature cells (Bruns and Ingram, 1973). Figure 6a is an example of a field of 10-day embryonic erythroid cells. Although unequivocal identification of the series or maturation stage of a cell that left a given patch

of membrane cannot be made, the following generalizations can be made with reference to adult cells (cf. Lucas and Jamroz, 1961; Edmonds, 1966; Ceresa-Castellani and Leone, 1969; Bruns and Ingram, 1973; Barrett and Dawson, 1974). Both spherical and oval cells are present, giving rise to both round and elliptical membrane patches; however, embryonic erythrocytes tend to be less elliptical than adult erythrocytes. Relatively few of these patches of plasma membrane possess intermediate filaments; those that do usually contain fewer filaments than are normally seen on adult erythrocyte membranes. These filaments often form hairpin loops, with the loop near the periphery of the membrane patch and its base near the center (Figure 6a). The stalks of these hairpin loops often appear to be helically coiled, such that the two antiparallel segments of the same filament form a double helix (Figure 6b). Another commonly observed feature on the membranes is closed circles of intermediate filaments; these circles have contour lengths of 1-10 μm (usually 3-4 μm), and often have one or two helical twists that result in a figure-8 pattern (Figure 6c and d).

Decoration of these filaments with antibodies specific for vimentin and synemin gives patterns similar to those observed for adult erythrocytes (shown for synemin in Figure 2e and f). However, as described above, the synemin periodicity of the embryonic filaments is 25-30% greater than that of the adult filaments.

Discussion

Synemin Periodicity

Previous biochemical studies showed that vimentin and synemin are the primary components of chicken erythrocyte intermediate filaments (Granger et al., 1982). The high molar ratio of vimentin to synemin (~50:1) suggested that vimentin forms the core polymer with synemin as an associated polypeptide. The results of the antibody decoration experiments presented here are consistent with this interpretation:

vimentin is present uniformly throughout the filaments, while synemin exists at regularly spaced, discrete foci along the filaments.

There are several possibilities for what determines the axial spacing of the synemin nodes on erythrocyte vimentin filaments. Theoretically, synemin (230,000 daltons) is large enough to lie along the filaments in a head-to-tail or overlapping fashion with a repeat distance of at least 180 nm. By analogy to tropomyosin (Caspar, Cohen and Longley, 1969), a two-strand, coiled-coil α-helix with subunits of 230,000 daltons would be about 300 nm long. If this were the case, then the lack of antibody decoration of the internodal segments of a filament could be explained by antigenic masking or complete lack of immunogenicity of that portion of the polypeptide in the rabbit in which the antibodies were made.

Alternatively, one of the minor components of the filament might be responsible for determining the synemin spacing, or perhaps a polypeptide that is solubilized and lost during the filament isolation procedure (Granger et al., 1982).

Another possibility is that the structure of the core filament dictates the site of synemin binding. A fundamental repeat distance of 31 nm has recently been observed for vimentin filaments using a monoclonal antibody (Blose et al., 1982), which is consistent with a 31 nm repeat observed for desmin filaments using optical diffraction (Small and Sobieszek, 1977). The decorated synemin repeat distance is probably at least five times this value; if vimentin itself is responsible for the observed synemin spacing, then it must be through some long-range, supramolecular structure of the filament. However, filament ultrastructure models can be constructed with little confidence at this time, due to uncertainties in the absolute (and therefore relative) axial repeat distances of vimentin and synemin (see Results), the molar ratio of vimentin to synemin (see Granger et al., 1982), the number of synemin polypeptides per decorated knob, and wide variation in the reported number of protofilaments in a given cross-section of the intermediate

filament core (for example, see Small and Sobieszek, 1977; Renner et al., 1981; Steinert, Idler and Goldman, 1980; Woodcock, 1980).

Even though the absolute numerical values derived for the spacing of synemin along the filaments should be regarded as approximate, the observed difference between adult erythrocytes and embryonic erythroid cells does not appear to be artifactual (see Results). The basis for this difference probably lies in some fundamental property of the filaments that changes during erythropoiesis; whether the difference is intrinsic to the filaments or a function of the cytoplasmic milieu, and whether this phenomenon is also characteristic of differentiating muscle cells (Granger and Lazarides, 1980) remains to be determined.

In previous studies, demonstration of the association of synemin with desmin- and vimentin-containing filaments in muscle cells and in avian erythrocytes was based on copurification and immunofluorescence data (Granger and Lazarides, 1980; Granger et al., 1982). Electron microscopic detection of this association has come about through the use of the antibody decoration technique in erythrocytes; synemin is otherwise not an apparent appendage or structural component of the filaments when examined only by shadowing (see Figures 1, 3, 4, 5 and 6) or the much higher resolution technique of negative staining (Cooke and Chase, 1971; Small and Sobieszek, 1977; Hubbard and Lazarides, 1979; Woodcock, 1980). Furthermore, decoration of erythrocyte filaments with vimentin antibodies results in a uniform coating of the filament, without obvious gaps where synemin might be located; this is probably a consequence of the relatively low resolving power of the platinum shadowing technique, though, since synemin antibodies are still capable of forming their characteristic knobs after the filaments have been decorated with anti-vimentin (data not shown). Nevertheless, considering the large size of the synemin polypeptide, these results are surprising, but not without precedent. A similar situation exists for vertebrate neurofilaments, in which a 200,000 dalton polypeptide is apparently

peripherally associated with the core filament (Willard and Simon, 1981). As with erythrocyte and muscle intermediate filaments, native vertebrate neurofilaments usually exhibit smooth contours when negatively stained (Schlaepfer, 1977; Eagles et al., 1980). Perhaps synemin and the large neurofilament polypeptide are normally in an extended conformation (see above) in close apposition to the core filament but become partially separated from the core upon binding of antibodies (see Willard and Simon, 1981). This interpretation, however, must be reconciled with the observation that the decoration pattern is not altered by fixation of the filaments before antibody application (see Results; Willard and Simon, 1981), and the fact that lateral arms or crossbridges are often seen when neurofilaments are examined in thin sections (Shelanski et al., 1971; Wuerker, 1970; Metuzals and Mushynski, 1974). Elucidation of conditions under which synemin can be visualized on desmin and vimentin filaments without the use of antibodies awaits further study.

Role of Synemin

The lateral registry and crossbridging of chicken erythrocyte intermediate filaments that is revealed by anti-synemin decoration is the first evidence of a function for synemin. That the synemin nodes in laterally-associated filaments coincide, even in preparations that have been fixed prior to decoration, implies that the observed crossbridging is not artificially induced by the bivalent antibodies. We have not discerned whether two filaments must be parallel or antiparallel in order to be crossbridged (assuming the filaments have polarity), or if other orientations are permitted. The laterally-associated filaments in the stalks of the hairpin loops observed in embryonic cells would have an antiparallel orientation, but this association may be completely independent of synemin. The electron micrographs of the decorated bridges suggest that the crosslinks are produced by self-association of the synemin polypeptides; this is consistent with the apparent tetramerization

of purified synemin in solution (Sandoval, Colaco and Lazarides, manuscript submitted for publication). In erythrocytes, synemin is always present along the filaments, yet not all of the synemins are involved at any one time in crossbridging (unless sonication has broken some of the links); regulation of the extent of crosslinking would modulate the flexibility of the cytoplasmic intermediate filament network. Elucidation of possible regulatory mechanisms (such as phosphorylation; Granger et al., 1982) and determination of the relationship between crosslinking and intermediate filament function await further study.

Implications of the Filament Distribution

Most hypotheses concerning the function of intermediate filaments are a result of their observed incidence and distribution, and therefore based on circumstantial evidence. Intermediate filaments form a network around and in close association with the nuclei of most higher eukaryotic cells; since nuclei may be enclosed in cages of intermediate filaments from which they are not easily dissociated, unambiguous determination of whether there is a specific association between intermediate filaments and nuclei is experimentally difficult. Despite the numerous electron microscope studies (including this one) that indicate attachment of intermediate filaments to the nucleus, the molecular and structural details of such an interaction have yet to be ascertained, and specificity has yet to be demonstrated.

One function that has been proposed for intermediate filaments in cultured cells and nucleated erythrocytes, based on thin sectioning and negative staining, is that of positioning or anchoring the nucleus in the cytoplasm (Harris and Brown, 1971; Lehto, Virtanen and Kurki, 1978; Small and Celis, 1978; Virtanen et al., 1979). Whereas these techniques give only two-dimensional views of the cytoskeleton, the shadowed replicas presented in this paper reveal more fully the extent of the erythrocyte intermediate filament network. In conjunction with immunofluorescence

data (Granger et al., 1982), this means of visualizing the cytoskeleton shows that the filaments surround the nucleus and appear to link it directly to the plasma membrane. Filaments also traverse the cytoplasm and link areas of the plasma membrane on opposite sides of the cell. The filaments are not distributed uniformly, in that they are firmly attached to the plasma membrane only in a defined region on each face of the erythrocyte disc that is not within 1-2 micrometers of the edge. The intermediate filament network may thus indeed function as a structural or integrating cellular matrix in the avian erythrocyte.

Examination of embryonic erythroid cells using the sonication/shadowing technique (this paper) as well as immunofluorescence (E. A. Repasky, B. L. Granger and E. Lazarides, unpublished observations) suggest that very young cells have few, if any, intermediate filaments in contact with the plasma membrane, and relatively few filaments lying on the nuclear membrane. As the cells mature, these nucleus-associated filaments may elongate and establish contacts with the plasma membrane, perhaps by some sort of looping out mechanism. The hairpin loops observed on the plasma membranes of sonicated embryonic cells often have a radial orientation, as if they were looping out from near the centralized nucleus. The closed circles often seen on these membranes might be a result of breakage of the stalks of these hairpins during sonication, with subsequent antiparallel overlap or joining of the two free ends to form a circle at the loop's original point of attachment to the membrane. The helical coiling of the stalks of the hairpins may simply be a reflection of the intrinsic helicity of the filaments themselves (Gilbert, 1972, 1975; Metuzals and Mushynski, 1974; Small and Sobieszek, 1977; Schlaepfer, 1977; Krishnan, Kaiserman-Abramof and Lasek, 1979; Blose et al., 1982); for example, supercoiling might simply result from a slight tightening or relaxation of the filament helices by changes in ionic environment during sample preparation.

The marginal band seems to play an essential role in the development of

the flat, discoid shape of nucleated erythrocytes (Barrett and Dawson, 1974), but has little if any role in maintaining the shape of mature erythrocytes (Behnke, 1970b; Barrett and Dawson, 1974; Bertolini and Monaco, 1976). It has been proposed that the elliptical shape of mature erythrocytes is the result of two forces: the outward force of the coiled microtubules of the marginal band, which favors a flat, round disc shape, and an asymmetric, inward force produced by the "trans-marginal band material (TBM)," which distorts this circle into an ellipse (Cohen, 1978). The exact nature of the TBM has not yet been determined; it was suggested that intermediate filaments might be a component of the TBM (Sloboda and Dickersin, 1980; Granger et al., 1982), but the results of the present study indicate that any interaction the intermediate filament network might have with the marginal band must be indirect, through the spectrin cytoskeleton lining the plasma membrane. Any direct interactions, if they occur in vivo, must be very tenuous. Regardless of how the elliptical disc shape forms, it now appears likely that the spectrin-based membrane cytoskeleton is primarily responsible for maintenance of this shape. This conclusion is drawn partly by analogy to the mammalian erythrocyte, whose biconcave shape seems to be determined primarily by this membrane cytoskeleton (see Sheetz and Singer, 1977; Johnson, Taylor and Meyer, 1980).

Microtubule Tracks

Mature chicken erythrocytes have an average of perhaps 10 or 15 microtubule revolutions in their marginal bands (Behnke, 1970b; Barrett and Dawson, 1974; Goniakowska-Witalińska and Witaliński, 1976), which is clearly greater than the number of "tracks" seen on the inner surfaces of membranes; this discrepancy is consistent with the bundled rather than ribbon-like arrangement of microtubules in the marginal band seen in thin sections (Behnke, 1970a,b; Barrett and Dawson, 1974) and implies that only a subset of the marginal band microtubules interact with the plasma membrane. The molecular basis for the lateral linkage and bundling

of microtubules within the marginal band (MAP-2?; Sloboda and Dickersin, 1980) may thus be different from that for the linkage of the band to the plasma membrane. Even though the composition of the tracks has not been determined, their presence suggests that nucleated erythrocytes may be a simple system for the study of microtubule-membrane interactions.

These tracks have not been noted previously in electron micrographs. In this study, they have proven useful as a frame of reference for the study of the topology of the plasma membrane. That they are more than just artifactual or passive remnants of microtubules is suggested by the observation that chick erythrocyte marginal bands that disappear upon cooling will usually reform in the correct position upon rewarming (Behnke, 1970b; Barrett and Dawson, 1974). Perhaps the tracks serve as a guide for this reformation; this would be further evidence of a stable anisotropy in the cytoskeleton of the erythrocyte membrane.

Neurofilament Analogy

Several analogies can be drawn between avian erythrocyte intermediate filaments and neurofilaments. Both consist of a core polymer to which high molecular weight polypeptides are peripherally and periodically bound (Willard and Simon, 1981). However, the axial periodicity of synemin on erythrocyte filaments is roughly twice that of the 200,000 dalton polypeptide on neurofilaments. The latter polypeptide is approximately 200 nm long and wraps helically around 140 nm of core filament with an overlap that gives an axial periodicity of 100 nm (Willard and Simon, 1981). Although no helicity or wrapping of synemin around the vimentin core has been revealed with the antiserum used in this study, this possibility cannot be ruled out. The core polypeptides (vimentin and neurofilament 68-70 K) are capable of polymerizing into intermediate-sized filaments in the absence of the associated polypeptides (Renner et al., 1980; Steinert et al., 1981; Moon et al., 1981; Geisler

and Weber, 1981a; Liem and Hutchison, 1982; Zackroff et al., 1982). The associated polypeptides may thus regulate filament length or stability (see Moon et al., 1981; Geisler and Weber, 1981a), mediate interactions with other cellular structures such as microtubules (Bertolini, Monaco and Rossi, 1970; Rice et al., 1980; Runge et al., 1981), or form interfilament crossbridges (Willard and Simon, 1981; this paper).

Significant differences between these two systems are also evident. As is true for the core polypeptides (Shaw, Osborn and Weber, 1981; Tapscott et al., 1981; Yen and Fields, 1981), synemin and the high molecular weight neurofilament polypeptides in chickens are electrophoretically and antigenically distinct (unpublished observations). Also, the molar ratio of synemin to its core polypeptide is much less (perhaps ten-fold less) than that of the large neurofilament polypeptides to their core subunit. The relative similarity in the axial repeat distances of these associated polypeptides does not reflect this molar ratio difference, though, suggesting that there must be fundamental structural differences between these two systems, related to their functions or the cell types in which they are found.

Functions hypothesized for synemin in erythrocytes may be applicable to synemin in smooth and skeletal muscle, where it is found with desmin as well as vimentin. Of all the types of intermediate filament subunits identified to date, vimentin and desmin appear most similar in terms of coexistence and copurification, peptide maps, amino acid sequence and association with synemin (Starger et al., 1978; Gard, Bell and Lazarides, 1979; Granger and Lazarides, 1979, 1980; Granger et al., 1982; Geisler and Weber, 1981b). Whether synemin mediates desmin and vimentin filament crosslinking in muscle cells, and whether it links the filaments to other cellular structures, remains to be determined.

Experimental Procedures

Antisera

Antibodies to synemin, vimentin and desmin were elicited in rabbits against chicken muscle proteins; each immunogen was ultimately purified by SDS-polyacrylamide slab gel electrophoresis prior to injection. The specificity of each antiserum has been demonstrated previously by two-dimensional immunoautoradiography (Granger and Lazarides, 1979, 1980; Granger et al., 1982).

Goat anti-rabbit IgG was conjugated with either fluorescein (purchased from Miles-Yeda; diluted 100-fold for use) or with ferritin (purchased from Cappel Laboratories; diluted 30-fold for use).

In one experiment, antisera were dialyzed against 10 mM sodium phosphate (pH 7.5) and applied to a DEAE-cellulose column (Whatman DE-52) equilibrated in the same buffer. The non-adsorbed fractions were pooled; fractions eluted subsequently with the same buffer containing 40 mM NaCl and then 140 mM NaCl, were also pooled and used without further dilution for filament decoration (see below).

Electron Microscopy

Specimen preparation techniques were essentially as described previously (Granger et al., 1982). Blood was collected from the wing veins of large White Leghorn chickens in 2-3 volumes of 5 mM HEPES, 155 mM choline chloride (brought to pH 7.1 at 20°C with Tris base; "HCCT") containing 0.01% sodium heparin. Blood from 10-day embryonic chickens was obtained by diffusion from carcasses into Earle's balanced salt solution or HCCT. Adult erythrocytes and embryonic erythroid cells were isolated and washed by 3-4 centrifugations (5 min at 1000-1500 x g) and resuspensions in HCCT, either at 20°C or 4°C, with removal of the buffy coat from each pellet by aspiration. The final cell pellet was resuspended in an equal volume of HCCT. Round glass coverslips that had been cleaned with acid, treated

with 0.1% or 1% Alcian blue 8GX and rinsed extensively with water were positioned horizontally and covered with a few drops of HCCT. A drop of cell suspension was carefully applied under the HCCT and allowed to spread out and settle onto the coverslip for 30-60 min at room temperature. Nonadherent cells were rinsed off in HCCT, and adherent cells were hypotonically lysed by immersion of the coverslips into a solution of cold 5 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 5 mM NaN_3, 1 mM TAME, 0.5 mM PMSF, 5 mM Tris-Cl (pH 7.5 at 0°C). Alternatively, for microtubule-preservation experiments, the cells were lysed in 5 mM $MgSO_4$, 2 mM EGTA, 1 mM DTT, 1 mM NaN_3, 1 mM TAME, 0.5 mM PMSF, 10 mM PIPES (pH 6.9), with all steps in the procedure carried out at room temperature.

After at least 10 min in several changes of hypotonic lysis buffer, coverslips were held in a face-up position on the bottom of a beaker filled with buffer, and disrupted with a sonicator probe tip positioned 3-4 cm overhead. Usually, a 4 mm diameter titanium tip was operated for 20 sec at 20 W. The duration and intensity of the sonication were adjusted to result in the disruption of approximately half of the cells. The extent of disruption could be monitored by viewing light reflected from the coverslip against a dark background, since intact ghosts scatter light, but membrane patches do not.

Coverslips were immersed in Buffer F (130 mM KCl, 5 mM NaCl, 1 mM NaN_3, 5 mM $MgCl_2$, 1 mM EGTA, 20 mM potassium phosphate, pH 7.5) and then either incubated with antisera or fixed for 1-16 hr at 20°C in Buffer F containing 1% glutaraldehyde (or in the pH 6.9 hypotonic lysis buffer containing 1% glutaraldehyde).

Typical antiserum incubations were as follows: The IgG-enriched fraction of serum that is precipitated by ammonium sulfate at 50% saturation at 0°C was diluted with Buffer F (containing 1 mM TAME and 0.5 mM PMSF) to give 1/30 of the serum concentration. Coverslips were incubated with 100 µl of diluted antisera for 30 min at room temperature, washed with Buffer F for 10-20 min,

and then either fixed as above or incubated as above with a secondary antiserum (goat anti-rabbit IgG; see above) prior to fixation.

After fixation, coverslips were desalted by perfusion with a continuous water gradient terminating in distilled water, and then dehydrated by perfusion with a continuous ethanol gradient terminating in anhydrous ethanol. Coverslips were dried from ethanol in a carbon dioxide critical point drier, and immediately shadowed with Pt-Pd (80:20) at an angle of 6-7° in a Kinney evaporation unit. Shadowing was unidirectional, pan-directional (rotary), or a combination of the two. Replicas were stabilized by carbon coating and removed from the coverslips by flotation on a solution of 5% HF. After washing with water, the replicas were either mounted directly on electron microscope grids or first cleansed of organic material (Figures 2a, 2e, 3c, 4a, 5a, 6a, 6d) by floating for 10-20 min on a solution of 5% sodium hypochlorite (household bleach). Replicas were examined with a Philips 201c transmission electron microscope operated at 60 kV, and photographed on 35 mm film.

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Figure Legends

Figure 1. Antibody Decoration of Chicken Erythrocyte Intermediate Filaments

Sonication of erythrocytes attached to a glass substrate leaves elliptical patches of adherent plasma membrane. Intermediate filaments associated with these patches can be visualized by transmission electron microscopy after low angle rotary shadowing with platinum (a). Incubation of patches with synemin preimmune serum prior to shadowing does not alter this image (b). Incubation with anti-vimentin (c) or anti-synemin (d) specifically decorates the corresponding antigen and reveals its spatial distribution within the filament. The dark segments of filament in a and b are above the plane defined by the remainder of the filaments on the patch. Bar = 5 μm.

Figure 2. Intermediate Filaments Decorated with Anti-Vimentin and Anti-Synemin

High power views of filaments from adult erythrocytes (a-d) and 10-day embryonic erythroid cells (e, f) decorated with anti-vimentin (a) and anti-synemin (b-f). In two cases (c and d), the anti-synemin decoration was amplified by further decoration with anti-IgG (c) or anti-IgG conjugated to ferritin (d). Note the registry of laterally associated filaments and existence of crossbridges between filaments decorated with anti-synemin (arrowheads in e and f). Bars = 500 nm.

Figure 3. Visualization of Marginal Band Microtubules and Their Tracks

Spatial segregation of the marginal band and intermediate filament network is always observed in these preparations (a). Removal of the marginal band microtubules by sonication or depolymerization reveals ridges or "tracks" on the inner surface of the plasma membrane (b, c). Correspondence of the microtubules (arrowhead) and tracks (arrow) is evident in b. Erythrocytes that adhere to the substrate only

(Figure 3 continued)

along an edge leave oblong patches of plasma membrane containing microtubules or tracks, but no intermediate filaments (b, c). Bars = 5 μm.

Figure 4. Variations in Degree of Disruption of Sonicated Chicken Erythrocytes

Shadowing was primarily unidirectional; micrographs were printed with reversed contrast to make platinum shadows appear dark. Filaments in b, c and d were decorated with vimentin antibodies prior to shadowing. Nuclei are electron-opaque (b) and must be digested from the replica to allow visualization of their surface features (a). Intermediate filaments can be seen spanning from the adherent area of plasma membrane to the nucleus (N) and overlying plasma membrane (PM) in a. Bars = 2 μm (a-c are at the same magnification).

Figure 5. Partially Disrupted Erythrocytes Showing Filaments That Traverse the Cytoplasm

Filaments span from the adhering to the overlying regions of plasma membrane (corresponding to the two faces of the erythrocyte disc) in these preparations that were rotary shadowed and bleached (a) or unidirectionally shadowed, not bleached, and printed with reversed contrast (b). PM, overlying plasma membrane; N, position of nucleus. Bars = 2 μm.

Figure 6. Sonicated Embryonic Erythroid Cells

(a) Low magnification view. (b) Detail of helically twisted loops of intermedaite filaments frequently seen in these cells. (c) Closed intermediate filament circles that have twisted into a figure-8 pattern. Bar = 5 μm in a, 1 μm for b-d.

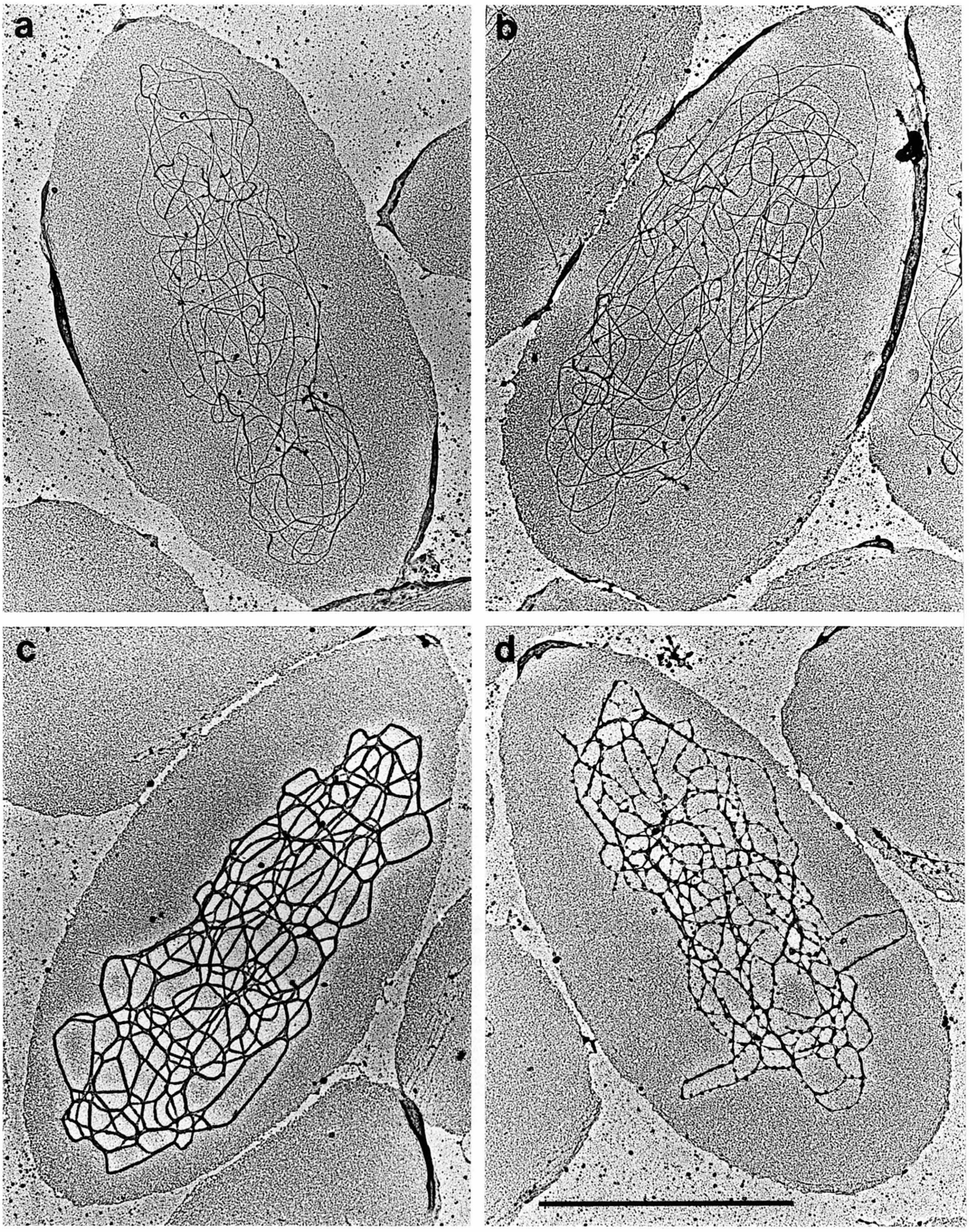

Figure 1

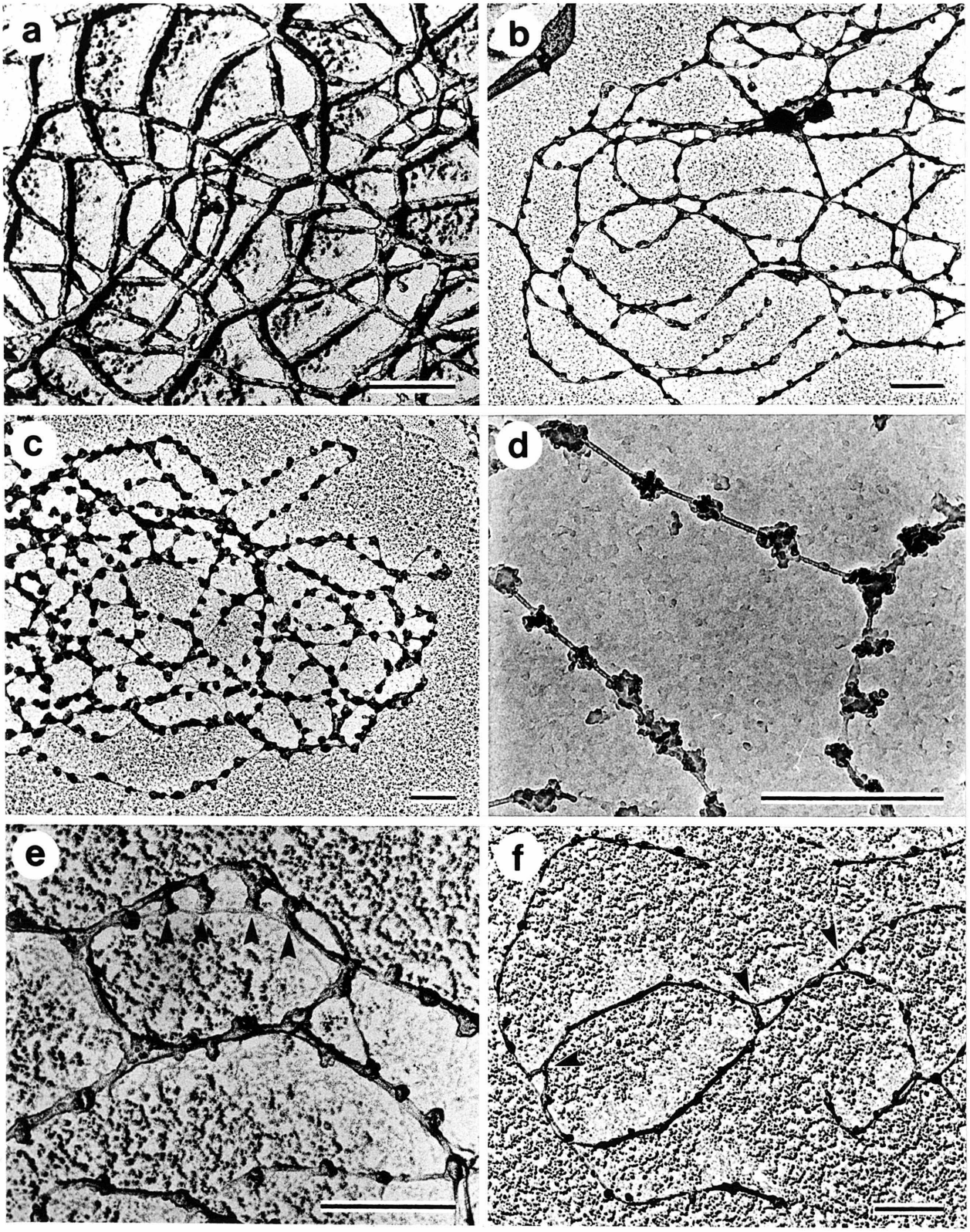

Figure 2

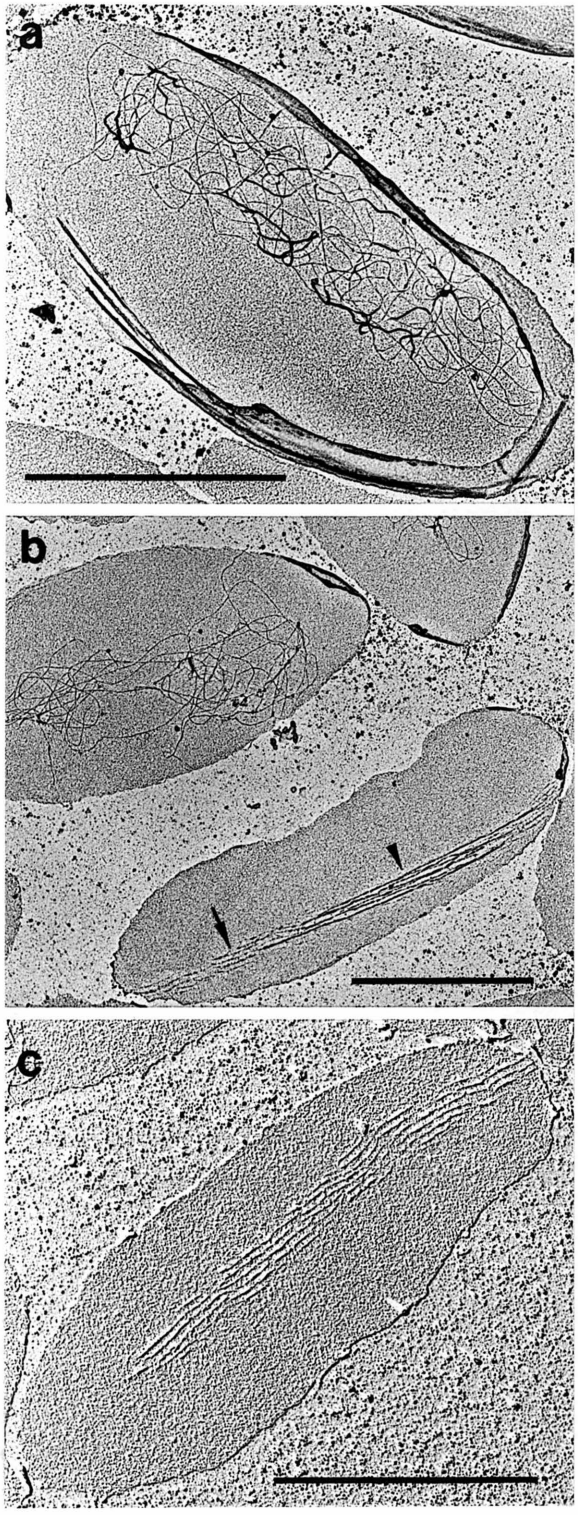

Figure 3

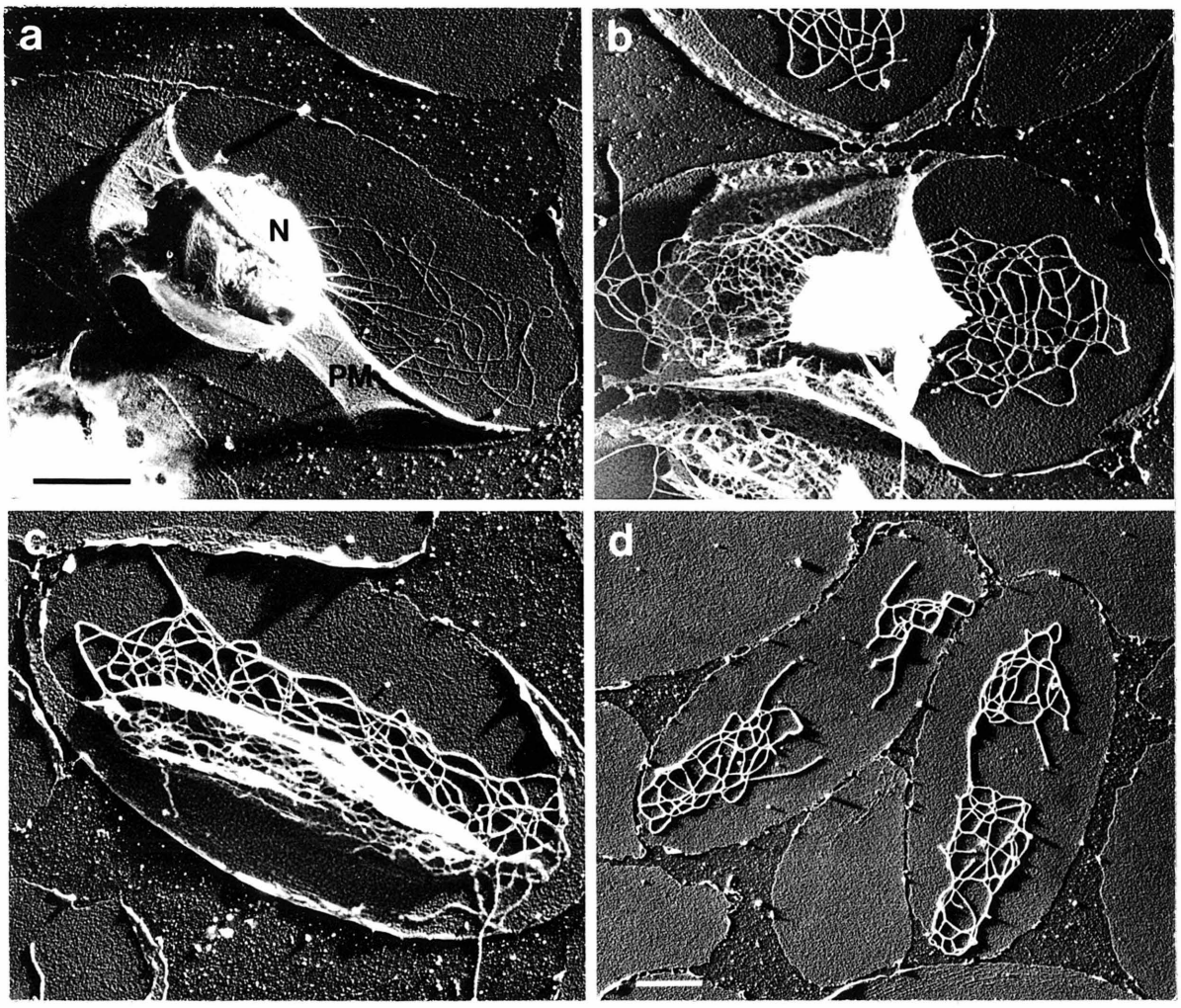

Figure 4

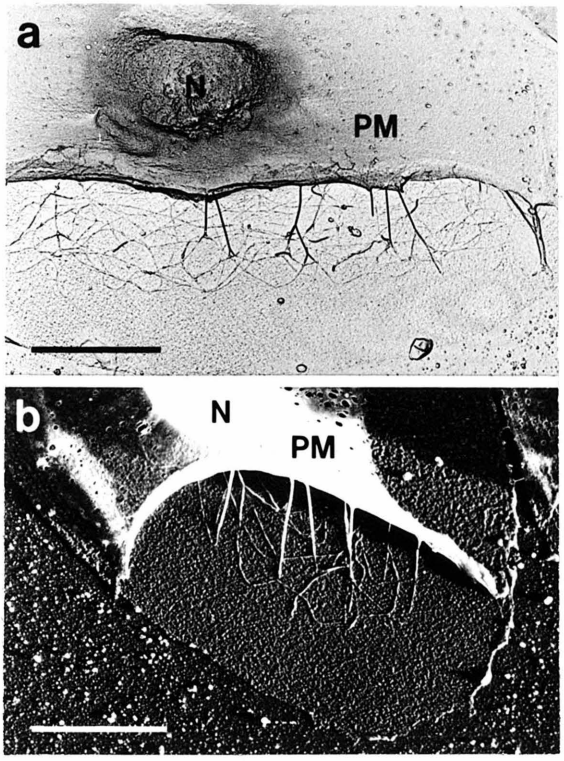

Figure 5

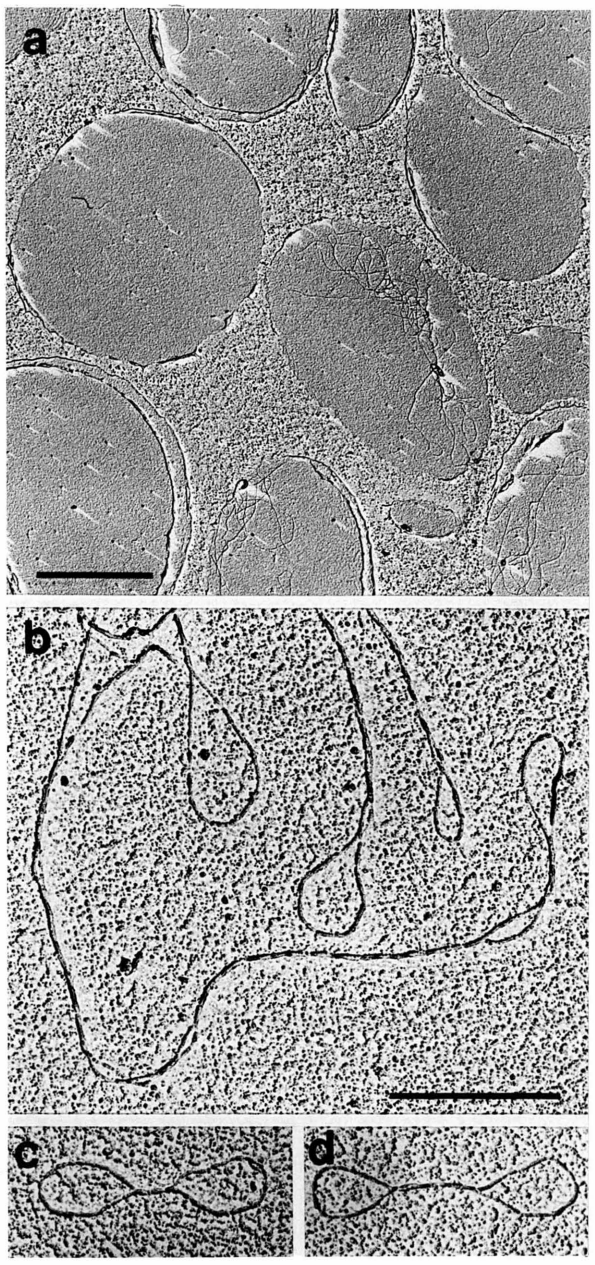

Figure 6

Chapter 7

Discussion

Functions of Intermediate Filaments

Most functions currently postulated for intermediate filaments are based on circumstantial evidence; that is, they are inferred solely from the observed cytoplasmic distribution of the filaments. For some cell types, these hypotheses seem intuitively reasonable; for others, they seem highly speculative, and largely without foundation. This chapter outlines some current thoughts about the functions of intermediate filaments.

Various means of perturbing a cell's intermediate filaments have been found, but have shed little light on their function. Intermediate filament mutants have not yet been identified, nor has there been any serious attempt to trace the filaments back through phylogeny. Polymerization in vitro does not seem to require energy or cofactors. No enzymatic activity has been ascribed to any of the filament subunits, and effects of posttranslational modifications have not been discerned. The functions of intermediate filaments have thus proven elusive when approached from a variety of different angles.

Intermediate filaments are most often thought to have a cytoskeletal role. Such a role seems almost certain for cytokeratin filaments in epithelial cells; these filaments form a mechanically continuous network through epithelial cell layers by associating with desmosomes (see Kelly, 1966; Drochmans et al., 1978), and thereby create an integrated, physically durable barrier between an organism and its external environment. In cornified epithelia, such as epidermis, the cells die during terminal differentiation; the keratin filaments become interlinked through disulfide bonds, and thus become part of a protective, acellular armor. Specializations of the epidermis that are composed predominantly of keratin-like polypeptides include callus, nails, claws, scales, feathers, hair, horns and beaks. Some of these "keratins", particularly those in avian specializations such as scales and feathers, are structurally quite different than the keratin polypeptides found in the epidermis (e.g., Kemp and Rogers, 1972); however, antigenic crossreactivity between mammalian hair keratins and

epithelial cytokeratins has been demonstrated (Weber, Osborn and Franke, 1980). Keratin filaments thus appear to be the most diverse and anomalous of all the classes of intermediate filaments, and those whose functions seem most clear.

The class of intermediate filaments associated with one of the most specialized cellular structures is that of the neurofilaments. Neurofilaments are uniquely found in the axons and dendrites of neurons. They are a component of slow axoplasmic transport, and have frequently been suspected of being in some way responsible for all axonal transport. One recent model supposes that the helical 200,000 dalton polypeptide rotates around the core filament and thereby acts as a worm screw to effect the linear transport of particles riding on the core filament "track" (Willard and Simon, 1981). Interactions between neurofilaments and microtubules (lateral bridges, regular geometric arrangements, in vitro associations) have frequently been noted (e.g., Bertolini, Monaco and Rossi, 1970; Rice et al., 1980; Runge et al., 1981b), but have given few clues about the functional implications of such phenomena. In certain cases, neurofilaments have an obvious structural role: in the marine worm, Myxicola, for example, the neurofilaments of the giant axon form a coherent, strong, flexible cord that can be physically removed from the axon by pulling at one end with forceps (see Gilbert, 1975). Whether axonal integrity is the primary function of this arrangement of neurofilaments, and whether this would also apply to much thinner axons (as in vertebrates), is not known. Neurofilaments are apparently not responsible for neurite outgrowth during neuronal differentiation, since they are not always present in such processes (see Debus et al., 1982; Sharp et al., 1982), yet they may exist in most axons to perform a stabilizing or transport role.

Another distribution of intermediate filaments that is indicative of function is seen in striated muscle cells. The feature of striated muscle that is responsible for the term "striated" is the consistent lateral registry of adjacent sarcomeres. At a given state of stretch or contraction, all of a muscle fiber's sarcomeres are

the same length. Their axial arrangement is defined by the myofibrils, in which the sarcomeres are in tandem array; the lateral registry of all of the myofibrils results in a cross-banding of the muscle fiber that has an axial repeat distance equal to the length of the sarcomere. The molecular basis fo this perfect lateral registry has long been speculated upon. Electron micrographs of thin sections of vertebrate skeletal and cardiac muscle have revealed filaments disposed transversely (at right angles to the myofibril axis) at the level of the Z-line (Sandborn et al., 1967; Lindner and Schaumburg, 1968; Page, 1969; Viragh and Challice, 1969; Ferrans and Roberts, 1973; Behrendt, 1977; Junker and Sommer, 1977; Eriksson and Thornell, 1979). Intermediate filaments were initially postulated to play no direct role in myofibrillogenesis, but to become deposited as transverse supportive elements at the level of the Z-lines (Kelly, 1969). In the flight muscle of bees, bridges connecting the Z-discs of adjacent myofibrils have been noted in micrographs (Ernst and Garamvölgyi, 1956; Garamvölgyi, 1962, 1965). Independently, the smooth muscle intermediate filament protein desmin was found by immunofluorescence to exist along the Z-lines of striated muscle cells, where it was hypothesized to interlink adjacent myofibrils (Lazarides and Hubbard, 1976). The concept of a Z-bridge was given support by the finding that the Z-discs of chicken skeletal muscle were physically linked to their laterally-adjacent Z-disc neighbors (Granger and Lazarides, 1978). This was shown by the observation that if permeabilized muscle was extracted with 0.6 M KI (which solubilizes and removes the myosin of the A-bands and actin of the I-bands), subsequent homogenization would cleave the fiber between planes of laterally-registered Z-discs, and liberate into the solution sheets of laterally-connected Z-discs. Desmin was found to be localized at the periphery of the Z-disc, with a Z-plane distribution complementary to that of the Z-disc matrix protein, α-actinin. Coupled with whole-mount electron micrographs showing filaments connecting Z-discs of adjacent myofibrils, these data suggested that desmin-containing intermediate filaments were responsible

for the lateral registry of myofibrils and striated appearance of muscle fibers. Subsequent work showed that vimentin coexisted with desmin at these locales, and was the first demonstration of two classes of intermediate filament in a single cell in vivo (Granger and Lazarides, 1979). Intermediate filaments in striated muscle may thus function as structural support or as elements that help to integrate the contractile activities of the separate myofibrils.

Since intermediate filaments in immature myotubes exhibit a seemingly random cytoplasmic distribution (Ishikawa et al., 1968), there must be a transition during myogenesis that gives rise to the Z-disc associated form. This was confirmed and studied using embryonic chicken myotubes grown in culture (Gard and Lazarides, 1980). Approximately 5-7 days after fusion of myoblasts into myotubes, and 1-3 days after α-actinin begins to coalesce into Z-discs, the intermediate filament proteins desmin and vimentin are gradually cleared from the cytoplasm and simultaneously begin to appear around the Z-discs. This roughly coincides with the establishment of myofibril registry, as if the intermediate filaments were promoting or stabilizing this higher level of organization (Gard and Lazarides, 1980).

Studies performed subsequent to this work, both with differentiating (Bennett et al., 1979; Peng, Wolosewick and Cheng, 1981) and mature skeletal muscle (Edström, Thornell and Eriksson, 1980; Nunzi and Franzini-Armstrong, 1980; Thornell et al., 1980; Pierobon-Bormioli, 1981; Richardson et al., 1981), as well as cardiac muscle (Forbes and Sperelakis, 1980; Chiesi et al., 1981), have also found evidence of intermediate filament-based lateral Z-bridges, and have corroborated many of the ideas presented above.

One function of mature skeletal muscle intermediate filaments that was postulated initially was linkage of the excitation-conducting transverse tubular system (T system) to the periphery of the Z-disc (Granger and Lazarides, 1978; Lazarides and Granger, 1978). Subsequent studies of muscles whose T systems were either poorly developed

and irregular, or positioned at the A-I junction rather than at the Z-line, revealed no correlation between the T tubules and the intermediate filament proteins (Granger and Lazarides, 1979). Whether there is any interaction between other membranous organelles, such as mitochondria and sarcoplasmic reticulum, and intermediate filaments in the interfibrillar space remains to be conclusively determined.

Intermediate filaments are far more abundant and apparent in smooth muscle than in mature striated muscle, yet their role in this tissue is less evident. They are primarily disposed along the long axis of the spindle-shaped smooth muscle cells, parallel to the actin and myosin filaments. They can be seen to associate with cytoplasmic and plasma membrane-bound dense bodies (Cooke and Chase, 1971; Uehara et al., 1971; Somlyo et al., 1971; Rice and Brady, 1972; Ashton, Somlyo and Somlyo, 1975). Dense bodies have been likened to myofibril Z-discs, in that they also contain α-actinin and have actin filaments, but not myosin filaments, emanating from them (Ashton et al., 1975; Schollmeyer et al., 1976; Geiger et al., 1981). It is thus tempting to extrapolate from skeletal to smooth muscle and hypothesize that intermediate filaments have a cytoskeletal or integrative role in these cells as well. The presence of desmin in cardiac intercalated discs (Lazarides and Hubbard, 1976) and intermediate filaments associating with these structures (Virágh and Challice, 1969; Ferrans and Roberts, 1973; Oliphant and Loewen, 1976; Behrendt, 1977) is reminiscent of keratin filaments associating with desmosomes, and similarly suggests a cytoskeletal role.

Perhaps the most tenuous link between structure and proposed function concerns intermediate filaments in cultured cells. Most primary cultures and cell lines exhibit a somewhat random array of intermediate filaments, either encircling the nucleus or oriented radially from the centralized nucleus towards the cell periphery. Early microscopy suggested a number of functions for the filaments (dePetris et al., 1962), such as being cytoskeletal (Brecher, 1975), or involved in cell spreading (Goldman and Knipe, 1973), locomotion (Felix and Sträuli, 1976) or organelle transport (Goldman

and Follett, 1970). The apparent fundamental association with nuclei has suggested that intermediate filaments may be responsible for anchoring or positioning the nucleus within the cell (Lehto, Virtanen and Kurki, 1978; Small and Celis, 1978; Virtanen et al., 1979). This seems naively plausible, but the possibility that the nucleus is merely trapped in the intermediate filament network cannot be ruled out; the observed effects and theoretical purposes of intermediate filament networks have so far proven to be difficult to distinguish experimentally (see also Woodcock, 1980; Laurila, Virtanen and Stenman, 1981; Chapter 6). Observations of intermediate filaments throughout the cell cycle have not indicated an active or essential role for intermediate filaments. During mitosis, for example, intermediate filaments form a ring or cage around the mitotic apparatus, where they may have the effect of positioning or confining the spindle (Blose, 1979, 1981; Aubin et al., 1980; Zieve, Heidemann and McIntosh, 1980).

Cytoplasmic microtubules exhibit a distribution in some cultured cells that is similar in overall appearance to the distribution of intermediate filaments (see Geiger and Singer, 1980). This has led to speculation of a functional interaction between these two systems (e.g., Goldman and Knipe, 1973; Wang and Goldman, 1978; Wang, Cross and Choppin, 1979). Treatments that effect a separation of cytoplasmic microtubules and intermeidate filaments in vitro, such as transformation (Hynes and Destree, 1978; Ball and Singer, 1981), vanadate (Wang and Choppin, 1981), cycloheximide (Sharpe et al., 1980; Ball and Singer, 1982), toxins (Sharpe et al., 1980) and cold (Hynes and Destree, 1978; Virtanen et al., 1980), as well as agents such as colcemid that alter the distribution of both (see Introduction), have shown that intermediate filaments are mutable, but have provided little insight into their functions. The results of such experiments are generally ambiguous anyway, since the specificity of the disrupting agents is uncertain; this problem has recently been overcome, however, by using specific antibodies.

Microinjection of antibodies specific for intermediate filament components causes the filament network to collapse, yet has little if any effect on mitosis, cytokinesis, spreading, locomotion or other salient cellular activities in vitro (Gawlitta, Osborn and Weber, 1981; Klymkowsky, 1981; Lin and Feramisco, 1981). The antibody-induced, perinuclear aggregate of intermediate filaments appears to be treated by the cell as a passive organelle, suggesting that in some cells in culture, at least, a spread network of intermediate filaments is not necessary for survival and growth. Similarly, the apparent absence of intermediate filaments in early mouse morulas and inner cell masses (Jackson et al., 1980; Paulin et al., 1980) indicates that intermediate filaments are not necessary for basic cellular functions, but may play a necessary role in differentiation.

Intermediate filaments have been postulated to interact with several cytoplasmic organelles other than nuclei and microtubules. A role in pigment granule migration in certain specialized epithelial cells has been proposed (e.g., Jimbow and Fitzpatrick, 1975) and disputed (Takeuchi and Takeuchi, 1979). Ribosomes are often seen to be associated with intermediate filaments (e.g., Pudney and Singer, 1979; Schliwa and vanBlerkom, 1981), suggesting a possible functional link to protein synthesis and dispersal. Intermediate filaments in cardiac conducting cells appear to bind glycogen particles (Rybicka, 1981) as if the filaments might regulate some aspect of metabolism; a similar conclusion can be drawn from the apparent association of creatine phosphokinase with some intermediate filaments (Eckert et al., 1980). Mitochondria appear to associate with tonofilaments in certain epithelial cells, where they are often observed closely apposed to desmosomes (Lundgren, 1974; Bernstein and Wollman, 1975; Lee, Morgan and Wooding, 1979); also, links have been observed between mitochondria and vimentin filaments (David-Ferreira and David-Ferreira, 1980; B. Granger, unpublished observations). The infancy of the intermediate filament field has not permitted determination of whether these isolated observations represent

specific adaptations in a limited number of cell types, or if they are generalizable to all intermediate filaments or even to a given class of filament.

The only posttranslational modification of intermediate filament proteins (aside from N-terminus blockage) that has been detected and studied is phosphorylation. Phosphorylation has been demonstrated for all classes of intermediate filament except glial filaments. It has been studied in relation to squid neurofilaments (Pant et al., 1978), vertebrate neurofilaments (Eagles et al., 1980; Runge et al., 1981a; Leterrier, Liem and Shelanski, 1981), keratin filaments (Sun and Green, 1978; Franke et al., 1981; Steinert, Wantz and Idler, 1982), desmin and vimentin filaments in myogenic cells (Gard et al., 1979; O'Connor, Balzer and Lazarides, 1979; O'Connor, Gard and Lazarides, 1981) and vimentin filaments in ovarian granulosa cells (Albertini and Kravit, 1981) and various cell lines (Cabral and Gottesman, 1979; Gard et al., 1979; Browning and Sanders, 1981; Steinert et al., 1982). In myogenic cultures, desmin and vimentin are phosphorylated in part by cyclic-AMP dependent protein kinases (O'Connor et al., 1981; D. Gard and E. Lazarides, submitted for publication; see also Granger et al., 1982; Browning and Sanders, 1981). Each of the most basic isoelectric variants of desmin and vimentin is not phosphorylated, and the multiple, more acidic variants probably differ in isoelectric point only because they have covalently bound phosphate. Unfortunately, no biochemical or structural change has been unambiguously correlated with phosphorylation of the intermediate filament proteins; thus, phosphorylation has not yet provided any clear evidence for the functions of intermediate filaments. However, the hormone dependence of the phosphorylation of muscle desmin and vimentin becomes apparent only in mature myotubes, suggesting that phosphorylation of intermediate filament subunits (and therefore the filaments themselves) may play some role in differentiation or morphogenesis (D. Gard and E. Lazarides, submitted for publication); this is consistent with the topological change that intermediate filaments undergo during myogenesis, as described earlier.

The roles of some of the intermediate filament associated proteins are beginning to be explored, which will presumably aid in the elucidation of the roles of the filaments themselves. Two of the associated proteins, synemin and the high molecular weight neurofilament protein, appear to have a crosslinking activity. Regulation of this activity might modulate the flexibility or state of dispersion of the intermediate filament network. These proteins may also be components of the bridges that have been seen spanning between intermediate filaments and microtubules (e.g., Rice et al., 1980), mitochondria (David-Ferreira and David-Ferreira, 1980) and actin filaments (Schliwa and vanBlerkom, 1981). Further investigation of the developmental regulation of paranemin, the 280,000 dalton polypeptide prominent in developing skeletal muscle (Breckler and Lazarides, 1982), and determination of which intermediate filament proteins and associated proteins it is coexpressed with, will also be clues to their function.

There are many exceptions to the generalizations concerning the cell type-specific distribution of the five classes of intermediate filament protein. For example, some epithelioid cells contain no keratin, some smooth muscles contain more vimentin than desmin, some contractile cells (myoepithelial cells) contain keratin but no desmin (Franke et al., 1980), some GFAP-containing cells are found outside the central nervous system (Jessen and Mirsky, 1980), and the neurofilament core polypeptide is found in avian erythrocytes (B. Granger, unpublished observations). Hopefully, as this cataloging of apparent anomalies continues, new patterns will emerge and functional correlates will become evident.

Many cell types contain vimentin at an early stage of differentiation. For example, in muscle cells (Gard and Lazarides, 1980), neurons (Tapscott et al., 1981a,b) and astroglia (Dahl et al., 1981; Schnitzer et al., 1981; Tapscott et al., 1981b; Yokoyama et al., 1981), vimentin is expressed initially but is largely or completely replaced by one of the other classes of intermediate filament as differentiation proceeds.

Similarly, cells in primary cultures often contain vimentin in addition to another filament subunit (Gard et al., 1979; Franke et al., 1979b; Chiu et al., 1981; Virtanen et al., 1981; Jacobs et al., 1982), as do established cell lines (Franke et al., 1979a,c; Paetau et al., 1979; Virtanen et al., 1981). The apparently ubiquitous presence of vimentin in proliferating cells in tissue culture has suggested that vimentin expression may represent an adaptation to culture conditions (Franke et al., 1979a,b.), but it may also results from a selective proliferation of vimentin-containing cells (Virtanen et al., 1981). Intermediate filament expression can indeed be modulated by humoral factors in vivo (Nickerson, Skelton and Molteni, 1970; Behrendt, 1977) as well as in vitro (Summerhayes et al., 1981; Duffy, 1982). However, vimentin expression is not required for cellular "housekeeping functions", since many cell types normally contain no vimentin; vimentin is also not required only for proliferation or differentiation, since the sole intermediate filament in some terminally differentiated cells is vimentin (e.g., Schwann cells (Autilio-Gambetti et al., 1982), lens cells (Ramaekers et al., 1980; Geisler and Weber, 1981) and avian erythrocytes (Granger et al., 1982)).

Perhaps the primary role of all intermediate filaments is to prevent disruption of the cell during periods of mechanical stress. This would thus be a corollary of the "mechanical integration" hypothesis (reviewed by Lazarides, 1980), and consistent with the probable function of keratin filaments in epithelial cells. For example, finite extensibility of intermediate filaments might define the limits to which a cell could be easily stretched, and thereby prevent the disastrous consequences of discontinuities in the plasma membrane. The extracellular matrix probably plays a similar role in many instances. In contrast to microfilaments and microtubules, which often appear relatively straight and stiff, intermediate filaments usually appear flaccid, as if they could resist tension or overextension, but not compression. Protection from overextension, or shock absorbance, might be a general effect of the seemingly random arrays of intermediate filaments in cultured cells and undifferentiated cells,

but a specific role of the more ordered arrays of filaments in differentiated cells. Without these filaments, the structural continuity of long axons might not be possible, astrocytes might not be effective supporting cells, and erythrocytes might disintegrate when deformed in small capillaries (perhaps the spectrin-based cytoskeleton has taken over this role completely in mammalian erythrocytes). When muscle cells shorten during contraction, the sarcolemma has a tendency to bulge out radially; in skeletal muscle, if the sarcolemma weren't attached to the myofibrils via intermediate filaments (see Pierobon-Bormioli, 1981), contraction might have the effect of destroying the continuity of the plasma membrane with the transverse tubular system. In smooth muscle, keeping the cells compact during contraction might help to keep the contractile filaments aligned and in close proximity (see Fay, 1976). This hypothesis presupposes that the diversity of intermediate filaments is a result of the specific needs of each cell type, related to the structure and potential stresses on a given cell type, as well as to the cellular structures that the filaments are required to specifically interact with to have this effect.

Speculation about the functions of intermediate filaments will acquire a firmer foundation as more data on their properties and distributions are collected. One approach that holds great promise for elucidating their functions is that of determining their molecular interrelationships and identifying their evolutionary precursors by using immunology and gene cloning technology to trace them back through phylogeny; functions may be apparent in more primitive systems, and such systems might prove to be experimentally more manipulable. Current technology is most likely adequate to solve the foremost mysteries of these structures that are built from some of the most abundant polypeptides in the cell.

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Chapter 8

Appendix

The Preparation and Assay of the Intermediate Filament Proteins Desmin and Vimentin

The following paper will appear in

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The Preparation and Assay of the Intermediate Filament Proteins Desmin and Vimentin

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The cytoplasm of many higher eukaryotic cells contains a filamentous system whose individual filaments have an average diameter of 100Å. Since their diameter is intermediate to those of the 60Å actin filaments and the 250Å microtubules in non-muscle cells and intermediate to those of the 60Å actin filaments and the 150Å myosin filaments in muscle cells, these filaments are referred to as intermediate filaments. Recent biochemical and immunofluorescence data have established intermediate filaments as a fibrous system, distinct from other filamentous systems, but composed of chemically heterogeneous subunits (for a review see Lazarides, 1980).[1] Different classes of intermediate filaments are characteristically found in different cell types: keratin filaments in epithelial cells, neurofilaments in neurons, glial filaments in glial cells, desmin filaments in muscle cells and vimentin filaments in cells of mesenchymal origin. It has recently become apparent, however, that even though these subclasses of intermediate filaments are generally associated with these particular cell types, many cell types have more than one type of filament subunit. For example, epithelial cells contain vimentin as well as keratin filaments,[2,3] glial cells contain both vimentin and glial filaments,[4,5] muscle cells contain vimentin and desmin[6,7] (as well as a newly identified intermediate filament protein referred to as synemin[8]) and many undifferentiated fibroblast-like cells (e.g. chick embryonic fibroblasts and baby hamster kidney (BHK-21) cells) contain desmin in addition to vimentin.[9,10] Distributions of filaments coexisting in the same cell are sometimes distinguishable by immunofluorescence, such as those of keratin and vimentin filaments in epithelial cells;[2,3,11] in other cases, as with desmin, vimentin and synemin in smooth and skeletal muscle, their localization is coincident.[6,7,8]

The general approach to identifying and characterizing subunits of intermediate filaments has been to isolate or enrich for them in a given cell or tissue type (as monitored by electron microscopy) and then assay the resulting proteins with electrophoresis to identify the major components. The production of antibodies to a presumptive

filament subunit then allows immunofluorescent visualization of its distribution in situ. If a network of cytoplasmic filaments is seen, compatible with the electron microscopic evidence, then that protein is tentatively identified as an intermediate filament subunit. To distinguish the immunofluorescent pattern of intermediate filaments from that of microtubules and microtubule associated proteins, cells can be observed after treatment with agents that depolymerize microtubules (e.g., Colcemid; see below). To date, this approach has yielded the complicated picture of intermediate filaments described above.

Below we describe in detail techniques for the isolation, biochemical characterization, and assay of desmin and vimentin from muscle cells. Assays involve identification of these two proteins on two-dimensional gels, production of specific antisera, and immunofluorescence. Testing the specificity of the antisera and detection of subunits in different cell types can be accomplished with the technique of immunoautoradiography.

Presence and General Properties of Desmin and Vimentin in Different Cell Types

Desmin and vimentin coexist in many cells types. A comparison of different cell types shows that the ratio of the two molecules is quite variable. For example, in cultured chicken embryo fibroblasts, 3T3 and CHO cells, vimentin predominates or is the sole filament subunit,[9,19,12] while in BHK-21 cells, desmin and vimentin, are present in similar amounts.[9,10,13] Avian erythrocytes contain vimentin but no detectable desmin.[14,15] Smooth, skeletal and cardiac muscle cells contain predominantly desmin, but also contain lesser amounts of vimentin;[6,7,8] an exception to this is vascular smooth muscle, which contains predominantly if not exclusively vimentin.[16] No cell type has yet been found that contains desmin but not vimentin with the possible exception of the Purkinje fibers in the heart.[17] However the extremely low quantities of one or the other subunit often makes detection difficult, so that one must exercise caution in concluding that a particular subunit is absent from a given cell type.

An indispensable tool for the characterization of desmin and vimentin in muscle and non-muscle cells is SDS polyacrylamide gel electrophoresis (SDS-PAGE). Although desmin and vimentin can be resolved and tentatively identified by their mobilities in SDS-PAGE, unambiguous identification and resolution of these proteins is possible if SDS-PAGE is combined with isoelectric focusing (IEF) in two-dimensional gels, as described by O'Farrell.[18] Proteolytic fragments of these molecules are characteristic and easily recognized on 2D gels, but virtually unidentifiable on 1D gels because of their comigration with other proteins.

Avian desmin usually appears on the 2D IEF/SDS PAGE as two isoelectric variants (α and β, with isoelectric points of 5.65 and 5.70) and a molecular weight of 50,000. Mammalian desmin usually appears as a single variant that is slightly higher in molecular weight and more acidic than the avian counterpart (Mr=51,000, pI=5.6).[19,20] Avian vimentin has a predominant variant with a pI of 5.3 (Mr 52,000) and a less abundant variant that is slightly more acidic.[9] Mammalian vimentin is also higher in molecular weight and more acidic than its avian counterpart (Mr=54,000, pI=5.2).[21]

The more acidic variants of avian desmin and vimentin are phosphorylated forms of the most basic variants.[22,23] The presence of phosphatase inhibitors during the preparation of cell extracts greatly enhances the presence of the acidic variants, suggesting that dephosphorylation by intrinsic phosphatases occurs during cell fractionation. α and β desmin copurify through several cycles of filament depolymerization and polymerization, indicating that they have similar solubility properties and are both integral components of intermediate filaments.[24]

In all cultured cell types examined thus far (except mature myotubes-see Fig. 3c), desmin and vimentin filaments are aggregated into cytoplasmic or perinuclear bundles upon prolonged exposure to the microtubule-depolymerizing drug Colcemid. The significance of this aggregation phenomenon has not been determined, but it appears

to be a characteristic feature of these filaments. It is useful in that it can be used to corroborate the identity of a given protein as an intermediate filament subunit or associated protein; i.e. any protein that is similarly rearranged by Colcemid is probably associated in some way with desmin or vimentin filaments. Synemin, for example, is a 230,000 dalton protein that co-localizes by immunofluorescence with desmin and vimentin filaments in cultured muscle cells, even if the cells are first treated with Colcemid; it therefore appears to be an intermediate filament associated protein. That synemin shares most if not all of its solubility properties with desmin and vimentin suggests that the association is a tight one.[8]

Desmin and vimentin have virtually identical solubility properties. Both remain insoluble under physiological conditions, as well as in the presence of high salt and nonionic detergents. Both appear to be soluble under certain conditions of low ionic strength, and at pH extremes (<pH 2 or >pH 10). Desmin and vimentin also copurify through cycles of depolymerization and polymerization.[8,9]

The relative insolubility of these proteins under normal conditions makes enrichment with respect to total protein in the starting material fairly simple---the tissue is extracted with high salt and detergent, for example, and the residue becomes rich with intermediate filaments. Enriched residues are easily assayed on two-dimensional gels for the presence of filament subunits. Complete purification from the enriched residue is more difficult, as solubilization and complete isolation appear to require denaturing solvents.

Since smooth muscle from chicken gizzard is the richest and most convenient source of desmin thus far known, use of this tissue for desmin isolation will be described in detail. The procedure can readily be applied to other tissue sources of desmin and vimentin, such as skeletal and cardiac muscle,[25] as well as other sources of smooth muscle, such as the muscular layers of blood vessels,digestive and urogenital tracts, and mammalian uteri.[26,27] It is also generally applicable in modified form

to cultured cells, and applies also to vimentin since its solubility properties are the same as those of desmin.

Purification of Desmin from Smooth Muscle

Purification of desmin from gizzard smooth muscle involves extraction of most other proteins from the tissue, followed by solubilization of desmin from the cytoskeletal residue with acetic acid.[24,26] Neutralization of the acetic acid solution leads to precipitation of the desmin; this precipitate can be redissolved in acetic acid and reprecipitated. The result of this cycling procedure is a preparation consisting primarily of desmin, with minor amounts of actin, vimentin and synemin.

As with any protein enrichment procedure, care should be taken to minimize proteolysis, denaturation and modification of the proteins. Processing is carried out at 0°-4°C, and appropriate protease inhibitors are added to solutions.

Tissue residues are extracted and washed by centrifugation; the volume ratio of supernatant to pellet and the frequency of washes should be maximized (to efficiently remove proteases and other extraneous material) within the limits of convenience.

The following is a typical protocol. Fresh or frozen gizzard muscle (500 g) is dissected free of mucosa and connective tissue. It is homogenized first in a meat grinder and then in a Waring blender, and suspended in 5-10 volumes of a buffer containing 140 mM KCl, 10 mM EGTA, 10 mM Tris, pH 7.5. The homogenate is pelleted (10,000 xg) and resuspended several times in this buffer (the concentration of EGTA can be reduced to 1 mM subsequent to the first or second spin). A large proportion of the desmin can be solubilized at this stage with low salt and EGTA. If this is desired, then the tissue pellet is washed two or three times with 10 mM Tris, 1 mM EGTA (pH 7.5) to remove salts and readily-solubilized proteins such as actin and tropomyosin. This is followed by 1-3 prolonged (0.5-1 day each) extractions

with 10 mM Tris, 10 mM EGTA (pH 7.5). Desmin in these latter extracts can be concentrated and enriched further by precipitation with ammonium sulfate at 35% saturation, and is useful for bulk preparations of desmin intended for antigen preparation, antibody adsorptions or peptide analyses.

More complete purification of desmin is achieved from the EGTA insoluble residue. The insoluble material is freed of most of the remaining actomyosin by extraction with high salt; for example, several washes with a solution containing 0.6 MKI, 10 mM $Na_2S_2O_3$, 10 mM Tris, 1 mM EGTA (pH 8) leaves little intracellular protein other than desmin. This desmin can be solubilized with urea (see below) or prepared for solubilization with acetic acid: the high salt residue is washed with water to reduce the salt concentration to less than 1 mM, extracted several times with acetone, air dried and stored at -20°C. For the final purification of desmin the acetone powder can be washed with water to remove additional actin and tropomyosin, or it can be suspended directly in 1 M acetic acid. The acetone powder is extracted 2-3 times with acetic acid, and the combined extracts are neutralized by dropwise addition of a 10 M NaOH solution. The precipitate that forms is collected by centrifugation (5-10,000 xg) and redissolved in acetic acid; this cycle is repeated 2-4 times, resulting in a preparation of fairly constant composition. An alternative to precipitation is gelation by dialysis of the acetic acid extract against water or a neutral buffer; this allows polymerization of the desmin into filaments, which can be collected by high speed centrifugation. Electrophoretic analysis of this material reveals that it is composed primarily of desmin, but also contains relatively constant but low amounts of actin, vimentin and synemin.

Huiatt et al. have reported a procedure for obtaining desmin in pure form, free of contaminating actin.[28] Gizzard muscle that has been extracted with high salt and nonionic detergent is dissolved in a solution containing 6 M urea. This desmin-rich cytoskeletal extract is subjected to chromatography on hydroxyla-

patite and DEAE-Sepharose in the presence of urea. The final purified desmin polymerizes into 100Å filaments upon dialysis against a physiologic buffer, but remains soluble when dialyzed against 10 mM Tris-acetate, pH 8.5. It is not clear whether these preparations also contain vimentin and synemin in relatively low amounts.

Desmin has also been purified from bovine pulmonary artery by extraction with acetic acid, solubilization with urea and chromatography on Bio-Rex-70 in urea.[27]

Modification of the above described method for the purification of desmin from smooth muscle can be used for the rapid enrichment of vimentin in cells grown in tissue culture. Cultured cells (e.g. chick embryonic fibroblasts, baby hamster kidney (BHK-21) cells or mouse 3T3 cells) are washed in phosphate buffered saline (150 mM NaCl, 10 mM sodium phosphate pH 7.4), scraped off the plates and collected in a clinical centrifuge. The cell pellet is then suspended on ice in a buffer containing 0.6 M KI (or KCl), 0.02 M Tris HCl pH 7.4, 5 mM $MgCl_2$, 1 mM EGTA, 1% TritonX-100. The insoluble material, which contains nuclear remnants and intermediate filaments, is collected in a clinical centrifuge, and washed once in the extraction buffer and once with distilled water. These extracts can then be analyzed by 2D IEF/SDS PAGE for the presence of vimentin or desmin.

One and Two Dimensional Polyacrylamide Gel Electrophoresis.

One-dimensional electrophoretic analysis of proteins is performed on high-resolution SDS-polyacrylamide slab gels (SDS-PAGE) by a modification of the discontinuous Tris-glycine buffer system.[29] The stacking gel contains: 5% acrylamide, 0.13% N,N'-methylene bisacrylamide. 0.125 M Tris-HCl, pH 6.8, and 0.1% SDS. The quantities of acrylamide and bisacrylamide in the separating (lower) gel are provided by a hyperbolic relationship: % acrylamide x % bisacrylamide = 1.3. Gels containing 12.5% acrylamide are used most often because of their high resolution in the molecular-weight range of actin and desmin. A 12.5% analytical gel contains:

12.5% acrylamide, 0.107% bisacrylamide, 0.386 M Tris-HCl, pH 8.7, and 0.1% SDS. Polymerization is catalyzed by the addition of 100 µl of 10% ammonium persulfate and 15 µl of N,N,N',N'-tetramethylethylenediamine/30 ml of gel solution. The same running buffer is used in both the upper and lower reservoirs: 0.025 M Tris base, 0.112 M glycine, and 0.1% SDS, final pH 8.5. Sample buffer (2X) contains 0.1 M dithiothreitol (or 1% 2-Mercaptoethanol), 0.1 M Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, and bromophenol blue. After electrophoresis, gels are stained at least 3 hr at room temperature in 47.5% ethanol,10% acetic acid, and 0.1% Coomassie Brilliant Blue R-250, and then destained in 12% ethanol, 5% acetic acid. A piece of polyurethane foam or a Kimwipe can be put in with the gel to serve as a dye sink. Gels are photographed over a light box with Polaroid PN-55 film, using an orange-colored filter to enhance contrast.

Two-dimensional electrophoresis is carried out according to the system of O'Farrell.[18] The first dimension (isoelectric focusing) is prepared and pre-run as described,[18] but with the following modifications: the gels (2.5 X 120 mm) contain 0.2% (W/V) Ampholines, pH range 3.5-10; 1% Ampholines, pH range 4-6; and 2% Ampholines, pH range 5-7 (each supplied as 40% W/V solution). Ampholines are not added to overlay solutions or to lysis buffers. Samples are dissolved in 8-10 M urea, 1-2% NP-40 and 1% 2-mercaptoethanol, overlayed directly with 0.02 M NaOH, and run at 450 V for 16 hr and then at 800 V for 1 hr. Most non-membrane proteins focus into sharper bands if NP-40 is eliminated from both the sample and the gel; this holds true even if the sample has been solubilized in SDS. To avoid proteolysis of desmin and vimentin during urea denaturation, samples are suspended in 1 volume of 1% SDS and immediately placed in a boiling water bath for no more than 1 min. They are then dissolved at room temperature in 10 volumes of 9 M urea and 1% 2-mercaptoethanol (and 12% NP-40, if desired) prior to isoelectric focusing.

Antibody Preparation

The primary concern in obtaining an antiserum specific for a particular peptide is purity of the antigen. Antigens differ in their immunogenicity, so that use of a poorly immunogenic antigen contaminated with a small amount of highly immunogenic antigen can lead to erroneous conclusions. This was often the case in the past, before the advent of techniques adequate for assessing antigen purity and antiserum specificity. For this reason, SDS polyacrylamide gel electrophoresis is routinely used as a final purification step for most antigens. Ideally, prior to SDS-PAGE the protein of interest should be purified as much as possible by other means (for example, differential extraction or precipitation, ion exchange chromatography), as whole cell extracts most likely contain many proteins with mobilities on SDS gels that are very similar to that of the desired protein. Also, most if not all proteins streak slightly in SDS-PAGE, and contamination from this phenomenon should be minimized.

For production of antibodies against desmin, we have used desmin that is present in the 10 mM Tris-10 mM EGTA extract of gizzard as well as desmin that has been partially purified by cycles of precipitation and solubilization in acetic acid, both as described above. Synemin is a by-product of the latter procedure. A rich source of vimentin is embryonic chicken skeletal muscle, from which high salt-detergent cytoskeletons can be made and run directly on SDS gels. Somewhat cleaner preparations of vimentin (and less likely to be contaminated with desmin and other muscle proteins) can be obtained from cytoskeletons of cultured fibroblasts or chicken erythrocytes. Triton-KCl cytoskeletons of embryonic chicken muscle for the purification of vimentin are prepared as follows: leg and breast muscles from 14-16 day old embryos are dissected out and homogenized in a Dounce homogenizer in 0.1 M KCl, 50 mM Tris HCl, pH 8.0, 5 mM EGTA, 1 mM 0-phenanthroline, 0.5 mM phenylmethylsulfonyl fluoride (PMSF). The muscle homogenate is washed (by pelleting at 10,000 xg and resuspension) twice in this buffer, filtered through cheese-cloth and extracted three times in 0.6 M KCl,

2 mM $Na_4P_2O_7$, 0.5% Triton X-100, 20 mM Tris-HCl, pH 7.5, 1 mM EGTA, 10 mM 2-mercaptoethanol, 0.5 mM 0-phenanthroline, 0.1 mM PMSF. The final insoluble residue is washed with water, dissolved in hot solubilization buffer (2% SDS, 50 mM Tris-HCl, pH 6.8, 0.5% 2-ME, 10% glycerol, 0.004% bromophenol blue) for 3 min and loaded onto preparative slab gels. Desmin purified by depolymerization and polymerization or from the EGTA soluble lyophilized material is similarly dissolved in solubilization buffer prior to electrophoresis.

After electrophoresis on preparative SDS gels, protein bands are visualized by staining 10-15 min in 0.25% Coomassie blue, 47.5% ethanol, 10% acetic acid and destaining in 12% ethanol, 5% acetic acid. There are various other ways to visualize bands or determine their positions, but this is probably the most precise way; exact localization is very important if there are other protein bands in close proximity. The band of interest is carefully excised with a razor blade, equilibrated in a physiologic salt solution, and homogenized in a motor driven Potter-Elvehjem Teflon-glass homogenizer. This last step is made easier and safer by using a laboratory jack with a large rubber stopper on top to apply pressure to the bottom of the glass vessel. Primary injections are usually given with Freund's complete adjuvant, while boosts contain no adjuvant. Alternatively, adjuvant can be omitted entirely. For the primary injection with adjuvant, the homogenate is coagulated with one-tenth volume of 10% $AlCl_3$, and then emulsified with 0.5-1 volume of adjuvant by repeated passage between two linked syringes. It is injected subcutaneously at several sites on the back of a female New Zealand White rabbit. Booster injections of just gel homogenate are administered at 2-6 week intervals thereafter. Small amounts of material can also be injected intradermally, intramuscularly, or into the footpads, if desired. The smallest particles in the gel homogenate (those sedimentable only by relatively high speed centrifugation) can be injected without adjuvant into an ear vein. Injections of 0.1-1 mg of protein in a volume of 1-10 ml are most common.

Blood is collected from the marginal ear vein 1-2 weeks after booster injections. The blood is allowed to clot 2-4 hr at room temperature and contract 12-24 hr at 4°C, and the serum is clarified by centrifugation. Gamma globulins are partially purified by precipitation at 0°C with ammonium sulfate at 50% saturation, dialyzed against Tris- or phosphate buffered saline containing 10 mM NaN_3 and 1 mM ε-amino-caproic acid (a plasmin inhibitor), and stored at -70°C. Antiserum specificities and titers are usually tested by double immunodiffusion analysis and indirect immuno-fluorescence; antidesmin activity is routinely assayed for by the labeling of Z lines in isolated chicken myofibrils, while antivimentin is tested for by the staining of intermediate filaments in cultures of chicken fibroblasts. Since double immuno-diffusion is not a sensitive test of specificity, the antisera are assayed by immuno-autoradiography for cross reaction with any protein other than the antigen of interest.

Immunoautoradiography

Antiserum specificity is assayed by immunoautoradiography, an extremely sensitive technique for determining which antigens in a given cell extract react with a given antiserum. Immunoautoradiography is a solid phase assay in which proteins are separated by high resolution SDS polyacrylamide gel electrophoresis and then immobilized in the gel; antiserum is applied to the gel, and those proteins that specifically bind antibody are detected by autoradiography using a radiolabeled tag.[30] The details of a modification of this technique are presented below.

This technique is applicable to many different gel systems, but we routinely use one-dimensional discontinuous slab gels and two-dimensional IEF/SDS gels (see above). Two-dimensional gels have a much greater resolving capacity, and are therefore preferred. In addition, they allow easier identification of proteolytic fragments and undissociated multimers of the proteins under study. Isoelectric focusing is most useful for acidic proteins, but basic proteins can be visualized on non-equilibrium pH gradient electrophoresis (NEPHGE) gels.[31] Alternatively, isoelectric focusing

gels can be run on the second dimension slab gel in conjunction with an aliquot of the original sample at the side, in order to display all of the proteins in a given system on the same slab. Whereas only one sample can be run on a two-dimensional gel, many different samples can be run on a single one-dimensional gel; this simplifies the task of following a scarce antigen through a purification procedure (by simultaneously assaying for the antigen in aliquots of different fractions), and also allows rapid screening of different cell types for immunoreactive forms of the antigen.

The size of the gel depends on the degree of resolution desired and the amount of reagent available (larger gels require more antiserum, etc). Gel thickness should be minimized to facilitate diffusion of reagents into and out of the gel, but must be sufficient to allow easy handling and prevent fragmentation during any of the manipulations. We routinely use gels that are 1.6 mm thick and consist of 12.5% acrylamide and 0.107% N,N' methylene bis acrylamide (MBA). We have not determined the extent of penetration of antibodies into these gels, but have found that gels of 15% acrylamide, 0.5% MBA do not work, suggesting that gels are not being strictly surface-labeled. Polypeptides are fixed in the gel by precipitation with ethanol and acetic acid; their resistance to elution and loss during processing in physiological salt solutions is probably a function of the size and solubility properties of the polypeptides, and perhaps also of the acrylamide and bis concentrations. We have noted the disappearance of very few proteins during processing, most notably a myosin light chain and tropomyosin. Gel porosity also affects the size range of proteins that are resolved in the separating gel. If proteins smaller than 20,000 daltons, for example, run with the dye front, and if the dye front is labeled with antibody, then a different technique must be used to display these proteins for immunoautoradiography (see below).

The protein sample that is run on the gel should be from the system under study. The technique is sensitive enough to generally allow whole cells or tissues

to be run on the gel, without having to enrich for the antigen of interest. Polypeptide components that are not present in sufficient quantity to be seen by Coomassie blue staining can be readily detected with immunoautoradiography. This has the added advantage of allowing one to avoid artifactual proteolysis of the antigen during processing, which is very difficult if not impossible to eliminate completely in any sort of non-denaturing isolation or enrichment procedure. Also, if the technique is being used to demonstrate specificity of an antiserum, then a positive result is more convincing if the antigen is a minor component of the sample. Intrinsic proteases can be inhibited in a variety of ways. Non-denaturing methods (i.e., the addition of certain chemical compounds, such as PMSF and EGTA, to the solutions used) are generally not completely effective. Since all of the proteins will be denatured prior to electrophoresis, a main concern in preparing a whole cell or tissue sample is to inactivate proteases without physically degrading or modifying other proteins. Many proteases are active in 8 M urea or in 1% SDS at room temperature, so these denaturing conditions are not stringent enough; boiling in SDS, however seems to be effective. Cultured cells can be washed free of medium, treated with ethanol to precipitate protein and extract lipid, scraped from the plates and pelleted in ethanol, and then boiled directly in SDS (or stored in a freezer or lyophilized until ready for use). Whole tissue can be pulverized to a fine powder in liquid nitrogen, thawed in ethanol, pelleted and treated as above. SDS does not seem to affect the isoelectric focusing of most proteins; we have had good luck with this approach even in the absence of NP-40 in the IEF gel and sample (see above).

The following protocol is used routinely in this lab. It is not necessarily the best procedure, since many parameters of the technique have not been investigated for efficacy or optimization, but it is very reproducible and has generally given us unambiguous results.

Polyacrylamide gels are run as described (above) and then fixed for 4-16 hr

in 50% ethanol, 10% acetic acid. All incubations and washes are carried out at room temperature on a rocking platform in 8" x 8" Pyrex baking dishes tightly covered with plastic food wrap or in polystyrene boxes with a fine nylon mesh underneath the gel to prevent sticking; solutions are removed exclusively by aspiration so that the gels always remain flat and never have a chance to tear. After fixation, gels are washed with several changes of distilled water to remove the ethanol and acetic acid, and then equilibrated in Buffer I [140 mM NaCl, 10 mM Tris-Cl (pH 7.5), 5 mM NaN_3, 0.1 mM EGTA, 0.1% gelatin (Difco; dissolved by heating the solution to 60°)]. Precipitates of gelatin occasionally form, but these do not affect the final result. From fixation to equilibration in Buffer I takes 1-2 days, depending on the frequency with which the solutions are changed. Gels are then incubated for 1 day with antiserum diluted in Buffer I. An antiserum that gives a strong precipitin line in double immunodiffusion can be diluted at least a thousand-fold; low titer sera may require less dilution. Whole sera, ammonium sulfate fractionated globulins, and DEAE-purified IgG have all been used successfully. Normally, we use serum fractionated with ammonium sulfate at 50% saturation. For a standard-sized two-dimensional gel, 100 µl of antiserum are diluted in 100 ml of Buffer I for the incubation; after 1 hay, the antiserum is removed and the gel is washed for 3 days in several changes (150-200 ml each) of Buffer I. Radioiodinated protein A (see below) is applied for 1 day, and then removed by 2 days of washing in Buffer I and 1 day in Buffer I without gelatin. Gels are stained in 0.1% Coomassie brilliant blue R-250, 47.5% ethanol, 10% acetic acid, destained in 12% ethanol, 5% acetic acid, and dried onto filter paper. Autoradiograms are made by exposure to Kodak X-Omat R XR5 film with a DuPont Cronex Lightning-Plus intensifying screen at -70° for a few hours to a few days, or, for increased resolution, at room temperature without an intensifying screen for up to a month.

Staphylococcus aureus protein A (Pharmacia) is iodinated by the chloramine T method,[32] using an excess of tyrosine to quench the reaction. All solutions are

made up in 0.5 M potassium phosphate, pH 7.5, and used at room temperature. One mCi of $Na^{125}I$ (high specific activity, carrier-free, in NaOH solution) is mixed with 100 µl of 0.5 M potassium phosphate (pH 7.5); 20 µl of protein A (5 mg/ml) and 20 µl of chloramine T (2.5 mg/ml) are then added. After 2 min, 150 µl of tyrosine (0.4 mg/ml) are added. The mixture is passed centrifugally[33] through a 3 ml bed of Sephadex G-25 equilibrated (prespun several times) in the KPO_4 buffer, and the void fraction is used for labeling; it is usable for at least a month when diluted ten-fold in Buffer I and stored at 4°. The centrifugal desalting step can be performed by packing a disposable 3 cc syringe barrel with Sephadex, hanging it by its flanges inside a conical centrifuge tube, and spinning it for about a minute at a medium speed of a tabletop clinical centrifuge. 1/3 to 2/3 of the ^{125}I ends up in the void fraction, and this is enough for 20-30 two-dimensional gels, each in 100 ml of Buffer I.

Several cautionary aspects of the technique of immunoautoradiography are worth mentioning. Protein A does not bind to all classes of antibody in a given species, and there is also wide variability between species.[34] It is conceivable that this could result in the failure to detect a given antibody specificity by immunoautoradiography. Similar considerations apply if a secondary antibody is used instead of protein A. It seems unlikely that this would be significant for most antisera, but would certainly be an important consideration if monoclonal antibodies were used.

Faint, nonspecific labeling of certain polypeptides is occasionally observed. Most notable is myosin heavy chain, which sometimes labels slightly with protein A. Stacking gels and perimeters of resolving gels retain more radioactivity than interiors or resolving gels; we have not determined the reason for this, but it is useful in that it serves as a clear reference for aligning the dried gel with the autoradiogram. An example of two-dimensional immunoautoradiography using anti-vimentin is shown in Figure 1.

Antigen for immunization is usually prepared in this laboratory by excision of a band from a polyacrylamide gel that has been briefly stained with Coomassie blue. The gel slice is homogenized and equilibrated in a physiological buffer prior to injection. The animal is thus immunized with Coomassie blue and polyacrylamide in addition to the purified protein. We have found that antibodies to Coomassie blue may be present in most if not all antisera. These findings are relevant to immunoautoradiography, in that they can be a source of interference, but have no effect in most other applications. Gels can be stained with Coomassie blue prior to application of antisera and subsequent processing for immunoautoradiography, but lengthy exposures can result in autoradiograms that show all of the proteins in the gel labeled very weakly. This could be due to nonspecific binding of immunoglobulins or protein A to the Coomassie blue stained proteins, or as suggested above, it could be a result of the dye acting as a weakly immunogenic hapten. Antibodies to polyacrylamide result in a uniformly labeled gel, and must therefore be removed. This can be accomplished by preincubation of the antiserum with a blank polyacrylamide gel homogenate that has been equilibrated in Buffer I, removal of the gel particles by centrifugation, and application of the supernatant to the gel to be labeled.

The problem of anti-polyacrylamide can also be overcome by transfer of the proteins from the gel to diazotized paper[35] or nitrocellulose paper[36] for immunoautoradiography. This approach is much less time-consuming than labeling in gels, due to reduced incubation and washing periods, but suffers from a slight loss in resolution and the difficulty of visualizing the exact positions of the transferred proteins. Different proteins may also be transferred with different efficiencies. Transfer to paper would, however, be useful for examining small polypeptides that are not resolved in polyacrylamide gels that are porous enough for immunoautoradiography. This would allow dissection of proteins with proteases and mapping of antigenic peptides, for example, to study the structural relationships of cross-reacting proteins.

The ultimate test of antiserum specificity is the ability of the purified antigen to block all antibody activity. This bypasses the argument that there are antibodies in the serum with specificities for antigenic determinants that are lost upon denaturation in the polyacrylamide gels. Blocking can be performed by pre-incubation of antisera with protein that has been purified by preparative SDS gel electrophoresis; protein bands are cut out, eluted in 0.2% SDS, dialyzed against distilled water and lyophilized prior to adsorption. Elution can be performed electrophoretically or by diffusion. Appropriate controls, such as adsorptions with unrelated proteins, must also be performed. Complete blocking by this technique suggests that there are not antibodies against determinants that are lost upon denaturation but are detectable in an assay such as immunofluorescence.

Immunofluorescence

A number of cytoskeletal proteins are assayed by indirect immunofluorescence. Since desmin, vimentin and synemin exhibit indistinguishable cytoplasmic distributions, their exact distributions can be compared by double immunofluorescence. In general, all of our antisera are elicited in rabbits and indirect immunofluorescence is performed using commercially-available fluorescein-conjugated IgG fraction of goat anti-rabit IgG as a labeled secondary antibody.

For direct immunofluorescence, antibodies are usually conjugated with rhodamine [using rhodamine B isothiocyanate (RBITC) or tetramethyl rhodamine isothiocyanate (TMRITC)]:[37,38] Immunoglobulin G is partially purified by precipitation with an equal volume of saturated ammonium sulfate, dialyzed against 40 mM NaCl, 10 mM sodium phosphate pH 7.5 and passed through a column of DEAE cellulose equilibrated in the same solution. The effluent (non-retained protein peak) is collected and concentrated by ultrafiltration to 7-10 mg protein/ml. It is then incubated with approximately 40 µg rhodamine-ITC/mg protein in 0.15 M sodium carbonate buffer pH 9.5, overnight at 4° with constant stirring. Unbound dye is removed by passage through a gel filtration

column (e.g., Sephadex G-25). Conjugated IgG is then further fractionated on DEAE cellulose; the conjugates eluting with 40 mM NaCl, 10 mM sodium phosphate, pH 7.5 are used for immunofluorescence (molar dye/protein ratio of approximately 2).

Antibodies to chicken cytoskeletal proteins are routinely assayed by indirect immunofluorescence on primary or secondary cultures of embryonic chicken skeletal muscle.[39] These cultures contain both skeletal muscle cells (myoblasts that fuse to form myotubes) and non-muscle (fibroblast-like) cells, so that muscle specific antisera are easily recognized. Cells are grown on collagen coated glass coverslips, and can be processed in a variety of ways for immunofluorescence. Cells can be fixed with formaldehyde or with an organic solvent such as ethanol, methanol or acetone; if fixed with formaldehyde, the membrane can be permeabilized with detergent or organic solvent. Typically, cells are washed very briefly in phosphate buffered saline (PBS; 150 mM NaCl, 5 mM NaCl, 10 mM Na_2PO_4 pH 7.4) and fixed by immersion of the coverslip in PBS containing 2-4% formaldehyde at 37° for 10 min. Alternatively, coverslips are immersed in PBS containing 0.1-0.5% formaldehyde, 0.5% Triton X-100 for 5 min at 37°, and subsequently in PBS containing formaldehyde as above. After fixation coverslips are washed in PBS or Tris buffered saline containing 0.5% Triton X-100, and all subsequent washes and incubations are made in this same buffer. If the antigen is an insoluble cytoskeletal component, cells can be extracted prior to fixation with 0.5% Triton X-100 and 0.6 M KCl or 0.6 M KI, or with 0.5% Triton X-100 alone (with the inclusion of 5 mM $MgCl_2$ to prevent cell detachment).

Coverslips are incubated with antisera for 30-60 min at 37°. Antisera are usually partially purified by ammonium sulfate fractionation, and occasionally by DEAE cellulose chromatography prior to immunofluorescence. Antisera are diluted appropriately in PBS depending on their titer; primary antisera are usually diluted 10-50 fold and secondary antisera 100-150 fold. Coverslips are washed at least 10 min after each antibody incubation. Double immunofluorescence is performed

using a modification of the indirect-direct staining method.[40] Fixed cells are incubated with primary antiserum followed by fluorsecein conjugated secondary antiserum, as described above for indirect immunofluorescence. Unreacted antigen-combining sites of the secondary antibodies are then blocked by incubation with normal rabbit serum (ideally the preimmune serum of the following, final antibody). Cells are then washed and incubated for 30-60 min at 37°C with rhodamine-conjugated antisera. (see Fig. 2). Cells are finally washed in PBS for 10-30 min, and mounted on a drop of Elvanol (or Gelvatol) or 90% glycerol. Cells are observed with a Leitz microscope equipped with phase and epifluorescence optics and filter modules H or K for fluorescein and N2 for rhodamine. Cells are photographed on Kodak Tri-X panchromatic film at ASA 1600 and developed in Diafine (Acufine, Inc.). Color images are recorded on Kodak Ektachrome 200 color slide film and developed commercially.

A related assay for intermediate filament associated proteins takes advantage of the fact that intermediate filaments in many cells characteristically aggregate into thick, sinuous cytoplasmic bundles when the cells are incubated with Colcemid (10^{-6}-10^{-5} M for 12-24 hr) (see Fig. 3b and c). If an antigen becomes rearranged in this manner under these conditions, then this is further evidence that it somehow is associated with or is a component of intermediate filaments.

Antisera are also routinely assayed on unfixed fragments of skeletal muscle, namely myofibrils and Z-disc sheets. Myofibrils are easily recognized arrays of sarcomeres with a distinct and identifiable topology; major proteins of myofibrils have been correlated with particular sections of these structures. Z-disc sheets are transverse sections of muscle fibers, representing individual planes of laterally-registered Z-discs.[41] Myofibrils and Z-disc sheets have together allowed the elucidation by immunofluorescence of central and peripheral domains of the Z-discs. The intermediate filament proteins, desmin, vimentin and synemin, are characteristically present at the periphery of each Z-disc, while α-actinin is found within each disc. Antisera

specific for filamin have also shown that filamin is a component of the peripheral domain.[42] Figure 3a shows a Z-disc sheet labeled with antidesmin.

Myofibrils and Z-disc sheets are prepared for immunofluorescence as follows: thin strips of skeletal muscle are tied to supports (e.g., wooden applicator sticks) to prevent contraction, and stored at -15° in a physiological salt solution containing 50% (v/v) glycerol and 1-5 mM EGTA. After a few weeks, myofibrils can be prepared by vigorous homogenization of the glycerinated tissue in this same solution in a spinning-blade homogenizer; progress of the homogenization is monitored frequently by phase-contrast microscopy in order to minimize the number of unfragmented fibers and maximize the length of the resulting myofibrils. Myofibrils can be purified somewhat by differential centrifugation in the glycerol solution to eliminate connective tissue, unhomogenized chunks of muscle, vesicles and soluble proteins. Myofibrils are also stored at -15° in the glycerol solution.

Z-disc sheets are prepared by extraction of small strips of glycerinated muscle with buffered (pH 7.5-8.0) 0.6 M KI containing 0.1 mM EGTA and 10 mM sodium thiosulfate to react with free iodine. This is usually performed for a week at 4° or a day at room temperature. The extracted tissue is homogenized in this solution in a small spinning-blade homogenizer (e.g., a Virtis "45"), and progress is monitored by phase contrast microscopy in an effort to maximize the number and size of the Z-disc sheets.

For immunofluorescence, a drop of myofibrils suspended in the glycerol solution, or a drop of Z-disc sheet homogenate in the KI solution, is spread on a glass coverslip. After rinsing of nonadherent material and equilibrating in the immunofluorescence buffer (the material will remain on the coverslips even in the presence of Triton X-100), the coverslips are treated as described above for fixed cells.

Summary

Desmin and vimentin share similar molecular properties and both appear to function as subunits of intermediate filaments. Their quantity and ratio varies in different cell types with some cell types containing predominantly desmin, others predominantly or exclusively vimentin. Desmin can be purified conveniently from gizzard smooth muscle by cycles of depolymerization and polymerization. Cytologically, desmin and vimentin can be identified in immunofluorescence with antibodies specific for each of them. Antibody specificity is assessed by immuno-autoradiography and adsorption controls coupled with immunofluorescence. Desmin and vimentin react characteristically with a filamentous system in the cytoplasm of myogenic and non-muscle cells grown in tissue culture which is distinct from actin filaments and microtubles. This filamentous network and the respective immunofluorescence of desmin or vimentin is induced to aggregate in cells exposed to colcemid. Desmin and vimentin are also components of myofibril Z-discs and in particular the periphery of the Z-disc.

Footnotes

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Figure Legends

Fig. 1. Two-dimensional immunoautoradiography. (A) An extract of chick embryo skeletal muscle was run on a two-dimensional gel [isoelectric focusing (IEF) from right (basic) to left (acidic) and sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS) from top to bottom] and subsequently processed with anti-vimentin and radioiodinated protein A. The stained, dried gel is in (A) and the corresponding autoradiogram is shown in (B). Some of the major cytoskeletal proteins are labeled. Arrows denote some of the proteolytic fragments of vimentin. (Fig. 1 from ref. 6).

Fig. 2. Double immunofluorescence. (A) Phase contrast micrograph of an embryonic chicken myotube after 7 days in culture. (B) Same myotube labeled with anti-α-actinin followed by a fluorescein-conjugated secondary antibody. α-Actinin is used as a marker for myofibril Z-lines. (C) Same myotube labeled with rhodamine-conjugated anti-desmin. Desmin is undergoing its transition from cytoplasmic filaments to a Z-line associated form at this time. Note asynchrony of transition in different parts of the same myotube. Arrowheads denote Z-lines. Bar = 10 μm. (Fig. 2 from ref. 7)

Fig. 3. Assays for desmin and vimentin by indirect immunofluorescence. (A) Z-disc sheet labeled with anti-desmin. Desmin is present at the periphery of each of the more than 500 Z-discs in this sheet. (B) Portion of a cultured chick embryo fibroblast stained with anti-vimentin. (C) A week-old myogenic culture treated with Colcemid and stained with anti-vimentin. Intermediate filaments in fibroblasts (F) aggregate in response to Colcemid, while Z-line associated forms of desmin and vimentin (in myotubes) resist aggregation. Bars = 10 μm. (Fig. 3a from ref. 1; Fig. 3c from ref. 7.)

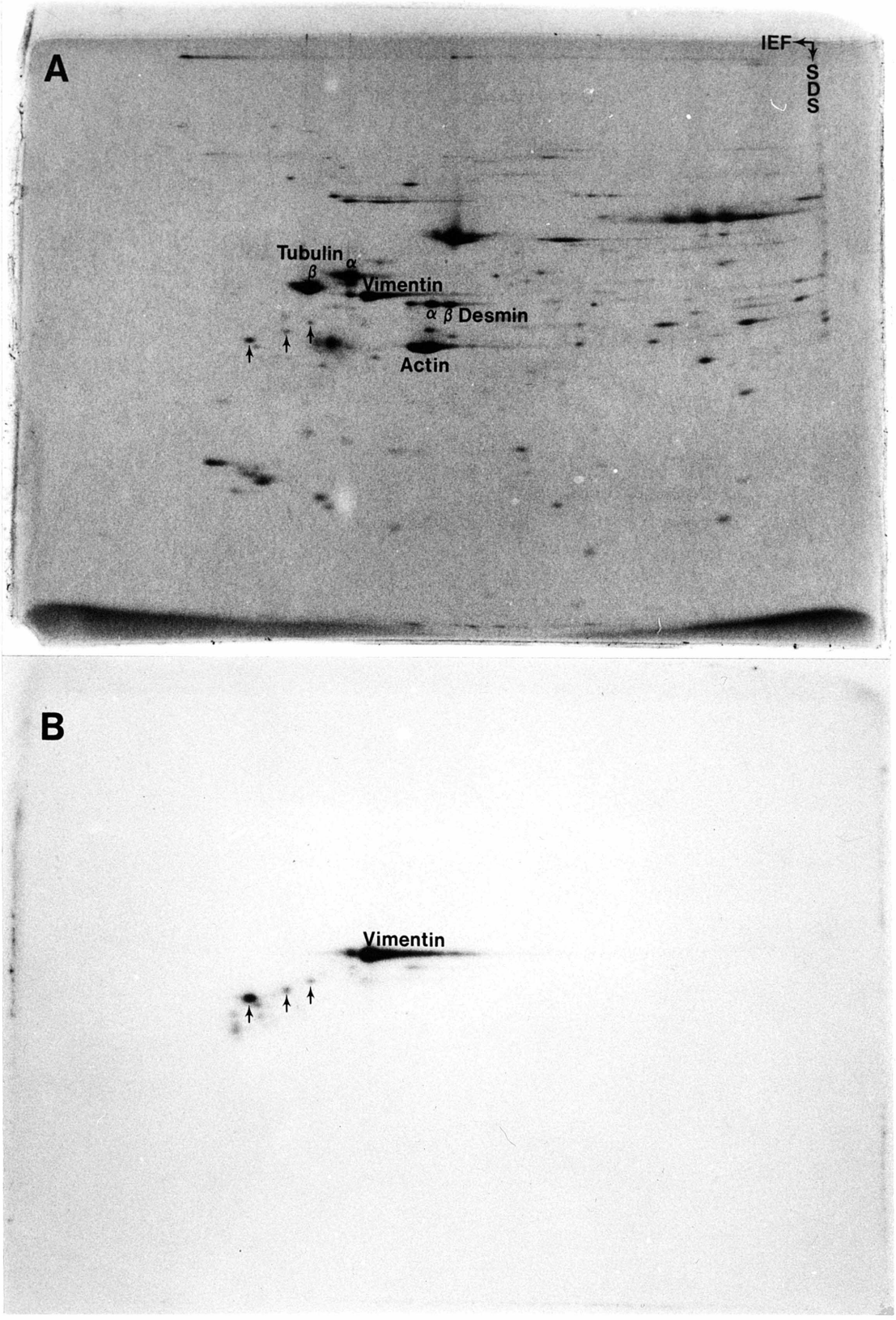

Figure 1

(from Granger and Lazarides, 1979)

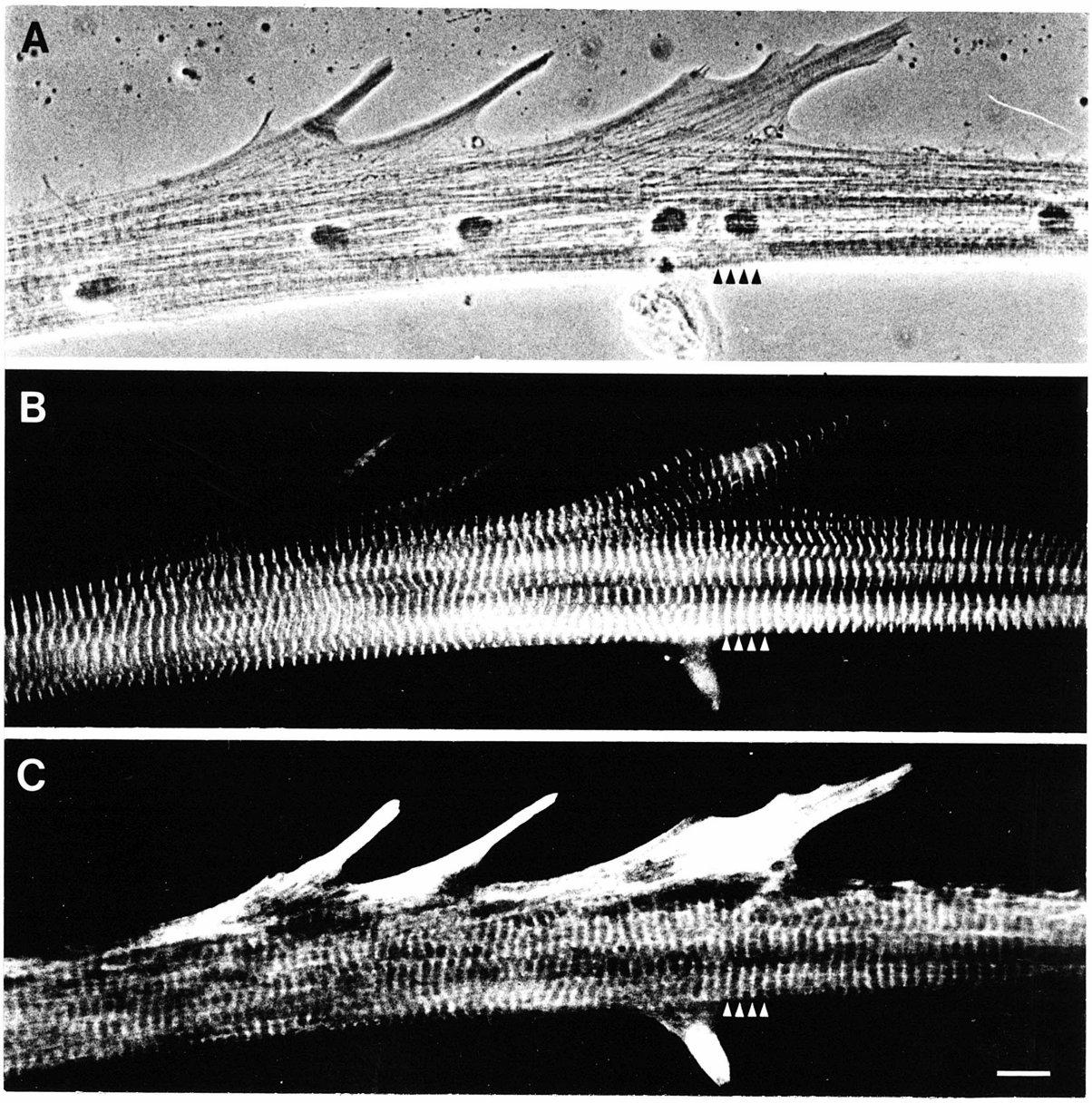

Figure 2

(from Gard and Lazarides, 1980)

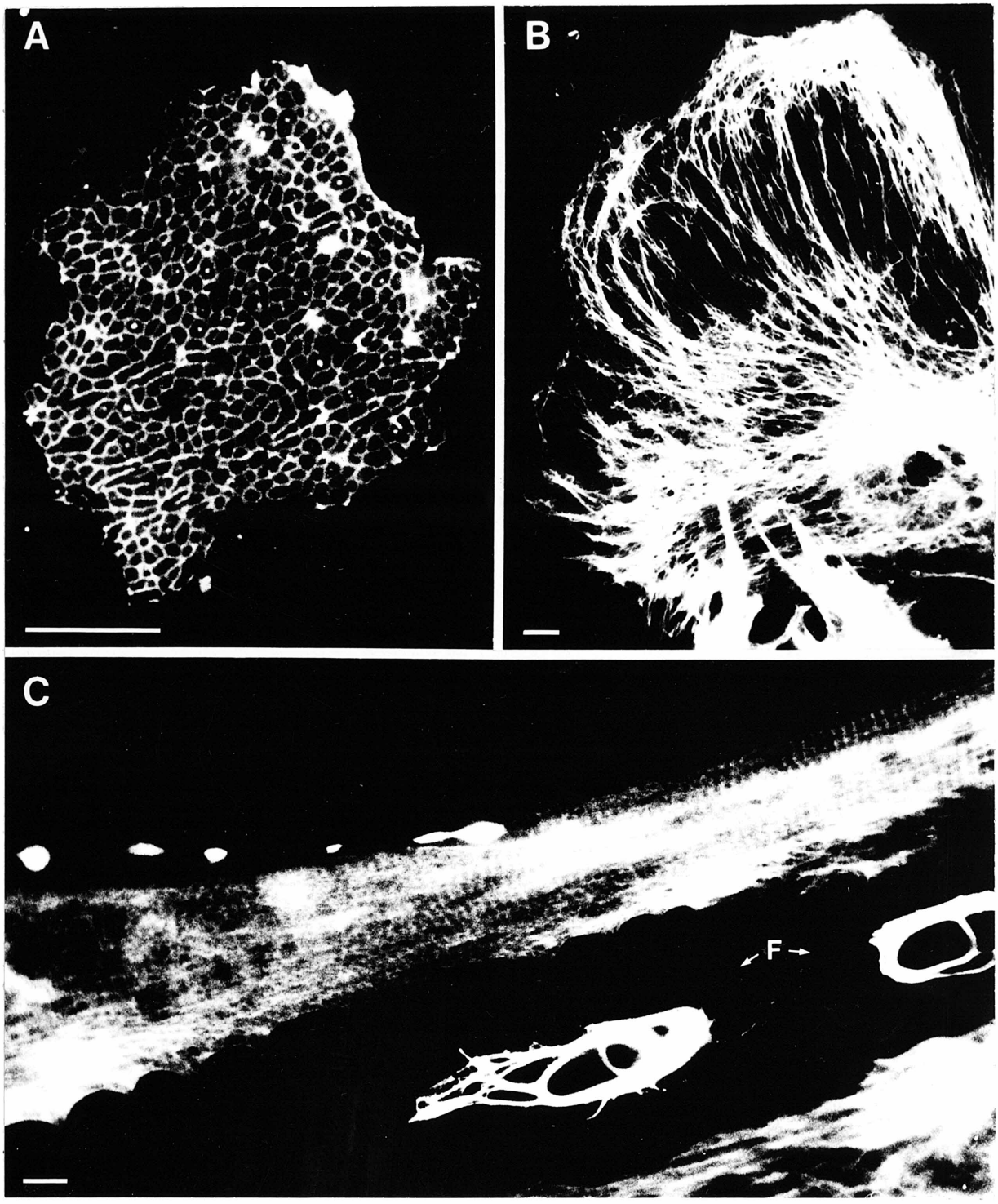

Figure 3

(a, from Lazarides, 1980; c, from Gard and Lazarides, 1980)