Intermediate Filaments and Myogenesis in vitro

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I would like to dedicate this thesis to my parents

Jesse and Ramona Gard

and to the memory of my grandfather

Jesse J. Gard, Sr.

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Abstract

This thesis describes my investigations into the composition and function of intermediate filaments (IF) during myogenesis in vitro. I have found that avian embryonic myotubes cultured in vitro contain two intermediate filament subunits, desmin and vimentin. Prior to myoblast fusion vimentin is the sole IF subunit protein detectable by electrophoretic and immunological techniques. The onset of desmin synthesis and its cytoplasmic accumulation appear to coincide with fusion of myoblasts into multinucleate myotubes. Immunofluorescence reveals dense networks of desmin- and vimentin-containing filaments in the sarcoplasm of immature myotubes; however, late in myogenesis antisera to both desmin and vimentin are observed to stain the Z-lines of myofibrils. Double immunofluorescence microscopy using antisera to α-actinin and desmin revealed that this association occurs after the assembly of α-actinin into Z-lines, at a time when individual myofibrils are being organized into bundles. Phosphorylation of intermediate filament proteins in muscle has been previously reported (O'Connor et al., Proc. Natl. Acad. Sci. U.S.A. **76**: 819-823, 1979). Using two-dimensional tryptic analysis I have found that desmin from embryonic myotubes is phosphorylated at multiple sites which correspond to sites phosphorylated by cAMP-dependent protein kinase in vitro. I have observed phosphorylation of desmin and vimentin in intact myotubes at all stages of myogenesis. However, treatment of mature (7 day and older) myotubes with 8-BrcAMP or isoproterenol results in a specific 2-3 fold increase in phosphorylation of these proteins, with a corresponding increase in $^{32}PO_4$ incorporation into one major phosphopeptide of desmin. Preliminary evidence indicates that treatment of 6-8 day myotubes with 8-BrcAMP or isoproterenol significantly inhibits the transition of intermediate filaments to the Z-line which occurs during normal myogenesis. These observations suggest that intermediate filaments containing desmin and vimentin are responsible for the organization of skeletal muscle myofibrils into an integral

contractile machinery, and that cAMP-dependent phosphorylation of desmin and vimentin plays an important role in the regulation of this function.

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Chapter 1

Introduction

Intermediate Filaments: General Background

Pioneering electron microscopists reported the existence of extensive networks of microscopic filaments and tubules coursing through the cytoplasm of (once) living cells. From these observations, and many that followed, arose the concept of a cellular skeleton, or "cytoskeleton", which provides a structural basis for cytoplasmic organization as well as many of the elements necessary for generation of mechanical force and cell motility. Since that time a great deal of effort has been expended in attempts to understand the composition and function of the cytoskeleton. To date, four filamentous systems have been described, and have been found to be ubiquitously distributed in diverse cell types throughout the animal kingdom: myosin filaments and actin-containing microfilaments which are responsible for force generation in muscle and many non-muscle cells; microtubules composed of tubulin which are involved in the motility of chromosomes during mitosis and are the major structural components of eukaryotic flagella; and a fourth class of filaments broadly categorized by the term intermediate filaments (for review, see Lazarides, 1980).

The intermediate filaments are a morphologically defined class of cytoplasmic filaments found in many diverse tissues of both vertebrates and invertebrates which are characterized by diameters of 8-12 nm. Typically they are described as long, unbranched filaments appearing round or occasionally square in cross-section. While collectively referred to by the generic term intermediate filament (IF), these filaments from distinct tissue sources are often classified according to their tissue of origin (i.e., neurofilaments or glial filaments) or by their constituent polypeptides (i.e., keratin filaments). This classification scheme has proved somewhat prophetic, for recent biochemical and immunological evidence suggests that the intermediate filaments are a heterogeneous group consisting of five distinct tissue-specific classes of filaments: axonal neurofilaments, glial filaments, keratin (tono) filaments found in epithelial cells, fibroblastic 10 nm filaments composed of vimentin, and muscle

intermediate filaments composed primarily of desmin. Each of these filament classes is composed of protein subunits which can be distinguished both biochemically and immunologically.

Intermediate Filaments in Epithelia: Keratins

Keratins and the keratin filaments are the major differentiation products of mammalian squamous epithelium (Frazer et al., 1972). Extraction of mammalian epithelium with physiological or low-salt buffers removes most cellular proteins, leaving behind a network of 8 nm keratin filaments which can be extracted as multi-chain subunits with denaturing agents such as urea (Steinert, 1975). The extensive disulfide cross-linking of keratin polypeptides during terminal differentiation requires the use of reducing agents for keratin extraction from fully differentiated tissue (Steinert, 1976; Sun and Green, 1978a). The resulting extract is enriched in 5-6 polypeptides with molecular weights ranging from 45-60,000 (Steinert, 1975; Steinert and Idler, 1975), termed the prekeratins, or cytokeratins. Removal of denaturing agents by dialysis results in the assembly of filaments similar to those observed in vivo (Steinert et al., 1976).

The amino acid compositions of the six prekeratin polypeptides are similar, but not identical (Steinert and Idler, 1975; Fuchs and Green, 1978). Based upon this observation and additional data from peptide mapping and in vitro translation of prekeratins (Fuchs and Green, 1978, 1979), it has been concluded that the prekeratins represent distinct gene products, and are not related by post-translational modification. Studies of differentiating epithelium indicate that changes in the expression of prekeratin polypeptides (and corresponding mRNA) occur during the course of epithelial cell differentiation. (Fuchs and Green, 1980).

Extensive immunological cross-reaction is observed between prekeratins from diverse mammalian species, though little correlation is observed between the

prekeratin polypeptides from different species (Sun and Green, 1978b). Antisera to prekeratins stain only true epithelial tissue in vivo (Franke et al., 1979a; Schmid et al., 1979; Sun et al., 1979). In vitro, only cells derived from epithelial sources are found to contain keratin filaments. In such cells keratin filaments are found to form extensive networks of individual or bundled cytoplasmic filaments, and are occasionally observed to associate with desmosomes (Brecher, 1975; Sun and Green, 1978b; Franke et al., 1978c, equating keratin filaments with the tonofilaments associated with desmosomes in vivo (Kelly, 1966). In contrast to other classes of IF, the distribution of keratin filaments is unaffected by the antimitotic drugs colcemid and colchicine, and keratin filaments are not observed to redistribute during mitosis (Franke et al., 1979c; Aubin et al., 1980). These characteristics and their somewhat lower diameter (8 nm, Steinert, 1976, as opposed to approximately 10 nm), make keratin filaments the least typical of the intermediate filaments.

The polymerization of prekeratins into filaments has been studied more extensively than that of other IF. In most studies keratins are solubilized in urea containing buffers, and removal of urea by dialysis results in filament assembly (Steinert, 1975; Steinert et al., 1976). Keratin filaments have been found to be obligate heteropolymers; no single prekeratin polypeptide is capable of filament formation. Copolymerization of two prekeratins invariably results in a 1:2 stoichiometry, suggesting that filaments are formed from a three-chain structural unit (Steinert et al.,1976).

Intermediate Filaments in the Nervous System: Glial and Neurofilaments

Fibrous astrocytes in mammalian brain contain many free cytoplasmic filaments, termed glial filaments, which appear similar to intermediate filaments from other tissue sources (Peters and Vaughn, 1967; Wuerker, 1970). Isolation of these filaments from gliosed brain results in the purification of a single protein

species with a molecular weight of 49-52,000 termed the Glial Fibrillary Acidic Protein (GFAP, Goldman et al., 1978; Rueger et al., 1980; for review see Bignami et al., in press). Dialysis of GFAP against low salt buffers results in its solubilization as multimers with molecular weights from 186-227,000, which can be reassembled into characteristic filaments by raising the ionic strength (Rueger et al., 1979). Antibodies raised against GFAP react specifically with glia in brain, suggesting immunological distinction from other IF subunit proteins (Bignami et al., 1972; Liem et al., 1978; Schachner et al., 1977; Yen and Fields, 1981).

Intermediate-sized filaments are one of the most common structural components of the axoplasm of both vertebrate and invertebrate neurons (Peters and Vaughn, 1967; Krishnan and Lasek, 1975; Metuzals, 1969). However, many of the early studies of mammalian neurofilaments (NF) prepared by the axonal flotation method were complicated by contamination with filaments of glial origin. This led to considerable controversy and confusion regarding the compositions of the filaments and suggestions that NF and glial filaments were composed of the same polypeptide subunit (Yen et al., 1976). Much of this controversy has subsequently been resolved, and it is now accepted that mammalian NF are composed of three polypeptides which are biochemically and immunologically distinct from GFAP. Often referred to as the neurofilament triplet, the molecular weights of these proteins have been reported as 68-73,000, 145-160,000 and 195-210,000 (Schlaepfer, 1978; Schlaepfer and Freeman, 1978; Liem et al., 1978; Schachner et al., 1978; Thorpe et al., 1979; Selkoe et al., 1979). Though some immunological cross-reaction between the triplet proteins has been reported (Willard and Simon, 1981), in vitro translation of mRNA from spinal cord indicates that the triplet proteins are all distinct translation products (Czosnek et al., 1980). These proteins have been found to represent a major portion of the slow component of axonal transport (Hoffman and Lasek, 1975; Lasek and Hoffman, 1976; Willard et al., 1980).

Monospecific antibodies to each of the neurofilament proteins have been observed to decorate isolated neurofilaments in vitro (Schlaepfer, 1977; Willard and Simon, 1981). However, it has not been determined whether all three polypeptides are necessary for filament assembly. In immunofluorescence microscopy antibodies to the NF triplet are found to stain only cells of neuronal origin, showing no cross-reaction with other cell types containing IF composed of GFAP, vimentin, desmin, or keratins (Liem et al., 1978; Bennett et al., 1978b; Schmid et al., 1979; Yen and Fields, 1981).

To circumvent the problem of glial filament contamination associated with mammalian NF preparations, several investigators turned to invertebrates as a source of NF. In the giant axon of the marine worm Myxicola infundibulum, intermediate-sized filaments are found in a tightly packed helical array which can easily be isolated by dissection (Krishnan and Lasek, 1975; Gilbert and Newby, 1975). These filaments have been found to be composed of two prominent polypeptides of 150-152,000 and 160,000 molecular weight (Gilbert and Newby, 1975). The giant axon of squid is also a good source of invertebrate neurofilaments (Metuzals, 1969). Early studies found a 70-80,000 dalton protein termed filarin as the major component of NF from Dosidicus gigas (Huneeus and Davison, 1970). More recently subunits of 60-64,000 and 190,000 have been isolated from axoplasm of Loligo paeli (Lasek and Hoffman, 1976). Purification of squid (Loligo paeli) NF by a process of assembly-disassembly in vitro has resulted in preparations enriched in a protein of 60,000 molecular weight, with minor amounts of 74,000, 100,000, and 220,000 molecular weight components (Zackroff and Goldman, 1980). Little is known about the relationship of vertebrate and invertebrate NF proteins.

Vimentin Filaments

Electron microscopy has revealed numerous intermediate-sized filaments in the cytoplasm of cells from many lines cultured in vitro (Goldman and Knipe, 1973).

Extraction of such cells with high salt and non-ionic detergents leaves a cytoskeletal residue enriched in intermediate filaments (Brown et al., 1976; Starger and Goldman, 1977; Starger et al., 1978). Analysis by SDS-polyacrylamide gel electrophoresis reveals a prominent 50-58,000 dalton protein which is immunologically and biochemically distinct from tubulin (Brown et al., 1976; Starger et al., 1978). Antisera to this protein, termed vimentin (Franke et al., 1978) , have been found to stain networks of cytoplasmic filaments in most cultured cell lines, similar in appearance to interphase arrays of microtubules. Generally filaments emanate from an indistinct perinuclear center and spread radially throughout the cell (Hynes and Destree, 1978). These filaments can be easily distinguished from microtubules, however, by the formation of characteristic perinuclear filament aggregates in cells treated with the microtubule depolymerizing drugs colcemid and colchicine (Hynes and Destree, 1978). Extensive surveys by immunofluorescence reveal that vimentin is present in nearly all cells cultured in vitro (Bennett et al., 1978a; Franke et al., 1979d). In vivo vimentin is found in a wide range of mesenchymal cells, but is absent in true epithelial cells and cells of neural origin (Schmid et al., 1979). Vimentin is found in adult muscle at levels much lower than desmin, the muscle-specific IF subunit. In developing muscle or neural tissue, expression of vimentin precedes the synthesis of the differentiated filament proteins (desmin and NF triplet; Jacobs et al., 1980; Tapscott et al., 1981), suggesting that vimentin may represent the major intermediate filament subunit of most undifferentiated cells.

Intermediate Filaments in Muscle: Desmin

The term "intermediate filament" was first applied to the numerous free cytoplasmic filaments observed in developing skeletal and heart muscle whose diameters (8-12 nm) are intermediate to those of actin thin filaments (4-6 nm) and myosin filaments (15 nm) or microtubules (25 nm) (Ishikawa et al., 1968). These filaments

with smooth wavy profiles were generally found oriented parallel to the long axis of the developing muscle fiber or myotube. While originally confused with assembling actin or myosin myofilaments, their insolubility under conditions known to extract actomyosin suggested that they represented a distinct population of cytoplasmic filaments (Rash et al., 1970a,b). The suggestion that these filaments represented an alternate polymeric form of tubulin, based upon apparent increases in the number of filaments due to treatment with colcemid or colchicine (Ishikawa et al., 1968; Holtzer et al., 1975), was also found to be incorrect (DeBrabander et al., 1975). It has subsequently been found that muscle intermediate filaments are composed of a protein subunit distinct from actin, myosin or tubulin.

While numerous in developing skeletal muscle, very few intermediate filaments are apparent in adult skeletal muscle (Page, 1969). Filaments of similar diameter and appearance are quite numerous in adult smooth muscle (Uehara et al., 1971; Cooke, 1976), however, providing a good source for the purification and characterization of the muscle intermediate filament subunit protein. As in skeletal muscle, it was found that IFs are insoluble under conditions which removed actomyosin and most other cytoplasmic proteins, leaving a cytoskeletal residue enriched in intermediate filaments (Cooke and Chase, 1971; Cooke, 1976; Small and Sobieszek, 1977). Subsequent extraction with 1 M acetic acid or 8 M urea results in solubilization of these filaments (Cook, 1976; Lazarides and Hubbard, 1976; Small and Sobieszek, 1977; Hubbard and Lazarides, 1979). Upon removal of urea by dialysis, or neutralization of acid extracts, a protein gel composed of intermediate-sized filaments is formed (Cook, 1976; Small and Sobieszek, 1977; Hubbard and Lazarides, 1979). By repeated cycles of assembly-disassembly in acid, many contaminants can be eliminated, resulting in enrichment of an acidic 50,000 dalton protein termed desmin (or skeletin), along with lesser quantities of actin (Hubbard and Lazarides, 1979; Small and Sobieszek, 1977). Further purification of desmin can be achieved by ion exchange or hydroxyapatite chromatography in the presence of urea, resulting in the separation of

desmin from contaminating actin (Huiatt et al., 1980). Purified desmin can assemble into characteristic intermediate filaments upon removal of the denaturing agent, suggesting that it is the structural subunit of smooth muscle intermediate filaments (Huiatt et al., 1980).

Two-dimensional gel electrophoresis revealed that avian gizzard desmin consists of two prominent isoelectric variants, termed α- and β-desmin (pIs of 5.65 and 5.7, respectively) (Lazarides and Balzer, 1978; Hubbard and Lazarides, 1979). Comparison of avian and mammalian desmins revealed that the mammalian counterpart is slightly more acidic and has a lower electrophoretic mobility than avian desmin (Izant and Lazarides, 1977; Lazarides and Balzer, 1978). Both peptide mapping (O'Shea et al., 1979; Gard et al., 1979) and, more recently, partial amino acid sequence analysis of avian and mammalian desmins (Geisler and Weber, 1981) reveal substantial homologies between these proteins.

Desmin has also been detected in both skeletal and cardiac muscle by immunological and electrophoretic techniques (Izant and Lazarides, 1977; Lazarides and Hubbard, 1976). By all criteria to date, desmin from all three muscle sources appears identical (Izant and Lazarides, 1977), suggesting that it is the major component of intermediate filaments in most muscle cells. Only some vascular smooth muscle appears to lack desmin, having instead intermediate filaments composed of vimentin (Franke and Warren, 1981; Gabbiani et al., 1981). No non-myogenic tissues in vivo have been found to contain desmin (Schmid et al., 1979), suggesting that it is a differentiation product specific to muscle.

In immunofluorescence microscopy of adult avian striated muscle antisera to desmin was found to stain the myofibril Z lines (Lazarides and Hubbard, 1976; Lazarides and Balzer, 1978). In transverse views of adult skeletal myofibril bundles desmin (and vimentin) is found at the periphery of each Z disc, forming a lattice which encircles and links Z discs of adjacent myofibrils (Granger and Lazarides, 1978, 1979). In cardiac muscle desmin antisera stains the myofibril Z lines, as well as

intercalated discs (Lazarides and Hubbard, 1976). Embryonic cardiac muscle cultured in vitro exhibits two patterns of antibody staining, one revealing striations corresponding to myofibril Z lines (E. Lazarides, personal communication), and a second displaying a cytoplasmic network of filaments similar to those observed with anti-vimentin sera in non-muscle cells (Lazarides, 1978). This observation suggested that some redistribution of desmin filaments might occur during the differentiation of cardiac muscle. The demonstration of a similar redistribution of desmin and vimentin intermediate filaments during skeletal myogenesis in vitro comprises Chapter 3 of this thesis.

Homology and Coexistence of Intermediate Filament Proteins

Despite their biochemically distinct composition, the five filament classes share several common traits in addition to similar morphologies, such as poor solubility in physiological strength buffers (for review, see Lazarides, 1980) and the formation of filament aggregates in cells treated with microtubule poisons such as colcemid (Ishikawa et al., 1968; Wisniewski et al., 1968; Croop and Holtzer, 1975; Hynes and Destree, 1978; Jacobs et al., 1980; keratin filaments alone remain unaffected by these drugs; Osborn et al., 1980). In addition, IF protein subunits have been found to have similar amino acid compositions, similar α-helical content (45-50%) placing them in the k m e f class of proteins (Day and Gilbert, 1972; Steinert et al.,1978; Huiatt et al., 1980), and all IF subunit proteins examined to date have been found to covalently incorporate phosphate (see below). These observations have suggested that the distinct IF subunit proteins from different tissues are related in evolution. Support for this concept has arisen recently in the form of significant homology (64%) in the partial amino acid sequences from porcine desmin and vimentin (Geisler and Weber, 1981).

The identification of biochemically distinct intermediate filament subunits in different tissues (i.e., neurofilaments in neuronal axons, keratins in epithelia, and

desmin in muscle) strongly suggest the tissue-specific expression of a single filament type in a given cell. To date this has proven to be an adequate generalization for fully differentiated cells in vivo (with the possible exception of muscle, which contains small amounts of vimentin in addition to desmin; Granger and Lazarides, 1979). This finding may have some clinical value, allowing the identification of tumor cell origin by screening with antisera to various IF proteins. However, it has been found that many cell lines cultured for extended periods in vitro possess more than one IF type (Franke et al., 1978; Franke et al., 1979b; Gard et al., 1979; Tuszynski et al., 1979; Jacobs et al., 1980; Osborn et al., 1980). In most cases reported, such cells possess vimentin in addition to the filament subunit characteristic of the cell's tissue of origin. For example, the epithelial cell lines PtK_2 and HeLa possess vimentin filaments as well as the keratin filaments normally found in epithelia (Franke et al., 1979c; Osborn et al., 1980). Expression of vimentin in such cultured cells may represent an adaptation or "dedifferentiation" necessary for growth in vitro.

The coexistence of distinct IF subunits in a single cell raises questions regarding the ability of these proteins to copolymerize into heteropolymer filaments. The identity of the filament networks stained by antisera to desmin or vimentin in cultured avian myotubes suggests such copolymerization (Gard and Lazarides, 1980; see Chapter 3). Recently it has been shown that desmin and vimentin from BHK hamster cells can form heteropolymers in vitro (Steinert et al., 1981b). In contrast, the distinct distributions of keratin and vimentin-containing filaments in HeLa and PtK_2 cells (Franke et al., 1979c; Aubin et al., 1980; Osborn et al., 1980) indicate that keratin-vimentin heteropolymers are not formed in vivo.

Assembly of Intermediate Filaments

Recent studies of intermediate filament structure and assembly have centered on the in vitro assembly properties of filaments from BHK cells (containing desmin and vimentin), or keratin filaments. Assembly of BHK filaments from low ionic

strength appears to follow kinetics compatible with a process of nucleation followed by elongation (Zackroff and Goldman, 1979). Chemical and proteolytic cleavage of BHK filaments and keratin filaments reveal significant similarities in their respective protofilament subunits. In each case protofilaments consist of a 3 polypeptide unit with coiled α-helical domains interrupted by non-helical regions, resulting in a particle 2-3 nm in diameter and 10-50 nm long (Steinert et al., 1980). Though corresponding data for glial filaments and neurofilaments have not been obtained to date, the formation of glial filaments from multimeric GFAP particles (Rueger et al., 1979), observations of protofilaments during disassembly of squid neurofilaments (Zackroff and Goldman, 1980), and ultrastructural studies of vertebrate neurofilaments (Schlaepfer, 1977) suggest a similar structure may also be found for these intermediate filament types.

Little is known about the assembly-disassembly process of intermediate filaments in vivo. As a rule, IF proteins are insoluble in physiological conditions of ionic strength and pH (for review, see Lazarides, 1980), and it is improbable that assembly in vivo can be modulated by changes in these two parameters. Soluble precursors of IF proteins have not been found. It is possible that filament assembly in vivo requires localized translation of IF protein mRNA near the growing end of filaments. Disassembly of filaments is also problematic. Many reports have documented the redistribution of intermediate filaments (in response to colcemid, mitosis or differentiation), but there are few cases of documented filament disassembly in vivo. The undirectional flow of neurofilament proteins in vertebrate axons (Lasek and Hoffman, 1976) suggests distinct sites of assembly and disassembly. It has been suggested that disassembly in vivo occurs through a process of Ca^{2+}-activated proteolysis which degrades filament subunits (Lasek and Hoffman, 1976). This suggestion is based upon the sensitivity of Myxicola and vertebrate neurofilaments to degradation by a Ca^{2+}-activated protease associated with the axonal cytoskeleton (Gilbert and Newby, 1975) and the finding of a Ca^{2+}-activated

protease which specifically degrades intermediate filament proteins (Nelson and Traub, 1981).

Functions of Intermediate Filaments

The most common role assigned to intermediate filaments is that of providing structural support. For example, it has been suggested that vimentin-containing filaments in cultured cells are responsible for the positioning and anchoring of cell nuclei and other cytoplasmic organelles (Hynes and Destree, 1978; Lehto et al., 1978; Small and Celis, 1978; Franke et al., 1978; Blose, 1979). During mitosis a cage of intermediate filaments is often observed to surround the mitotic apparatus (Hynes and Destree, 1978; Zieve et al., 1980), and it has been suggested that this filamentous barrier may serve to exclude other organelles from interferring with mitosis (Zieve et al., 1980). It has also been suggested that many key metabolic processes may occur in association with the cytoskeleton and intermediate filaments (Lenk et al., 1977).

The neurofilaments represent one of the major structural elements of neuronal axons. Along with tubulin and actin, the neurofilament proteins comprise the bulk of the slow component of axonal transport (Hoffman and Lasek, 1975; Lasek and Hoffman, 1976; Willard and Simon, 1981). It has been postulated that these elements may provide the structural elements of a neuronal transport system (Willard and Simon, 1981).

The keratins have probably the most well defined structural role. As the major differentiation product of squamous epithelia, these proteins are the major component of mammalian epidermis, providing a limiting barrier between an organism and its environment (Frazer et al., 1981).

In smooth muscle desmin-containing intermediate filaments form a cytoskeletal matrix which may serve a structural role in the organization of the contractile machinery (Cooke and Chase, 1971; Uehara et al., 1971; Cooke, 1976; Small and

Sobieszek, 1977). In skeletal and cardiac muscles intermediate filaments may serve two distinct functions. First, dense networks of intermediate filaments may provide a structural framework during the early stages of myogenesis and myofibril assembly. Late in myogenesis desmin- and vimentin-containing filaments may be directly responsible for the organization and integration of individual myofibrils into a cohesive contractile unit. The observations upon which this model is based, and data suggesting phosphorylation of IF proteins as a regulatory mechanism, are presented in the following chapters of this thesis.

Phosphorylation of Intermediate Filament Proteins

Many structural or "cytoskeletal" proteins, including actin, tubulin, microtubule associate proteins, myosin light chains, spectrin, filamin and others, are subject to modification by covalent addition of phosphate (Steinberg, 1980; Eipper, 1972; Piras and Piras, 1975; Sloboda et al., 1975; Cleveland et al., 1977; Chacko et al., 1977; Pinder et al., 1977; Davies et al., 1977; Wallach et al., 1978; Mak et al., 1978; Weatherbee et al., 1979). It is becoming increasingly apparent that protein phosphorylation may play an integral role in regulation of cellular architecture.

One feature apparently common to all intermediate filaments is the ability of their subunit polypeptides to incorporate covalently bound phosphate. In extracts of squid giant axon radiolabeled phosphate from $^{32}P(\gamma)$-ATP is incorporated into the 200,000 dalton neurofilament component, along with incorporation into a 400,000 dalton protein which also copurifies with neurofilaments (Pant et al., 1978). Similarly, both neurofilament components in Myxicola are phosphorylated in vitro by a kinase activity endogenous to the neurofilament preparations (Shecket and Lasek, 1978; Eagles and Gilbert, 1979). In crude neurofilament preparations from mammalian sources an endogenous kinase incorporates radiolabeled phosphate into all three neurofilament triplet proteins (Shecket and Lasek, 1978). Further purification results in loss of this kinase activity; however, the 150,000 molecular weight neuro-

filament component can be phosphorylated by a microtubule-associated cAMP-dependent protein kinase (Leterrier et al., 1981). Two prekeratin polypeptides have been found to be the major phosphate acceptors in human keratinocytes cultured in vitro (Sun and Green, 1978a). Both desmin and vimentin have been observed to incorporate radiolabeled phosphate in intact muscle, cultured muscle, and non-muscle cells incubated with inorganic ^{32}P-phosphate (O'Connor et al., 1979; Steinberg and Coffino, 1979; Cabral and Gottesman, 1979; Steinert et al., 1981a,b). In cultured avian myotubes both desmin and vimentin exhibit single basic non-phosphorylated species and one or more acidic phosphorylated variants (O'Connor et al., 1979). Phosphorylation of the glial filament subunit GFAP has not been reported to date. However, the presence of multiple isoelectric variants of GFAP (Rueger et al., 1980), as observed with desmin, phosphorylated prekeratins, vimentin, and other phosphoproteins, (Traugh and Porter, 1976) may be indirect evidence of GFAP phosphorylation.

A growing body of evidence suggests that phosphorylation of intermediate filament proteins in many cell types is modulated by cAMP. In S49 mouse lymphoma cells phosphorylation of vimentin is stimulated by cAMP or β-adrenergic hormones, and a mutant cell line with defective cAMP-dependent protein kinase is also deficient in vimentin phosphorylation (Steinberg and Coffino, 1979). Vimentin has also been identified as a hormonally-dependent phosphoprotein in C6 mouse glioma cells (Browning and Sanders, 1981). In Chapters 4-7 of this thesis I present evidence which strongly suggests that phosphorylation of desmin and vimentin in muscle is modulated by cAMP, and that cAMP-dependent phosphorylation of IF proteins may regulate the distribution and function of intermediate filaments during myogenesis in vitro.

A Summary of cAMP Roles in Cell Growth and Differentiation

The cyclic nucleotide adenosine 3', 5' cyclic monophosphate (cAMP) was first found to play an important role in the regulation of cell metabolism during investigations of hormonally stimulated glycogenolysis in liver and muscle (for review, see

Langan, 1973). It has subsequently been shown that cAMP serves as an intracellular mediator, or "second messenger", of many physiologically important hormonal responses. It is currently accepted that all cAMP effects in eukaryotes result from activation of cAMP-dependent protein kinases (for review, see Corbin and Lincoln, 1978), and subsequent phosphorylation of cellular protein substrates (Greengard, 1978). A second cyclic nucleotide, guanosine 3', 5' cyclic monophosphate (cGMP) has also been implicated in many cellular regulatory processes, often acting in opposition to cAMP (for review, see Goldberg et al., 1973; Goldberg et al., 1975).

A considerable amount of evidence has accumulated which suggests that cyclic nucleotides are intimately involved in the regulation of cell morphology, the cell cycle, and cell growth and cellular differentiation. It has been well established that intracellular levels of cAMP vary in concert with the cell cycle (for review, see MacManus et al., 1978; Friedman et al., 1976). cAMP levels fall to their lowest values during late G_2 and mitosis, and reach a peak during late G_1, just prior to initiation of DNA synthesis (G_1/S border). The activity ratio of cAMP-dependent protein kinase is also found to peak during late G_1 (Russell, 1978). Many conditions yielding increased cell growth such as serum stimulation and hormonal stimulation result in decreases in intracellular cAMP, while decreased growth due to serum starvation or high cell density results in increases in cellular cAMP (for review, see Pastan et al., 1975). In general it has been found that rapidly growing cells have lower cAMP levels than more slowly growing cells (for review, see Ryan and Heidrick, 1974). These observations have led to the conclusion that high cAMP levels act to inhibit cell proliferation.

Transformed cells which exhibit altered morphology, loss of contact inhibition of movement and loss of density-dependent growth inhibition exhibit lower intracellular cAMP levels than their non-transformed counterparts (Ryan and Heidrick, 1974; Pastan et al., 1974). Addition of exogenous cAMP analogues or phosphodiesterase inhibiters to these transformed cells results in return to more normal

morphology, decreases in motility, and lower cell saturation density (inhibited growth). Unfortunately, in some cells these same effects of "reverse transformation" have been obtained with several metabolic products of the commonly used analogue dibutyryl cAMP, raising questions about the validity of these effects (Ryan and Heidrick, 1974).

In many cell types high intracellular cAMP levels are associated with cessation of growth and motility, and ultimately cell differentiation. For example, in cultured neuroblastoma cells elevation in intracellular cAMP levels is associated with neurite outgrowth and increases in size of cell nuclei and the cell soma, processes which correlate with cell differentiation (Prasad and Kumar, 1974). In muscle, elevation of cAMP is associated with the early stages of differentiation and myoblast fusion (see below).

Myogenesis In Vitro

Adult vertebrate skeletal muscle fibers are multinucleate syncytia which arise by fusion of mononucleate precursor cells termed myoblasts. Myoblasts from embryonic chick muscle can be explanted and cultured in vitro (Cooper and Konigsberg, 1961). Initially a population of mononucleate myogenic cells, or presumptive myoblasts, these cells undergo a few rounds of cell division and then begin a process of differentiation remarkably similar to the process of myogenesis in vivo (Cooper and Konigsberg, 1961; O'Neill and Stockdale, 1972). After completing a terminal mitosis myoblast DNA synthesis terminates, and the cells take on a bipolar spindle shape (Konigsberg et al., 1978). Actin-containing microprocesses are extended from the cell body which fuse with similar processes from neighboring cells. Fused cells then merge into multinucleate myotubes characteristic of the muscle syncytia in vivo (O'Neill and Stockdale, 1972).

Coincident with cell fusion, or soon after, the myogenic cells exhibit increases in the synthesis of many muscle-specific proteins and isozymes (Coleman and

Coleman, 1968; Patterson and Strohman, 1972; Emerson and Beckner, 1975; Devlin and Emerson, 1978; Zani et al., 1978). As muscle protein synthesis proceeds, myofilaments begin to appear throughout the cytoplasm. Paired thick (myosin) and thin (actin) myofilaments are seen during the earliest stages of assembly (Shimada and Obinata, 1978). At a later stage myofilaments are observed to aggregate into loosely organized bundles, thought to be myofibril precursors (Shimada and Obinata, 1978). As differentiation proceeds, these bundles take on the appearance of mature myofibrils with characteristic striations. During the later stages of development myotubes in culture exhibit well organized systems of myofibrils, a rudimentary transverse tubule system, and peripherally located nuclei as observed in vivo. Spontaneous contractions of cultured muscle are also commonly observed.

cAMP and Myogenesis

Changes in intracellular cAMP have been followed during the early stages of myogenesis in vitro. Four to six hours prior to myoblast fusion there is a transient increase in cAMP levels of approximately one hour duration (Zalin, 1973; Zalin and Montague, 1974). This increase is followed by a decline to basal levels. A smaller spike in cAMP is coincident with the onset of myoblast fusion (Zalin and Montague, 1974). Prolonged treatment of myogenic cultures with agents yielding increases in intracellular cAMP (Prostaglandin E_1, dibutyryl cAMP, theophylline) results in inhibition of myoblast fusion; fusion then occurs following release from treatment (Wahrman et al., 1973; Zalin, 1976; Zalin, 1977). Measurements of cAMP-dependent kinase levels show increased kinase activity coincident with peak cAMP levels (Zalin and Montague, 1974). These observations suggest that cAMP is involved in regulation of the initial events of myogenesis; however, little is known about the role of cyclic nucleotides during the latter stages of myogenic differentiation.

The ease of muscle culture in vitro and the accessibility of cells for experimental manipulation make this an excellent system for the study of

intermediate filaments during myogenesis. The following chapters of this dissertation present the results of investigations of the composition, function and regulation of intermediate filaments during myogenesis in vitro, carried out by myself and my collaborators. Several of these studies have been previously published and are included as final manuscripts containing introductory material and discussion relevant to the topic at hand. In the final chapter, I have presented a brief overview of the possible role and regulation of intermediate filaments in myogenesis.

References

Aubin, J. E., Osborn, M., Franke, W. W., and Weber, K. (1980) Exp. Cell Research **129**, 149-165.

Bennett, G. S., Fellini, S. A., and Holtzer, H. (1978a) Differentiation **12**, 71-82.

Bennett, G. S., Fellini, S. A., Croop, J. M., Otto, J. J., Bryan, J., and Holtzer, H. (1978b) Proc. Natl. Acad. Sci. U.S.A. **75**, 4364-4368.

Bignami, A., Dahl, D., and Rueger, D. C. (in press) in Advances in Cellular Neurobiology, Vol. 1.

Bignami, A., Eng, L. F., Dahl, D., and Uyeda, C. T. (1972) Brain Research **43**, 429-435.

Blose, S. H. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 3372-3376.

Brecher, S. (1975) Exp. Cell Res. **96**, 303-310.

Brown, S., Levinson, W., and Spudich, J. A. (1976) J. Supramolecular Structure **5**, 119-130.

Browning, E. T. and Sanders, M. (1981) J. Cell Biol. **90**, 803-808.

Cabral, F. and Gottesman, M. M. (1979) J. Biol. Chem. **254**, 6203-6206.

Chacko, S., Conti, M. A., and Adelstein, R. S. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**, 129-133.

Cleveland, D. W., Hwo, S.-Y., and Kirschner, M. (1977) J. Mol. Biol. **116**, 207-225.

Coleman, J. R. and Coleman, A. W. (1968) J. Cell Physiol. **72**, (Supl. 1), 19-34.

Cooke, P. H. and Chase, R. H. (1971) Exp. Cell Res. **66**, 417-425.

Cooke, P. (1976) J. Cell Biol. **68**, 539-556.

Cooper, W. G. and Konigsberg, I. R. (1961) Anat. Rec. **140**, 195-206.

Corbin, J. D. and Lincoln, T. M. (1978) in Advances in Cyclic Nucleic Research, **9**, 159-170, Eds. George, W. J. and Ignarro, L. J. (Raven Press, New York).

Croop, J. and Holtzer, H. (1975) J. Cell Biol. **65**, 271-285.

Czosnek, H., Soifen, D., Wisniewski, H. M. (1980). J. Cell Biol. **85**, 726-734.

Davies, P., Shizuta, Y., Olden, K., Gallo, M., and Pastan, I. (1977) Biochem. Biophys. Res. Comm. **74**, 300-307.

Day, W. A. and Gilbert, D. S. (1972) Biochim. Biophys. Acta. **285**, 503-506.

DeBrabander, M., Aerts, F., Van De Veire, R., and Borgers, M. (1975) Nature Vol. 253, 119-120.

Devlin, R. B. and Emerson, C. P., Jr. (1978) Cell **13**, 599-611

Eagles, P. A. M. and Gilbert, D. S. (1979) J. Physiol. (London) **287**, 10P.

Eipper, B. A. (1972) Proc. Natl. Acad. Sci. U.S.A. **69**, 2283-2287.

Emerson, C. P., Jr. and Beckner, S. K. (1975) J. Mol. Biol. **93**, 431-447.

Frank, E. D. and Warren, L. (1981) Proc. Natl. Acad. Sci. U.S.A. **78**, 3020-3024.

Franke, W. W., Appelhaus, B., Schmid, E., Freudstein, C., Osborn, M., and Weber, K. (1979a) Differentiation **15**, 7-25.

Franke, W. W., Schmid, E., Bretkcreutz, D., Lüder, M., Boukamp, P., Fusenig, N. E., Osborn, M., and Weber, K. (1979b) Differentiation **14**, 35-50.

Franke, W. W., Schmid, E., Osborn, M., and Weber, K. (1978) Proc. Natl. Acad. Sci. U.S.A. **75**, 5034-5038.

Franke, W. W, Schmid, E., Weber, K., and Osborn, M. (1979c) Exp. Cell Res. **118**, 95-109.

Franke, W. W., Schmid, E., Winter, S., Osborn, M., and Weber, K. (1979d) Exp. Cell Res. **123**, 25-46.

Frazer, R. D. B., MacRae, T. M., and Rogers, G. E. (1972) The Keratins, Their Chemistry, Structure, and Biosynthesis. (Thomas, Springfield).

Friedman, D. L., Johnson, R. A., and Zeilig, C. E. (1976) In Advances in Cyclic Nucleic Research, Vol. 7, pp.69-114, Eds. Greengard, P. and Robison, G. A., (Raven Press, New York).

Fuchs, E. and Green, H. (1978) Cell **15**, 887-897.

Fuchs, E. and Green, H. (1979) Cell **17**, 573-582.

Fuchs, E. and Green, H. (1980) Cell **19**, 1033-1042.

Gabbiani, G., Schmid, E., Winter, S., Chaponnier, C., Chastonay, C. D., Vandekerckhove, J., Weber, K., and Franke, W. W. (1981) Proc. Natl. Acad. Sci. U.S.A. **78**, 298-302.

Gard, D. L., Bell, P. B., and Lazarides, E. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 3984-3898.

Gard, D. L. and Lazarides, E. (1980) Cell **19**, 263-275.

Geisler, N. and Weber, K. (1981) Proc. Natl. Acad. Sci. U.S.A. **78**, 4120-4123.

Gilbert, D. S. and Newby, B. J. (1975) Nature **256**, 586-589.

Goldberg, N. D., Haddox, M. K., Nicol, S. E., Glass, D. B., Sanford, C. H., Kuehl, F. A., and Estensen, R. (1975) In Advances in Cyclic Nucleotide Research, Vol. 5, pp. 307-330, Eds. Greengard, P. and Robison, G. A. (Raven Press, New York).

Goldberg, N. D., O'Dea, R. F., and Haddox, M. K. (1973) In Advances in Cyclic Nucleotide Research, Vol, 3, pp. 155-224, Eds. Greengard P. and Robison, G. A. (Raven Press, New York).

Goldman, J. E., Schaumburg, H., and Norton, W. T. (1978) J. Cell Biol. **78**, 426-440.

Goldman, R. D. and Follett, E. A. C. (1970) Science **169**, 286-288.

Goldman, R. D. and Knipe, D. M. (1973) Cold Spring Harbor Symposium on Quantitative Biology **37**, 523-534.

Granger, B. and Lazarides, E. (1978) Cell **15**, 1253-1268.

Granger, B. and Lazarides, E. (1979) Cell **18**, 1053-1063.

Greengard, P. (1978) Science **199**, 146-152.

Hoffman, P. N. and Lasek, R. J. (1975) J. Cell Biol. **66**, 351-366.

Holtzer, H., Croop, J., Dienstman, S., Ishikawa, H., and Somlyo, A. (1975) Proc. Natl. Acad. Sci. U.S.A. **72**, 513-517.

Hubbard, B. D. and Lazarides, E. (1979) J. Cell Biol. **80**, 166-182.

Huiatt, J. W. Robson, R. M., Arakawa, N., and Stromer, M. H. (1980) J. of Biol. Chem. **255**, 6981-6989.

Huneeus, F. C. and Davison, P. F. (1970) J. Mol. Biol. **52**, 415-428.

Hynes, R. O. and Destree, A. T. (1978) Cell **13**, 151-163.

Ishikawa, H., Bischoff, R., and Holtzer, H. (1968) J. of Cell Biol. **38**, 538-555.

Izant, J. G. and Lazarides, E. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**, 1450-1454.

Jacobs, M., Choo, F., and Thomas, C. (1980) Cell Biology International Reports **4**, 778.

Kelly, P. (1966) J. Cell Biol. **28**, 51-72.

Konigsberg, I. R., Sollmann, P. A., and Mixter, L. O. (1978) Dev. Biol. **63**, 11-26.

Krishnan, N. and Lasek, R. J. (1975) J. Cell Biol. **67**, 226a.

Langan, T. A. (1973) Advances in Cyclic Nucleotide Research, Vol. 3, pp. 99-153, Eds. Greengard, P. and Robison, G. A. (Raven Press, New York).

Lasek, R. J. and Hoffman, P. N. (1976) Cell Motility, pp. 1021-1050, (Cold Spring Harbor Laboratory, New York).

Lazarides, E. (1978) Exp. Cell Res. **112**, 265-273.

Lazarides, E. (1980) Nature **283**, 249-256.

Lazarides, E. and Balzer, D. R., Jr. (1978) Cell **14**, 429-438.

Lazarides, E. and Hubbard, B. D. (1976) Proc. Natl. Acad. Sci. U.S.A. **73**, 4344-4348.

Lehto, V.-P., Virtanen, I., and Kurki, P. (1978) Nature **272**, 175-177.

Lenk, R., Ranson, L., Kaufman, Y., and Penman, S. (1977) Cell **10**, 67-78.

Leterrier, J.-F., Leim, R. K. H., and Shelanski, M. (1981) J. Cell Biol. **90**, 755-760.

Liem,R. K. H., Yen, S.-H., Salomon, G. D., and Shelanski, M. L. (1978) J. Cell Biol. **79**, 637-645.

MacManus, J. P., Boynton, A. L., and Whitfield, J. F. (1978) Advances in Cyclic Nucleotide Research, Vol. 9, pp. 485-492, Eds. George, W. J. and Ignarro, L. G. (Raven Press, New York).

Mak, A., Smillie, L. B., and Bárány, M. (1978) Proc. Natl. Acad. Sci. U.S.A. **75**, 3588-3592.

Metuzals, J. (1969) J. Cell Biol. **43**, 480-505.

Nelson, W. J. and Traub, P. (1981) Eur. J. Biochem. **116**, 51-57.

O'Connor, C. M., Balzer, D. R., and Lazarides, E. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 819-823.

O'Neill, M. C. and Stockdale, F. E. (1972) J. Cell Biol. **52**, 52-65.

Osborn, M., Franke, W., and Weber, K. (1980) Exp. Cell Res. **125**, 37-46.

O'Shea, J. M., Robson, R. M., Huiatt, T. W., Hartzer, M. K., and Stromer, M. H. (1979) Biochem. and Biophys. Res. Comm. **89**, 972-980.

Page, S. G. (1969) J. Physiol. **205**, 131-145.

Pant, H. C., Shecket, G., Gainer, H., and Lasek, R. J. (1978) J. Cell Biol. **78**, R23-R27.

Pastan, I., Anderson, W. B., Carchman, R. A., Willingham, M. C., Russell, T. R., and Johnson, G. S. (1974) Cold Spring Harbor Conferences on Cell Proliferation, Vol. 1, pp. 563-570, Eds. Clarkson, B. and Baserga, R. (Cold Spring Harbor Laboratory, New York).

Pastan, I. H., Johnson, G. S., and Anderson, W. B. (1975) Ann. Rev. Biochem. **44**, 491-522.

Pinder, J. C., Bray, D., and Gratzer, W. B. (1977) Nature (London) **270**, 752-754.

Patterson, B. and Strohman, R. C. (1972) Dev. Biol. **29**, 113-138.

Peters, A. and Vaughn, J. E. (1967) J. Cell Biol. **32**, 113.

Piras,R. and Piras, M. M. (1975) Proc. Natl. Acad. Sci. U.S.A. **72**, 1161-1165.

Prasad, K. N. and Kumar, S. (1974) Cold Spring Harbor Conferences on Cell Proliferation, Vol. 1, pp. 581-594, Eds. Clarkson, B. and Baserga, R. (Cold Spring Harbor Laboratory, New York).

Rash, J. E., Shay, J. W., and Biesele, J. J. (1970) J. Ultrastructural Research **33**, 399-407.

Rash, J. E., Biesele, J. J., and Gey, G. D. (1970) J. Ultrastructural Research **33**, 408-435.

Rueger, D. C. Huston, J. S., Dahl, D., and Bignami, A. (1979) J. Mol. Biol. **135**, 53-68.

Rueger, D. C., Dahl, D., and Bignami, A. (1980) J. Cell Biol. **87**, 177a.

Russell, D. H. (1978) Advances in Cyclic Nucleotide Research, **9**, 493-506, Eds. George, W. J. and Ignarro, L. J. (Raven Press, New York).

Ryan,W. L. and Heidrick, M. L. (1974) Advances in Cyclic Nucleotide Research, Vol. 4, pp. 81-116, Eds. Greengard, P. and Robison, G. A. (Raven Press, New York).

Schachner, M., Hedley-Whyte, E. T., Hsu, D. W., Schoonmaker, G., and Bignami, A. (1977) J. Cell Biol. **75**, 67-73.

Schachner, M., Smith, C., and Schoonmaker, G. (1978) Develop. Neurosci. **1**, 1-14.

Schlaepfer, W. W. (1977) J. Ultrastructure Research **61**, 149-157.

Schlaepfer, W. W. (1977) J. Cell Biol. **74**, 226-240.

Schlaepfer, W. W. (1978) J. Cell Biol. **76**, 50-56.

Schlaepfer, W. W. and Freeman, C. A. (1978) J. Cell Biol. **78**, 653-662.

Schmid, E., Tapscott, S., Bennett, G. S., Croop, J., Fellini, S. A., Holtzer, H., and Franke, W. W. (1979) Differentiation **15**, 27-40.

Selkoe, D. J., Liem, R. K., Yen, S.-H., and Shelanski, M. L. (1979) Brain Res. **163**, 235-252.

Shecket, G. and Lasek, R. J. (1978) The Cytoskeletal and Contractile Networks of Non-muscle Cells, p. 48, Ed. Goldman, R. (Cold Spring Harbor Laboratory, New York).

Shimada, Y., Fischman, D. A., and Moscona, A. A. (1967) J. Cell Biol. **35**, 445-453.

Shimada, Y. and Obinata, T. (1978) J. Cell Biol. **72**, 777-785.

Sloboda, R. D., Rudolph, S. A., Rosenbaum, J. L. and Greengard, P. (1975) Proc. Natl. Acad. Sci. U.S.A. **72**, 177-181.

Small, J. V. and Sobieszek, A. (1977) J. Cell Sci. **23**, 243-268.

Small, J. V. and Celis, J. E. (1978) J. Cell Sci. **31**, 393-409.

Starger, J. M. and Goldman, R. D. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**, 2422-2426.

Starger, J. M., Brown, W. E., Goldman, A. E., and Goldman, R. D. (1978) J. Cell Biol. **78**, 93-109.

Steinberg, R. A. and Coffino, P. (1979) Cell **18**, 719-733.

Steinberg, R. A. (1980) Proc. Natl. Acad. Sci. U.S.A. **77**, 910-914.

Steinert, P. M. (1975) Biochem. J. **149**, 39-48.

Steinert, P. M. and Idler, W. W. (1975) Biochem. J. **151**, 603-614.

Steinert, P. M., Idler, W. W. and Zimmerman, S. B. (1976) J. Mol. Biol. **108**, 547-567.

Steinert, P. M. (1978) J. Mol. Biol. **123**, 49-70.

Steinert, P. M., Zimmerman, S. B., Starger, J. M., and Goldman, R. D. (1978) Proc. Natl. Acad. Sci. U.S.A. **75**, 6098-6101.

Steinert, P. M., Idler, W. W., and Goldman, R. D. (1980) Proc. Natl. Acad. Sci. U.S.A. **77**, 4534-4538.

Steinert, P. M., Idler, W. W., and Wantz, J. L. (1981) Federation Proceedings **40**, 1547.

Steinert, P. M., Idler, W. W., Cabral, F., Gottesman, M. M., and Goldman, R. (1981) Proc. Natl. Acad. Sci. **78**, 3692-3696.

Sun, T.-T. and Green, H. (1978a) J. Biol. Chem. **253**, 2053-2060.

Sun, T.-T. and Green, H. (1978b) Cell **14**, 469-476.

Sun, T.-T., Shih, C., and Green, H. (1979) Proc. Natl. Acad. Sci. **76**, 2813-2817.

Tapscott, S. J., Bennett, G. S., and Holtzer, H. (1981) Nature **292**, 836-838.

Thorpe, R., Delacourte, A., Ayers, M., Bullock, C., and Anderton, B. H. (1979) Biochem. J. **181**, 275-284.

Traugh, J. A. and Porter, G. G. (1976) Biochemistry **15**, 610-616.

Tuszynski, G. P., Frank, E. D., Damsky, C. H., Buck, C. A., and Warren, L. (1979) J. Biol. Chem. **13**, 6138-6143.

Uehara, Y., Campbell, G. R., and Burnstock, G. (1971) J. Cell Biol. **50**, 484-497.

Wahrmann, J. P., Winand, R., and Luzzati, D. (1973) Nature NB **245**, 112-113.

Wallach, D., Davies, P., Bechtel, P., Willingham, M., and Pastan, I. (1978) Advances in Cyclic Nucleotide Research **9**, 371-380, Eds, George, W. J. and Ignarro, L. J. (Raven Press, New York).

Weatherbee, J., Luftig, R. B., and Weihing, R. R. (1979) J. Cell Biol. **83**, 343a.

Willard, M., Simon, C., Baitinger, C., Levine, J., and Skene, P. (1980) J. Cell Biol. **85**, 587-596.

Willard, M. and Simon, C. (1981) J. of Cell Biology **89**, 198-205.

Wisniewski, H., Shelanski, M. L., and Terry, R. D. (1968) J. Cell Biol. **38**, 224.

Wuerker, R. B. (1970) Tissue Cell 2, 1-9.

Yen, S. ,Dahl, D., Schachner, M., and Shelanski, M. L. (1976) Proc. Natl. Acad. Sci. U.S.A. **73**, 529-533.

Yen, S.-H. and Fields, K. L. (1982) J. Cell Biol. **88**, 115-126.

Zackroff, R. V. and Goldman, R. D. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 6226-6230.

Zackroff, R. V. and Goldman, R. D. (1980) Science **208**, 1152-1155.

Zalin,R. J. (1973) Exp. Cell res. **78**, 152-158.

Zalin,R. J. and Montague, W. (1974) Cell 2, 103-108.

Zalin,R. J. (1976) Dev. Biol. **53**, 1-9.

Zalin,R. J. (1977) Dev. Biol. **59**, 241-248.

Zani, B., Cossv, G., Adamo, S., and Molinaro, M. (1978) Differentiation **10**, 95-100.

Zieve, G. W., Heidemann, S. R., and McIntosh, J. R. (1980) J. Cell Biol. **87**, 160-169.

Chapter 2

Coexistence of desmin and the fibroblastic intermediate filament subunit in muscle and nonmuscle cells: identification and comparative peptide analysis

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ABSTRACT Extraction of chicken embryo fibroblasts (CEF) or baby hamster kidney (BHK) cells with 1% Triton X-100 and 0.6 M KCl leaves an insoluble cytoskeletal residue composed primarily of the 52,000 m.w. subunit of intermediate filaments (F-IFP). In addition, CEF cytoskeletons exhibit a minor component with a molecular weight of 50,000, identified as α-desmin, one of the two major isoelectric variants of the intermediate filament subunit from smooth muscle. BHK cytoskeletons contain the 50,000 m.w. mammalian desmin variant. Cytoskeletons prepared from chicken embryonic myotubes contain F-IFP, and both α- and β-desmin. These data suggest that two distinct 10 nm filament subunits coexist in a single cell.

One-dimensional peptide analysis of F-IFP and desmin from avian and mammalian cells reveals significant interspecies homology, as well as homology between F-IFP and desmin from the same species. Peptide analysis of ^{32}P-labeled intermediate filament subunits suggest that there is considerable similarity in the phosphorylation sites of these proteins. These results indicate that F-IFP and desmin might be evolutionally related.

INTRODUCTION

Intermediate or 10 nm filaments have been observed in a multitude of cell types including neural, glial, muscle, and fibroblastic cells of higher vertebrates (1-9). While these filaments share several morphological and biochemical characteristics which suggest a common origin, recent studies suggest that the protein subunits of the filaments from different tissue sources are biochemically and immunologically distinct (6-8, 10-12, 14). To date, at least four distinct subclasses of 10 nm filaments can be identified: glial filaments, neurofilaments, muscle intermediate filaments, and 10 nm filaments in fibroblastic cells. The purified protein subunits of each of these filament types have distinct apparent molecular weights, and are immunologically distinguishable (2, 4-12, 14). The filament subunit from fibroblastic cells, with a molecular wieght of 52,000-55,000 has been studied in Triton X-100 extracted cytoskeletons of normal embryonic fibroblasts, and has recently been isolated from filament caps formed in spreading or colcemid treated baby hamster kidney cells (1, 12, 15). Intermediate filaments isolated from avian smooth muscle are composed of a 50,000-55,000 m.w. protein termed desmin (2, 5, 6, 9, 11), which has also been biochemically and immunologically identified in skeletal and cardiac muscle (6, 13, 14).

A fifth class of intermediate-sized filaments, characteristic of epithelial and epidermal cells, is composed of keratins. While the keratin filaments (or tonofilaments) are similar in diameter to the intermediate filaments of other cell types, they are often found in wavy bundles within the epithelial cytoplasm (15-17), or are associated with desmosomes (16). These morphological characteristics, and the role of keratin filaments in the terminal differentiation of epidermal cells distinguish them from other intermediate filament types (17).

The existence of these biochemically and immunologically distinct, cell type specific subunits suggests that a cell might contain only one class of intermediate filament, determined by its tissue of origin. Recent reports, however, indicate that some cell types possess both fibroblastic 10 nm filaments and keratin tonofilaments (15). Thus, it seems possible that two distinct intermediate filament subunits coexist in a given cell type. Here we present biochemical and immunological evidence that some nonmuscle cell types possess both the fibroblastic (F-IFP) and muscle (desmin) intermediate filament subunits. Similarly, avian skeletal myotubes possess both F-IFP and desmin. These data support the conclusion that a given cell may possess more than one class of intermediate filaments. Additionally, we present evidence suggesting that F-IFP and desmin, though antigenically and biochemically distinct, exhibit significant peptide homologies.

MATERIALS AND METHODS

Cells. Cultures of non-myogenic chicken embryo fibroblasts (CEF) were obtained by serial subculturing of fibroblastic cells derived from embryonic thigh during the preparation of myogenic cultures (see below). Three to four passages on non-collagenized petri plates are sufficient to eliminate all myogenic cells as determined by the lack of α-actin in lysates analyzed by gel electrophoresis. Fibroblasts are maintained in Eagles minimum essential medium (all culture media are from Grand Island Biological Company, Grand Island, New York) supplemented with 10% horse serum, 2% embryo extract, and antibiotics; cells were subcultured by brief trypsinization.

Baby hamster kidney 21 (BHK) cells were grown in monolayer culture in Glasgow Minimal Essential Medium supplemented with 10% tryptose phosphate and 10% calf serum.

Primary cultures of embryonic chick myogenic cells were prepared according to the method described by O'Neill and Stockdale (18), with the indicated modifications. Ten-day embryonic thigh muscles were dissected free of skin and bone, and were dissociated with trypsin at a final concentration of 0.05% for 30 min. Further dissociation was accomplished by repeated pipetting. The cell suspension was filtered through 5-ply sterile cheesecloth, and cells were washed twice by centrifugation (500 g for 5 min) followed by resuspension in growth medium. Cells were then preplated twice for 10 min to remove adherent fibroblasts (used for CEF cultures above), and then plated on collagen-coated petri plates at densities of 0.8-1.5 x 10^6 cells per 100 mm plate.

Growth medium consists of Eagles MEM supplemented with 15% horse serum, 1 x non-essential amino acids, 5% chick embryo extract, and antibiotics (100 mg/ml streptomycin, 100 µl/ml penicillin). To prevent overgrowth of contaminating fibroblasts, cultures were fed on day 3 with growth medium containing 10 µM cytosine arabinoside (CalBiochem, La Jolla, California).

Plastic petri plates (Falcon Labware, Oxnard, California) were collagen-coated with a brief wash of soluble calf-skin collagen (Worthington Biochemical Corp., Freehold, New Jersey) diluted to 0.5 mg/ml, followed by air-drying under a germicidal lamp.

Preparation of Triton-KCl Cytoskeletons for IEF/NaDodSO$_4$-PAGE. Triton-KCl cytoskeletons of BHK and CEF cells and myotube cultures were prepared by a modification of the procedure outlined by Starger et al. (12). Confluent cultures of BHK or CEF cells were primarily used, and no differences were observed in the cytoskeletal composition of spreading or colcemid-treated cells. Cells were washed twice with PBS (171 mM NaCl, 3 mM KCl, 6 mM NaK phosphate, 2 mM EGTA, pH 7.4), and then scraped from the plates with a rubber policeman. The

cell suspension was then pelleted at approximately 500 x g for 5 min. The cell pellet was lysed in 1 ml of lysis buffer (1% TX-100, 0.6 M KCl, 1 mg/ml p-tosyl-L-arginine methyl ester HCl (TAME), 0.1 mM phenylmethyl-sulfonyl fluoride (PMSF), 0.1 mM O-phenanthroline in PBS) per 100 mm plate of cells. This lysate was then digested with 0.1 mg/ml DNase I, in the presence of 10 mM Mg^{++}, for 15 min. The cytoskeletal residue was then pelleted at 1000 x g for 5 min and reextracted twice with 0.5 ml lysis buffer/plate, followed by several washes in PBS supplemented with protease inhibitors. The cytoskeletons were then solubilized in 50-300 µl of isoelectric focusing buffer (see below).

Preparation of Crosslinked Cytoskeletons for Electrophoretic Analysis and Electron Microscopy. Crosslinked cytoskeletons were prepared using a modification of the procedure of Bell et al. (19). Monolayers of BHK cells were rinsed once with Dulbecco's Phosphate Buffered Saline (DPBS, Grand Island Biological Company) and then crosslinked by adding a freshly prepared solution of 4 mg/ml of dimethyl-3, 3'-dithiobispropionimidate dihydrochloride (DTP) in DPBS. Cells were incubated in DTP for 10 min at 37°C, and the crosslinking reaction was quenched by replacing the DTP with 0.05 M NH_4Cl in PBS for 5 min. Cells were then rinsed twice with PBS, and extracted with 0.5% Triton X-100 in PBS for 5 min at room temperature. Following two rinses with PBS, the cytoskeletons were either fixed for electron microscopy (19), or solubilized in electrofocusing buffer.

Preparation of Cytoskeletons for Immunofluorescence Microscopy. Cells for immunofluorescence microscopy were grown on 18 mm circular glass coverslips. These cells are rinsed once in PBS, and then extracted for 1.5-2 min in a lysis buffer containing 1% Triton X-100, 0.6 M KI, and 0.37% formaldehyde. A small amount (approximately 1 mM) sodium thiosulfate is also added to the lysis buffer

to prevent oxidation of iodide to iodine. Cells are then briefly rinsed in PBS and are fixed in PBS containing 3.7% formaldehyde for an additional 7 min. All of the above steps are carried out at 37°C. The subsequent processing for immunofluorescence including the preparation and characterization of desmin antibody has been published previously (6, 20).

Two-dimensional Isoelectric Focusing-NaDodSO$_4$-Polyacrylamide Gel Electrophoresis (IEF/NaDodSO$_4$-PAGE). Two-dimensional electrophoresis follows a modification of the O'Farrell procedure (21) as described by Hubbard and Lazarides (11). Isoelectric focusing sample buffer contains 8 M urea, 1% NP-40, 0.5% 2-mercaptoethanol (2-ME) and protease inhibitors (PMSF, phenanthroline).

One-dimensional Peptide Mapping by Limited Proteolysis. Peptide mapping by limited proteolysis follows the procedure outlined by Cleveland et al. (22). Proteins to be digested were isolated by two-dimensional IEF/NaDodSO$_4$-PAGE; gels were stained briefly with Coomassie Brilliant Blue, and the spots of interest cut out with razor blades. These protein bands were then equilibrated in 100 mM Tris-Cl, pH 6.8, containing 2 mM EGTA and frozen until use. Gel slices were placed in wells atop the mapping gel and overlayed with differing amounts (see figure legends) of Staphylococcus aureus protease V8 (SA-V8, obtained from Miles Research Products, Elkhart, Indiana). Electrophoresis was carried out at 20 mA until the dye front reached the top of the resolving gel, at which point the power was turned off for 25 min; electrophoresis was then continued at 35 mA until the dye front reached the bottom of the gel.

The gel system used for peptide mapping consists of a 3.5 cm stacking gel of 5% acrylamide, 0.13% bisacrylamide as described previously (11), and a 15% resolving gel (23), modified by the inclusion of 8 M urea. Gels are stained overnight, destained, and photographed as previously described (11).

RESULTS

Protein Composition of Triton-KCl Cytoskeletons of CEF Cells. Extraction of chicken embryo fibroblasts (CEF) with 1% Triton X-100 leaves an insoluble cytoskeletal residue, composed primarily of actin filaments and 10 nm filaments (1, see below). Inclusion of 0.6 M KCl in the extraction buffer facilitates the extraction of actin, leaving predominately 10 nm filaments. Comparison of two-dimensional isoelectric focusing-NaDodSO$_4$ polyacrylamide gel electrophoreograms of this cytoskeleton with an 8 M urea whole cell extract shows enrichment of a small number of protein species in the residual cytoskeleton (see Fig. 1). The major polypeptide species possesses a molecular weight of 52,000, and has been tentatively identified as the fibroblastic intermediate filament subunit (F-IFP) as described by Brown et al. in similar CEF cultures (1), and by Starger et al. in BHK cells (12). Two peptides with molecular weights of 50,000 are also apparent in the cytoskeletal sample. The more basic of these, slightly more basic than actin, has been identified as α-desmin by coelectrophoresis of CEF cytoskeletons with desmin isolated from avian smooth muscle (11, data not shown). The more acidic 50,000 m.w. protein yields a one-dimensional peptide profile similar to α-desmin (data not shown), and thus represents a new desmin variant. Previous reports indicate that desmin from smooth muscle and skeletal muscle myofibrils consists of two isoelectric variants, α and β (5, 6); no β desmin is identifiable in cytoskeletons from CEF cells. Small quantities of β,γ actin also remain in the cytoskeletal residue, though the majority of actin is solubilized during the extraction. Several degradation products of F-IFP are apparent, forming a diagonal line extending to the lower left (acidic, lower molecular weight) of F-IFP. The presence of these degradation products is not affected by the inclusion of TAME, PMSF, or phenanthroline,

but is eliminated by heating samples to 100°C in 1% $NaDodSO_4$ and 0.5% 2-ME for 3 min prior to IEF (data not shown).

Immunofluorescence of chick embryo fibroblasts with rabbit antisera raised against chicken gizzard desmin reveals little antibody specific fluroescence. However, if the CEF cells are pretreated with 5 μM colcemid for 16 hr prior to fixation and antibody staining, the induced 10 nm filament cap is weakly positive for desmin-specific antibody. This fluorescence is more easily visualized in cells which have been extracted with TX-100 and KI prior to fixation (see Fig. 2), which is analogous to the preparation of cytoskeletons for electrophoretic analysis. Absorption of the antiserum with purified desmin (11) blocks the specific fluorescence (not shown). No cross-reaction of desmin antisera with F-IFP is detectable in immunolabeling of IEF/$NaDodSO_4$ gels (not shown), in spite of the homology exhibited by these proteins (see below).

Cytoskeletons from BHK Cells. Analysis of BHK cytoskeletons prepared using the reversible crosslinker dimethyl-3,3'-dithiobispropionimidate (DTP) prior to extraction with TX-100 yields results similar to those described above for CEF cells (Fig. 3a). F-IFP and β,γ actin are clearly identifiable, as well as two proteins with molecular weights of 50,000. The more basic of these two proteins has been identified as the mammalian desmin species by coelectrophoresis with mammalian and avian muscle desmin. The single mammalian desmin species differs slightly from avian α-desmin in both molecular weight and isoelectric point (14). The more acidic protein of 50,000 molecular weight in BHK cytoskeletons corresponds to the new acidic variant of desmin described above for CEF cells. It is interesting to note the lack of the "diagonal" proteins in crosslinked cytoskeletons. Electron microscopy of crosslinked cytoskeletons reveals numerous 10 nm filaments within the cytoplasm (Fig. 3b).

Cytoskeletons from Skeletal Muscle Myotubes. Triton cytoskeletons prepared from cultures of skeletal muscle myotubes low in contaminating fibroblasts were compared to 8 M urea extracts of whole myotubes (Fig. 4), and to CEF cytoskeletons. The protein constituents of the myotube cytoskeleton are quite similar to those obtained from fibroblasts (compare Figs. 1 and 4). The two major features which distinguish myotube cytoskeletons are the presence of both α and β desmin, and the predominance of the muscle specific α-actin variant. IFP, the diagonal proteins, and the new acidic desmin variant are all identifiable in quantities which cannot be accounted for by a slight (< 5%) contamination with mononucleate fibroblastic cells.

Comparative Peptide Analysis of Desmin and IFP. One-dimensional peptide analysis of cytoskeletal proteins was performed using protease V8 from Staphylococcus aureus. Due to the similarity in the electrophoretic mobilities of IFP and desmin, it was found necessary to isolate these proteins from two-dimensional IEF/NaDodSO$_4$-PAGE gels. Extensive homology is evident between avian (CEF) F-IFP and mammalian (BHK) F-IFP, with 11 of 14 generated peptides exhibiting closely similar molecular weights (Fig. 5, lanes A-D). Similarly, avian and mammalian desmin exhibit several similar peptides (Fig. 5, E-H) implying some interspecies homology between these proteins, though less than the homologies exhibited by the F-IFPs. Homologous peptides are also apparent when desmin and IFP from the same cells (either CEF, myotubes, or BHK) are compared (Fig. 5, C-F). Neither IFP nor desmin showed homology with actin or tropomyosins. Examination of the phosphopeptides generated from ^{32}P-labeled F-IFP and desmin (24) further supports the conclusion that these proteins are homologous. The autoradiogram of the peptide mapping gel (Fig. 6) shows that the patterns of phosphopeptides of these two proteins from both myotubes and BHK cells are nearly identical; phosphopeptides from α, β tropomyosin (24) are not homologous to either IFP or desmin.

DISCUSSION

Coexistence of Desmin and F-IFP in CEF, BHK, and Muscle Cells. We have demonstrated that nonmuscle cells of both avian (CEF) and mammalian (BHK) species contain desmin, and that avian skeletal muscle myotubes contain the fibroblastic intermediate filament protein. These results are interesting in several regards. First, the presence of identifiable desmin in CEF and BHK cells indicates that desmin is not a muscle-specific protein, having a wider distribution than originally believed (5, 6, 11, 14). A protein tentatively identified as desmin has also been observed in cytoskeletons of mouse 3T3 cells (unpublished observations). Second, the observation that these cell types contain both desmin and F-IFP indicates that two proteins capable of forming morphologically similar filaments may coexist in the same cell. Analogous results have been obtained by Franke et al., who have reported the coexistence of keratin filaments and fibroblastic (termed vimentin) filaments in PTK_2 and HeLa cells (15). Closer examination of the results published by Brown et al. also reveal desmin in CEF cytoskeletons (1). Analysis of BHK 10 nm filament caps by Starger et al. revealed the presence of two major components of 55,000 and 54,000 m.w., which exhibited some peptide homologies (12). Data presented above suggest that these components represent F-IFP and desmin, respectively, which migrate with molecular weights of 52,000 and 50,000 in our gel systems.

The relationship between the desmin variants described to date remains to be established. Desmin from avian smooth and skeletal muscle myofibrils consists of two isoelectric variants, α and β (5, 14). In this paper, we identify a third, more acidic, variant of desmin present in CEF cells and skeletal myotubes. Recently we have reported that α desmin and the new acidic variant are phosphorylated both in in vitro differentiating myotubes, and in embryonic muscle in vivo (24). β Desmin is not phosphorylated in myotubes, and is not observed in CEF cells.

It is possible that β desmin represents a nonphosphorylated precursor to the more acidic variants, with the addition of phosphate responsible for the observed differences in isoelectric point. A similar situation may exist for F-IFP, where multiple phosphorylated variants and a single more basic variant which is not phosphorylated has been described (24). A new acidic variant of mammalian desmin is apparent in BHK cells, which also possess the mammalian species of desmin previously described in rat and guinea pig myofibrils (14). As in avian cells, the acidic desmin variant is phosphorylated, while the basic mammalian desmin species is not phosphorylated, suggesting that it is analogous to avian β desmin (Gard and Lazarides, unpublished data).

Peptide Homologies between Desmin and F-IFP. Amino acid compositions reported for intermediate filament subunits from many sources (including glial, neural, epidermal, muscle, and fibroblastic cells) suggest that these proteins might be similar (8, 11, 12), though they are biochemically and immunologically distinct (6-12). The analysis above indicates that there is significant homology between the proteolytic peptides of F-IFP from BHK and CEF cells, implying some sequence homology between these proteins. F-IFP from mouse 3T3 cells also exhibits significant homology to that from CEF and BHK cells (Gard and Lazarides, unpublished data). Similarly, desmin isolated from avian and mammalian sources exhibit peptide homologies. Also of interest is the intra-species homology seen between F-IFP and desmin from either avian myotubes or BHK cells, which is more evident in the analysis of the phosphopeptides from F-IFP and desmin. Since both filament proteins (F-IFP and desmin) are phosphorylated in both BHK cells and avian myotubes (24; unpublished observation), we used this property to focus our peptide analysis on regions of possible functional significance. The high degree of similarity between the phosphopeptides of F-IFP and desmins from both BHK cells and avian myotubes

strengthens the conclusion that desmin and F-IFP have some homologous sequences, and in particular suggests that these homologies span the sites of phosphorylation. Though these data are not yet conclusive, they suggest that the subunits of 10 nm filaments form a family of evolutionally related proteins.

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REFERENCES

1. Brown, S., Levinson, W. & Spudich, J. A. (1976) J. Supramol. Struct. **5**, 119-130.
2. Cooke, P. (1976) J. Cell Biol. **68**, 539-556.
3. Goldman, R. (1971) J. Cell Biol. **51**, 752-762.
4. Ishikawa, H., Bischoff, R. & Holtzer, H. (1968) J. Cell Biol. **38**, 538-558.
5. Izant, J. G. & Lazarides, E. (1974) Proc. Natl. Acad. Sci. USA **74**, 1450-1454.
6. Lazarides, E. & Hubbard, B. D. (1976) Proc. Natl. Acad. Sci. USA **73**, 4344-4348.
7. Liem, R. K. H., Yen, S.-H., Salomon, G. D. & Shelanski, M. L. (1978) J. Cell Biol. **79**, 637-646.
8. Schachner, M., Hedley-Whyte, E. T., Hsu, D. W., Schoonmaker, G. & Bignami, A. (1977) J. Cell Biol. **75**, 67-73.
9. Small, J. V. & Sobieszek, A. (1977) J. Cell. Sci. **23**, 243-268.
10. Bennet, G. S., Fellini, S. A., Croop, J. M., Otto, J. J., Bryan, J. & Holtzer, H. (1978) Proc. Natl. Acad. Sci. USA **75**, 4364-4368.
11. Hubbard, B. D. & Lazarides, E. (1979) J. Cell Biol. **80**, 166-182.
12. Starger, J. M., Brown, W. E., Goldman, A. E. & Goldman, R. D. (1978) J. Cell Biol. **78**, 93-109.
13. Granger, B. L. & Lazarides, E. (1978) Cell **15**, 1253-1268.
14. Lazarides, E. & Balzer, D. R., Jr. (1978) Cell **14**, 429-438.
15. Franke, W. W., Schmid, E., Osborn, M. & Weber, K. (1978) Proc. Natl. Acad. Sci. USA **75**, 5034-5038.
16. Sun, T.-T. & Green, H. (1978) Cell **14**, 469-476.
17. Rice, R. H. & Green, H. (1977) Cell **11**, 417-422.
18. O'Neill, M. C. & Stockdale, F. E. (1972) J. Cell Biol. **52**, 52-65.
19. Bell, P. P., Jr., Miller, M. M., Carraway, K. L. & Revel, J.-P. (1978) in Scanning Electron Microscopy (SEM, Inc., O'Hare, IL), Vol. II, pp. 899-906.

20. Lazarides, E. (1976) J. Cell Biol. **68**, 202-219.

21. O'Farrell, P. H. (1975) J. Biol. Chem. **250**, 4007-4021.

22. Cleveland, D. W., Fischer, S. G., Kirschner, M. W. & Laemmli, U. K. (1977) J. Biol. Chem. **252**, 1102-1106.

23. Laemmli, U. K. (1970) Nature **227**, 680-685.

24. O'Connor, C. M., Balzer, D. R. & Lazarides, E. (1979) Proc. Natl. Acad. Sci. USA, in press.

FIGURE LEGENDS

FIG. 1. Comparison of whole cell extracts with Triton-KCl cytoskeletons of chick embryo fibroblasts. Isoelectric focusing was from right (basic) to left (acidic) in all two-dimensional gels presented. The whole cell extract (A) contains tubulin (Tb), fibroblastic intermediate filament protein (IFP), and β,γ-actin (Ac) as well as many unidentified proteins. No desmin can be identified in the whole cell extract. The insoluble residue of Triton-KCl extraction is enriched in IFP, actin, and two desmin variants (Ds). The more basic variant (middle bracket) has been identified as α-desmin (see text, Fig. 4). The acid variant (dotted bracket) has not been identified in adult muscle. No β desmin (right bracket) is detectable in CEF cells. The proteins referred to in the text as the "diagonal" proteins are seen to the acidic side of IFP, and with lower molecular weights.

FIG. 2. Immunofluorescence of Triton-KI cytoskeletons of colcemid-treated CEF cells. Cells were incubated for 16 hr in 5 μM colcemid, and cytoskeletons examined by phase (A) and immunofluorescence microscopy (B) using antibodies raised against smooth muscle desmin (800 x mag.). The colcemid induced aggregation of intermediate filaments into a perinuclear filament cap is apparent. Desmin-specific fluorescence is localized to this structure.

FIG. 3. Analysis of DTP crosslinked cytoskeletons of BHK cells. (A): The cytoskeletal residue of BHK cells crosslinked with DTP prior to Triton extraction contains predominantly IFP, β,γ-actin, and desmin (Ds). Note the lack of diagonal proteins in the crosslinked cytoskeleton (see text). (B): Thin sections of the cytoskeleton reveal numerous intermediate filaments with diameters of 7-8 nm (x 62,000).

FIG. 4. Comparison of whole cell extracts and Triton-KCl cytoskeletons of cultured embryonic myotubes. Whole cell extracts of 7 day myotubes contain tubulin (Tb), IFP, α and β desmin (Ds), α, β, γ-actins, α, β-tropomyosins, and many unidentified protein species. The cytoskeletal residue (B) consists of IFP, α, β desmin (Ds) and actin (Ac). Note that α-actin is the major actin species of the muscle cytoskeleton.

FIG. 5. One-dimensional peptide analysis of cytoskeletal proteins using S. aureus protease V8. (A,B): Hamster IFP using 0.02 μg and 0.1 μg protease, respectively; (C,D): Chick IFP; (E,F): Chick desmin (myotube); (G,H): Hamster desmin. Significant homology is evident between IFP from hamster and chick (compare A,B with C,D). Desmins from the two species also show several homologous peptides (compare E,F with G,H). IFP and desmin from chick show less homology, although several similar peptides are apparent.

FIG. 6. One-dimensional analysis of phosphopeptides from IFP and desmin. Cultured chick myotubes and BHK cells were labeled for 20 hr with 200 μCi of $^{32}PO_4$ (carrier free, New England Nuclear) per plate. Cytoskeletal proteins were isolated and subjected to peptide analysis. Significant homologies are evident in the phosphopeptides of chick F-IFP (A,B; 0.01 μg and 0.1 μg SA-V8, respectively), and chick α-desmin (C,D); β-desmin (E,F) is not phosphorylated (with slight contamination by α-desmin). Hamster F-IFP (G) and the hamster acidic desmin variant (H, see text) also show striking homology to each other, and to the analogous chick proteins. The pattern of phosphopeptides from chick myotube tropomyosin (I,J) is distinct.

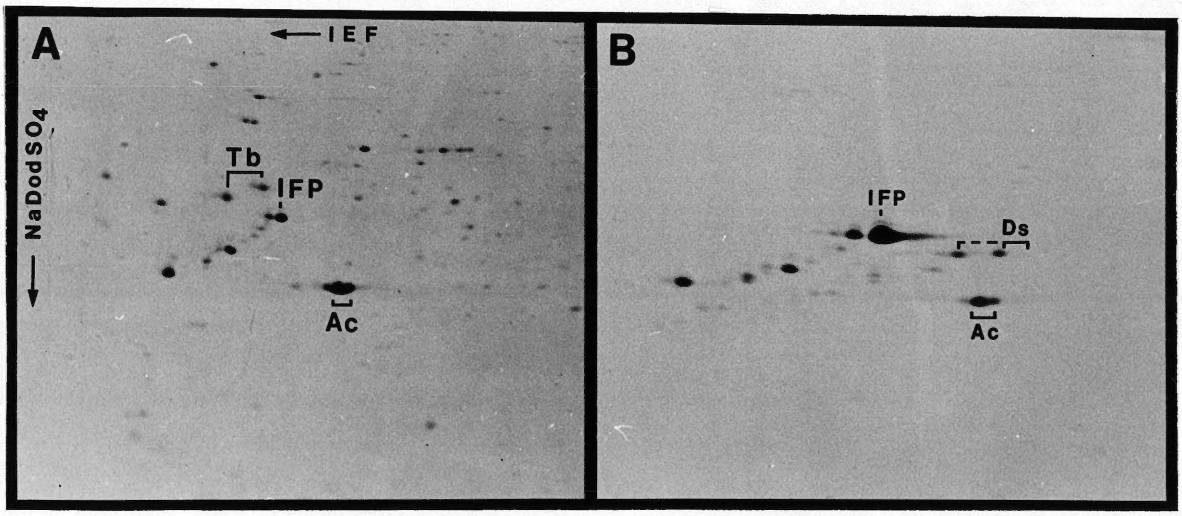

Figure 1

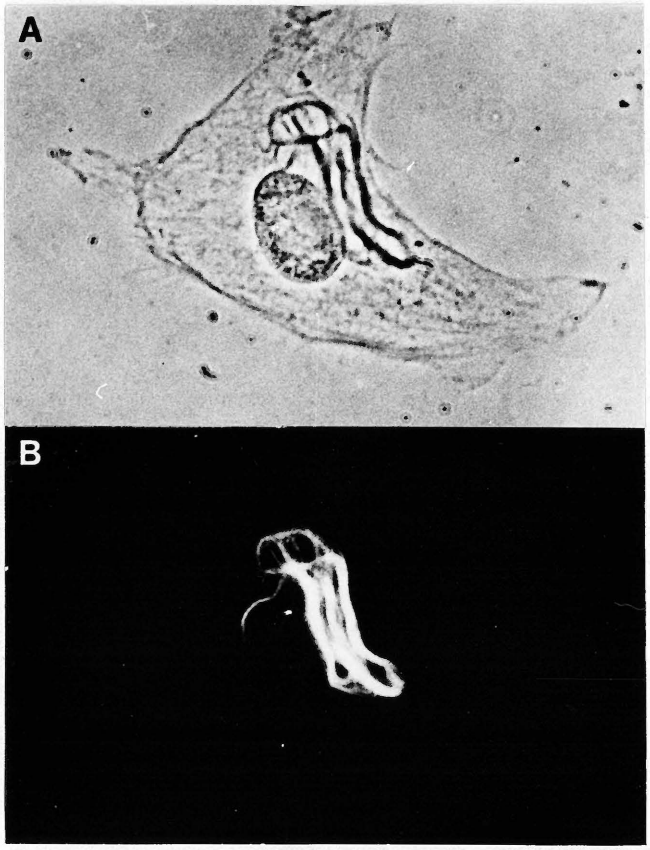

Figure 2

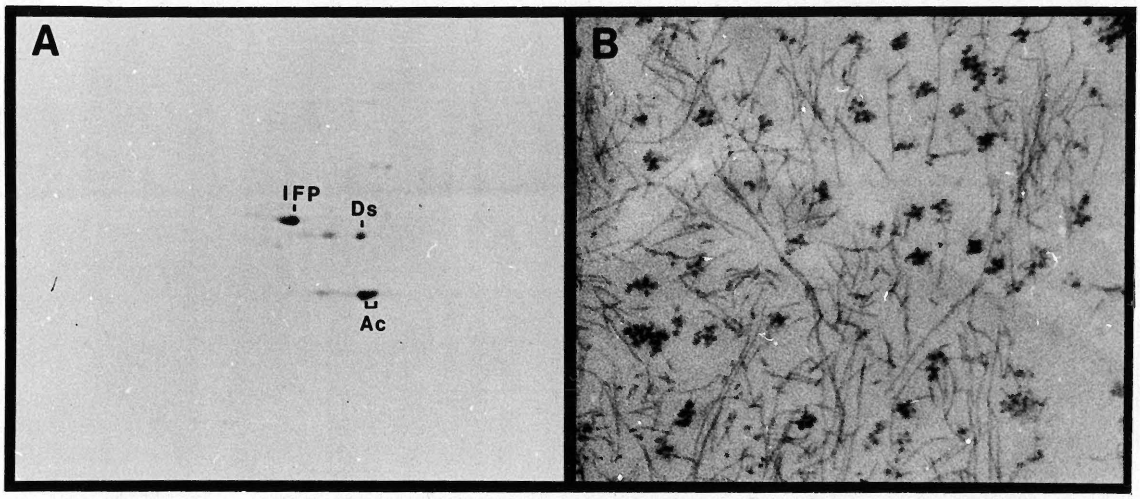

Figure 3

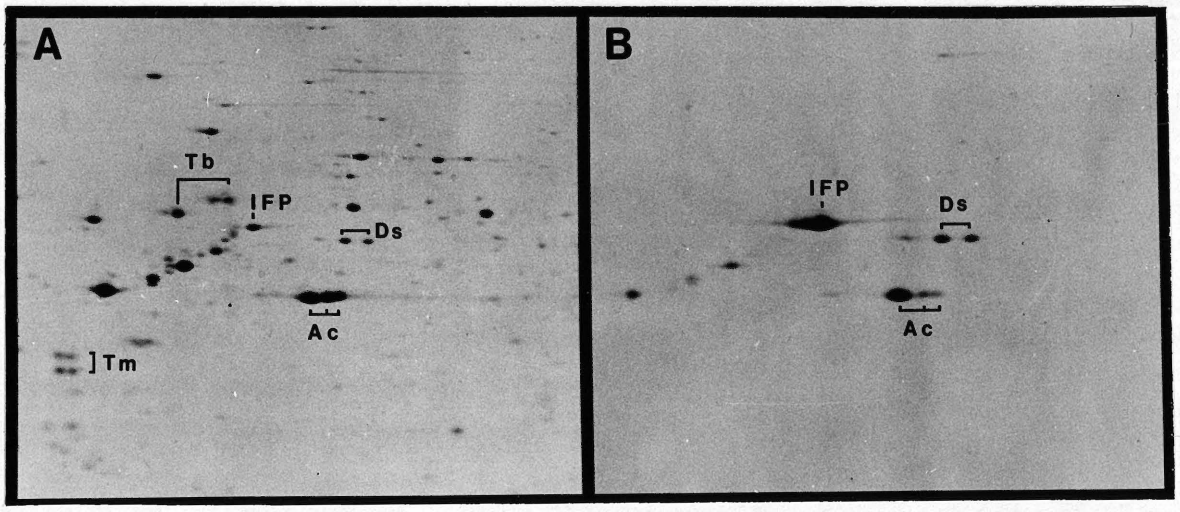

Figure 4

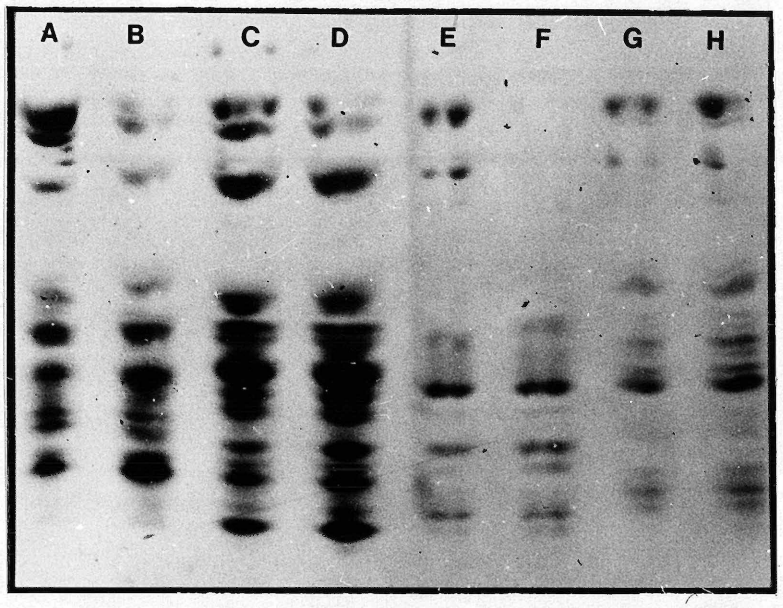

Figure 5

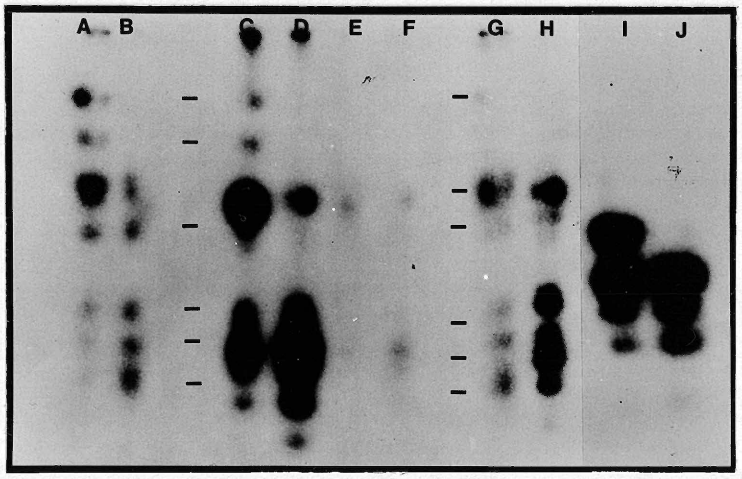

Figure 6

Chapter 3

The synthesis and distribution of desmin and vimentin during myogenesis in vitro

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Summary

Electrophoretic and autoradiographic analysis of the incorporation of ^{35}S-methionine into newly synthesized proteins during myogenesis reveals that presumptive chicken myoblasts synthesize primarily one intermediate filament protein: vimentin. Upon the onset of fusion, desmin synthesis is initiated. Synthesis rates of both filament subunits increase during the first three days in culture, relative to the total protein synthesis rate. The observed increase in the rate of desmin synthesis (at least 10 fold) is significantly greater than that observed for vimentin, and is responsible for a net increase in the cellular desmin content relative to vimentin. Both filament subunits continue to be synthesized through at least 20 days in culture. Immunofluorescent staining using desmin and vimentin specific antisera supports the conclusion that desmin is synthesized only in fusing or multinucleate cells. These results indicate that the synthesis of the two filament subunits is not coordinately regulated during myogenesis.

The distributions of desmin and vimentin in multinucleate chicken myotubes are indistinguishable, as determined by double immunofluorescence techniques. In early myotubes, both proteins are found in an intricate network of free cytoplasmic filaments. Later in myogenesis, several days after the appearance of α-actinin containing Z line striations, both filament proteins become associated with the Z lines of newly assembled myofibrils, with a corresponding decrease in the number of cytoplasmic filaments. This transition corresponds to the time when the α-actinin containing Z lines become aligned laterally. These data suggest that the two intermediate filament systems, desmin and vimentin, play an important role in the lateral organization and registration of myofibrils and that the synthesis of desmin and assembly of desmin-containing intermediate filaments during myogenesis is directly related to these functions. These results also indicate that the Z disc is assembled in at least two distinct steps during myogenesis.

Introduction

Intermediate filaments have been observed in many cell types of diverse origins (Brecher, 1975; Brown, Levinson and Spudich, 1976; Blose and Chacko, 1976; Liem et al., 1978; Schachner et al., 1977; Sun and Green, 1978; Franke et al., 1978; Hynes and Destree, 1978a), including all three vertebrate muscle types (Cooke, 1976; Uehara, Campbell and Burnstock, 1971; Campbell et al., 1971; Ishikawa, Bischoff and Holtzer, 1968; Kelly, 1969; Viragh and Challice, 1969; Ferrans and Roberts, 1973; Rash, Biesele and Gey, 1970; Rash, Shay and Biesele, 1970; Ericksson and Thornell, 1979). Biochemical and immunological evidence suggests that this morphologically-defined filament class may actually represent as many as five filament classes composed of distinct subunit proteins: neurofilaments, glial filaments, keratin filaments, fibroblastic intermediate filaments and muscle intermediate filaments (for a review see Lazarides, 1979). Desmin, the 50,000 m.w. subunit of avian smooth muscle intermediate filaments (Lazarides and Hubbard, 1976; Small and Sobieszek, 1977; Hubbard and Lazarides, 1979), has also been identified in both cardiac and skeletal myofibrils, and in skeletal myotubes grown in vitro (Lazarides and Hubbard, 1976; Izant and Lazarides, 1977; Granger and Lazarides, 1978; Gard, Bell and Lazarides, 1979). Immunological localization of desmin in embryonic cardiac cells and skeletal myotubes in vitro indicates that desmin forms an intricate array of filaments in both of these cell types (Lazarides, 1978; Campbell et al., 1979; Bennett, Fellini, and Holtzer, 1978), corresponding, at least in part, to the intermediate filaments observed in ultrastructural studies of these cells. In mature skeletal and cardiac muscle; however, desmin is found in the Z discs of isolated myofibrils (Lazarides and Hubbard, 1976; Lazarides, 1978). When viewed in cross section, desmin is observed at the periphery of the Z discs, where it forms a transverse network encircling and interlinking adjacent Z discs (Granger and Lazarides, 1978).

These observations indicate that there must be a redistribution of desmin, from free cytoplasmic filaments to the myofibril Z disc during the differentiation of both skeletal and cardiac muscle cells.

Recently, it has become apparent that many cell types possess more than one identifiable class of intermediate filament (Franke et al., 1978; Franke et al., 1979; Gard et al., 1979). We have reported that myotubes differentiating in vitro possess, in addition to desmin, a 52,000 m.w. protein identified as the subunit of intermediate filaments found in many cells of mesenchymal origin (Brown et al., 1976; Starger et al., 1978) and which has been termed vimentin (Franke et al., 1978). This protein is distinguishable from desmin by molecular weight, isoelectric point, and antigenicity (Gard et al., 1979; Granger and Lazarides, submitted for publication). Biochemical and immunological evidence demonstrate that vimentin is also a component of isolated adult myofibrils, where its distribution coincides with that of desmin at the periphery of the Z discs (Granger and Lazarides, submitted for publication). These results raise many questions regarding the biochemical composition of intermediate filaments in differentiating muscle, and the distributions, associations, and functional specificities of the two intermediate filament subunits.

In this paper we present our investigations on the synthesis and distribution of desmin and vimentin during the process of myogenesis in vitro. We find that the synthesis of the two filament subunits is not coordinately regulated; however, their cytoplasmic distribution coincides throughout myogenesis. Using double immunofluorescence, and α-actinin as a marker for newly assembling myofibrils, we show that both intermediate filaments do indeed change their cytoplasmic distribution and become associated with Z discs at some point subsequent to the assembly of α-actinin into these structures. These results shed new light on the process of Z disc assembly and the function of intermediate filaments during myogenesis,

and provide an in vitro system for elucidating the molecular events that may bring about the lateral registration of myofibrils.

Results

Synthesis of Desmin and Vimentin during Myogenesis

Primary chicken myoblasts labeled for short periods of time with ^{35}S-methionine immediately after dissociation from 10-day-old embryonic thigh (see Figure 1A) synthesize several major proteins which can be detected on two-dimensional gels, corresponding to the tubulins (α, β), nonmuscle actins (β, γ), and a 52,000 m.w. protein which we have identified as vimentin (Gard et al., 1979). Of particular interest is the lack of synthesis of desmin (m.w. 50,000). In some instances, desmin synthesis can be faintly detected in overexposed autoradiograms. In these cases, however, the presence of this protein is accompanied by the presence of detectable synthesis of α- and β-tropomyosin and α-actin, suggesting a slight contamination by post-fusion myotubes. Within 6 hr of plating (Figure 1B) the synthesis of both the α and β desmin variants can be detected, though they are only faintly visible in the stained gel (not shown). Synthesis of α-actin is also apparent by 6 hr indicating that fusion of myoblasts and synthesis of muscle-specific proteins have commenced. These data suggest that desmin synthesis occurs primarily post-fusion, a conclusion further supported by immunofluorescence data (see below). The increase in the rates of desmin synthesis and the accumulation of desmin continue through the first three days in culture, by which time the two isoelectric variants of this molecule represent a significant fraction of the protein being synthesized by differentiating myotubes (Figure 1D). During this time, vimentin, as well as tubulin, appear to be synthesized continuously. Quantitation of the radioactivity incorporated into individual proteins (see Materials and Methods) indicate that the synthesis of vimentin represents approximately

0.8% of the total protein synthesis in myoblasts, and 1.7% of the total protein being synthesized in secondary myotubes 3 days after subculturing. During this same time period, the synthesis of desmin (relative to total protein synthesis) increases more than 10-fold, until it comprises 0.5% of the total synthetic activity of 3-day-old myotubes. Total actin (α, β and γ variants) and tropomyosin (α and β) synthesis also increase dramatically, as reported in a number of previous studies (Whalen et al., 1976; Devlin and Emerson, 1978) while tubulin and several other proteins show only minor fluctuations in their synthetic rates. Synthesis of both desmin and vimentin is detectable in 20-day-old cultures of fibroblast-free myotubes (not shown), indicating that both of these proteins continue to be made late in the myogenic process. Stained gels of 1 hr and 7-day myogenic cultures (shown in Figure 2) reveal a significant increase in the desmin content during early myogenesis; by 7 days desmin represents one of the major proteins observed.

We have recently reported the presence of a third, more acidic variant of desmin in cultured myotubes (O'Connor, Balzer and Lazarides, 1979; Gard et al., 1979). This variant is apparent in the stained gels showing the protein components in the later stages of myogenesis (Figure 2b). No label is incorporated into this desmin species during 1 or 2 hr pulses with ^{35}S-methionine; however, significant labeling of this species is detectable after a chase period of 24 hr (Figure 3). The increase in the ratio of ^{35}S-methionine in the acidic variant to ^{35}S in the other desmin species during long (1-4 day) chase periods suggests that this acidic species is derived from a precursor molecule, presumably α- or β-desmin (data not shown). This result is consistent with our earlier observation that α-desmin and the acidic variant are phosphorylated both in vivo and in vitro (O'Connor et al., 1979), suggesting that the acidic variant represents a phosphorylated form of desmin, distinct from α-desmin.

Immunofluorescent Localization of Desmin during Myogenesis in vitro

The distribution of desmin during myogenesis in vitro, determined by indirect immunofluorescence microscopy using antisera against chicken gizzard desmin, is shown in Figure 4 (the corresponding distributions of vimentin are shown for later reference). Primary cultures of chicken embryonic thigh consist of two predominant cell types: fibroblasts, and characteristically bipolar presumptive myoblasts. At the earliest times observed (18 hr primary cultures), neither mononucleate cell type exhibits significant desmin-specific fluorescence; desmin fluorescence is observed only in multinucleate myotubes, and in aggregates of fusing cells at this time, indicating that desmin synthesis occurs primarily post-fusion. Within 18 hr of subculturing, a large proportion of myoblasts have either fused, or are aligning laterally or end-to-end in preparation for cell fusion (see Table 1, Materials and Methods section). An example of an aggregate of fusing myoblasts is shown in Figure 4A. Such cell aggregates often contain cells which stain with anti-desmin, and cells which do not, supporting the conclusion that desmin synthesis commences at the onset of fusion. Within 48 hr after subculturing, the majority of the cells have fused into multinucleate myotubes, which display a fine network of desmin-containing filaments, as seen in Figure 4B. In older myotubes (3-4 days, Figure 4C), desmin filaments are often packed at such a high density that their filamentous nature is obscured, resulting in a highly-fluorescent solid staining of the sarcoplasm. In such cells, the filament density often diminishes at the myotube ends, allowing the visualization of the intricate network of desmin filaments (see Figure 5). Examination of 3-day myotubes with phase microscopy indicates that they contain many longitudinally-oriented phase-dense filament bundles (see Figures 4C), which have been previously shown to contain actin (Lazarides and Weber, 1976; Moss, Asch and Schwartz, 1979). Often there is no discernible association between the desmin filament network

and these presumed myofibril precursors. However in some cells, an increase in desmin filament density is observed to coincide with these actin filament bundles (Figure 4C, and also Figure 7). These concentrations of desmin-containing filaments appear as brightly fluorescent streaks superimposed upon the dense cytoplasmic staining.

Between the fifth and seventh days in culture, a striking redistribution of desmin takes place within the myotubes. During this time many cells begin to exhibit a transversely striated pattern of desmin-specific fluorescence (see Figure 4E). Correlation of these striations with phase microscopy, and the distribution of α-actinin (see below) indicates that the desmin-containing striations correspond to the myofibril Z lines.

Double Immunofluorescent Localization of Desmin and α-Actinin

Direct comparison of the distribution of desmin and assembling myofibrils in the same cell is afforded by the use of double immunofluorescent staining for desmin, and the Z line protein α-actinin. Immunofluorescence using directly conjugated antisera to skeletal muscle α-actinin reveals that α-actinin is first distributed in focal deposits along the non-striated phase-dense actin filament bundles previously described (see Figure 6A-B). The first recognizeable α-actinin-containing Z lines, visible as early as three days after culturing, appear to evolve through an aggregation and redistribution of α-actinin along these presumptive myofibrils. Desmin in such cells is still distributed as a fine filamentous network filling the sarcoplasm (Figure 6C). By five days, many myotubes exhibit numerous individual myofibrils, visible by both phase microscopy (not shown), and α-actinin Z line fluorescence (for example, see Figure 7). Anti-desmin staining reveals that the majority of desmin in these cells comprises an intricate filamentous network, as described above. However, concentrations of desmin filaments are often observed to correspond to the assembling myofibrils,

or to organizing myofibril bundles. Figure 7 shows a five-day myotube exhibiting such a concentration of desmin filaments, with faintly visible striations. In seven-day myotubes, phase and α-actinin fluorescence microscopy reveal that the majority of myofibrils have been organized into bundles, as evidenced by the alignment of sarcomeres (Z lines) in adjacent myofibrils. Many of these cells exhibit a striated pattern of desmin fluorescence, as seen in Figure 8, which corresponds to the phase-dense, α-actinin-containing Z lines. By 15 days in culture, the striated pattern of desmin-specific fluorescence in many cells is nearly indistinguishable from that obtained with the α-actinin antibody (Figure 9). Little evidence remains of the cytoplasmic network of desmin filaments which predominate early in myogenesis.

Double Immunofluorescent Localization of Desmin and Vimentin

The results presented above indicate that desmin synthesis begins at the time of myoblast fusion, while vimentin is synthesized in both mononucleate myoblasts and multinucleate differentiating myotubes. We have used double immunofluorescence to directly correlate the distribution of vimentin with that of desmin during the various stages of myogenesis. In contrast to their lack of desmin, mononucleate myoblasts contain a typical pattern of intermediate filaments which stain with vimentin antibody (Figure 4). This filament network often appears to radiate from a perinuclear center in both myoblasts and fibroblasts (not shown). The pattern of filaments in fibroblastic cells appears more complex than that in myoblasts, probably due to the larger and more complex shape of the former cell type. As myoblasts fuse, desmin-specific fluorescence first becomes apparent (see above). Comparison of the images of desmin fluorescence and vimentin-specific fluorescence using double-labeling reveals that the distribution of newly synthesized desmin

coincides with that of vimentin (Figure 4A). Examination of older myotubes at higher magnification reveals no discernible differences in the localization of vimentin and desmin (Figure 5), within the resolution limits of light microscopy. Figure 4E indicates that vimentin-specific Z line striations appear in conjunction with the appearance of desmin at the Z line.

Desmin in Fibroblastic Cells

We have previously reported that α-desmin is present in cultured fibroblasts free of contaminating myogenic cells (Gard et al., 1979). Under the conditions used in this study, desmin-specific fluorescence is not detectable in normal fibroblasts, but can be faintly detected in the colcemid-induced filament aggregates of these cells, confirming our earlier results (such a cell is seen in Figure 10).

Effects of Colcemid on Desmin and Vimentin Distribution

To further characterize the relative distribution of desmin and vimentin, we subjected 5-day myotubes to 5 μM colcemid for an incubation period of 18 hr, resulting in the formation of large aggregates of sarcoplasmic intermediate filaments (Croop and Holtzer, 1975; Holtzer et al., 1975). To visualize the filament aggregates, or "caps" more clearly, we extracted the myotubes with 1% Triton X-100 and 0.6 M KCl, which solubilizes the majority of the sarcoplasmic protein. Double immunofluorescence staining reveals that the resulting filament aggregates consist of both desmin and vimentin, with no detectable differences in their distributions (Figure 10A-B). In more mature (7-day-old) myotubes, shown in Figure 10C-D, treatment with colcemid has no effect on the association of desmin and vimentin with the myofibril Z lines while free cytoplasmic filaments form characteristic aggregates.

Discussion

Synthesis of Desmin and Vimentin during Myogenesis

Changes in the patterns and rates of protein synthesis in myogenic cells have been well documented, with particular emphasis placed upon the regulation of synthesis of proteins involved in contraction (Devlin and Emerson, 1978; Zani et al., 1978; Patterson and Strohman, 1972; Rubinstein et al., 1976; Whalen et al., 1976; Emerson, 1977; Emerson and Beckner, 1975). Several studies have indicated that the synthesis of four major myofibril components—actin, myosin, tropomyosin, and troponin—may be coordinately controlled (Rubinstein et al., 1976; Devlin and Emerson, 1978). In this investigation we have focused our attention on the synthesis and distribution of desmin and vimentin during myogenesis. We have recently reported that skeletal muscle myotubes contain both desmin and vimentin (Gard et al., 1979), and that these two proteins are minor constituents of skeletal muscle myofibrils (Lazarides and Hubbard, 1976; Izant and Lazarides, 1977; Granger and Lazarides, 1978; Granger and Lazarides, submitted for publication). Our results indicate that vimentin is one of the major protein species synthesized in mononucleate myoblasts derived from embryonic thigh, while desmin synthesis is undetectable. That vimentin is the predominant intermediate filament subunit detected in this cell population is consistent with the undifferentiated fibroblastic state of these cells. Upon initiation of cell fusion, which occurs as early as 6 hr after plating, synthesis of desmin becomes detectable. During the first three days in culture, when myoblast proliferation and fusion are both occurring, there is at least a 10-fold increase in the rate of desmin synthesis (relative to total protein synthesis), with a corresponding 2-3-fold increase in the relative rate of vimentin synthesis. The net result of this difference is an increase in the ratio of desmin to vimentin during differentiation. These findings suggest that the synthesis of

desmin and vimentin is not coordinately regulated, contrary to previously published conclusions (Devlin and Emerson, 1978; vimentin is identified as protein number 9 by these authors). Synthesis of both intermediate filament proteins continues throughout myogenesis in vitro, and is detectable in differentiated myotubes for as long as 20 days in culture. This result, and the presence of desmin and vimentin in adult myofibrils (Granger and Lazarides, submitted for publication), suggests that the intermediate filament subunits play important and continuing roles during myogenesis.

Distribution of Desmin and Vimentin

Immunofluorescent localization of desmin and vimentin during myogenesis supports the conclusions derived above. In the early stages of myogenesis, primary myoblasts stain only with antisera directed against vimentin. As cells begin to aggregate, desmin-specific fluorescence is first observed in fused, or fusing cells, suggesting that synthesis of desmin and desmin deposition is initiated with cell fusion.

In multinucleate myotubes, both anti-vimentin and anti-desmin are observed to stain intricate networks of cytoplasmic filaments. This result is significant in several regards. First, it confirms our previous report that desmin and vimentin coexist in myotubes in vitro (Gard et al., 1979; in this paper vimentin is referred to as F-IFP). Second, close examination reveals that the distributions of desmin and vimentin in double immunofluorescence staining are indistinguishable. This coincidental localization of the two proteins observed in all stages of myogenesis after fusion, and their coaggregation in colcemid-treated cells, suggests that the two filament subunits are intimately associated. One possibility suggested by these results is that desmin and vimentin copolymerize into the same filaments. Since intermediate filaments composed of vimentin are present in pre-fusion myoblasts, fusing myoblasts may assemble newly synthesized desmin subunits into a preexisting network of vimentin filaments. Incorporation of desmin into these filaments might

provide a mechanism by which the cell alters its filament network to accommodate the specific functions needed for myogenesis.

Desmin and Vimentin Associate with the Z Lines of Myofibrils

During the maturation of myotubes in vitro, there is a significant redistribution of both desmin and vimentin. This is first observed as an increase in the staining density of antisera against these proteins around assembling (or assembled) myofibrils. Later, the cytoplasmic filament network is replaced by a transversely striated pattern, corresponding to the myofibril Z lines. Double immunofluorescence using anti-desmin and anti-α-actinin antibodies indicates that the appearance of desmin (and vimentin) at the Z line occurs several (2-3) days after the assembly of phase-dense, α-actinin-containing Z lines. It is at this time that individual myofibrils become aligned and organized into bundles with the sarcomeres of adjacent fibrils in register. We have previously reported that desmin and vimentin are present at the Z lines of adult myofibrils, in the form of a transverse network of collar-like rings which encircle and interlink each Z disc in a myofibril bundle (Lazarides and Hubbard, 1976; Granger and Lazarides, 1978; Granger and Lazarides, submitted for publication). It seems likely, then, that intermediate filaments composed of desmin and vimentin may be directly involved in this alignment process.

The molecular basis for the redistribution of desmin and vimentin during myogenesis remains undetermined. However, some sort of molecular change must take place that allows desmin and vimentin to associate with the Z disc. Biochemically, there are no detectable changes in either the molecular weights or isoelectric points of either protein, suggesting a controlling factor more subtle than direct modification of existing proteins. Both desmin and vimentin exist as phosphorylated and non-phosphorylated variants; so far no direct correlation between phosphorylation and

state of assembly has been detected (O'Connor et al., 1979). Whether the association of desmin and vimentin with the Z lines involves newly synthesized protein, or a redeployment and condensation of existing filaments at the Z line also remains unresolved. In fact, the ultrastructural organization of these two proteins remains unclear. Very little evidence for an association between intermediate filaments (as opposed to filament subunits) and skeletal muscle myofibrils has been reported. However, in cardiac muscle where a similar transition from cytoplasmic filaments to Z line desmin occurs (Lazarides, 1978; Lazarides, unpublished observations), intermediate filaments are often seen encircling and interconnecting adjacent Z discs (Viragh and Challice, 1969; Ferrans and Roberts, 1973; Behrendt, 1977; Eriksson and Thornell, 1979). We infer from these studies that desmin and vimentin associated with skeletal muscle Z lines are also in the form of intermediate filaments, although such filaments have been observed only rarely (Page, 1969).

During the preparation of this manuscript, a report was published describing the distributions of desmin and vimentin during myogenesis (Bennett et al., 1979). These authors found that vimentin, though present in myoblasts and early myotubes, disappears within 10-14 days of culturing. This result, based solely upon immunofluorescence, is in conflict with our findings that vimentin synthesis continues through 20 days in culture and that vimentin is found in a distribution identical to desmin throughout myogenesis in vitro including its association with the myofibril Z lines. It is unclear why this discrepancy exists, but we believe that the data presented above, and the identification of vimentin in adult skeletal myofibrils (Granger and Lazarides, submitted for publication) clearly demonstrate the presence of vimentin throughout myogenesis.

Conclusion

Our results demonstrate that two distinct intermediate filament subunits, desmin and vimentin, coexist throughout myogenesis. However, their synthesis does not appear to be coordinately controlled and one of them, desmin, is specifically synthesized after the onset of fusion. The immunofluorescence results indicate that during myogenesis the Z disc is assembled in at least two distinct steps, as evidenced by the presence of α-actinin in association with these structures and the absence of desmin or vimentin early in myogenesis and the presence of all three proteins later in myogenesis. This transition of desmin and vimentin to the Z discs coincides with the lateral registration of myofibrils; taking into consideration our previous results on the localization of desmin and vimentin at the periphery of the Z disc in adult myofibrils, these results imply that desmin and vimentin containing intermediate filaments may be responsible for the lateral alignment and organization of myofibrils and may provide the molecular basis for the cross-striated appearance of skeletal (and cardiac) muscle.

Materials and Methods

Preparation of Myogenic Cultures

Primary cultures of embryonic chicken thigh muscle were prepared according to the method of Konigsberg et al. (1978) from 10-day-old embryos. A modification of their procedure was used in the preparation of secondary cultures. Primary cells (18 hr, at 10^7 cells/100 mm plate) were washed twice with Earle's balanced salt solution (EBSS), and trypsinized with 5 ml trypsin in EBSS (0.025 mg/ml) at 37°C for 3-5 min. Trypsin digestion was terminated by the addition of 5 ml growth medium, consisting of Eagles minimal essential medium (MEM) supplemented with 15% horse serum, non-essential amino acids, 5% chick embryo extract, and antibiotics (100 µg of streptomycin and 100 U of penicillin per ml). The cells were then gently pipetted from the plates, pelleted at 500 x g, and washed once with growth medium. These cells were then preplated twice according to the procedures of O'Neill and Stockdale (1972). For immunofluorescence, cells were plated at a density of 2.5-4 x 10^5 cells/60 mm petri plate, with 3-4 collagen-coated coverslips per plate. For ^{35}S-methionine labeling, cells were plated at a density of 1-4 x 10^6 cells/100 mm collagen-coated plate, as indicated. These procedures yielded cultures that were 90-95% free of contaminating fibroblasts. To prevent overgrowth of fibroblasts during long-term cultures, cells were fed on day 3 with complete medium, Eagles MEM containing 10% horse serum and 2% embryo extract, containing 10 µM cytosine arabinofuranoside (ARA-C). Cultures were then fed at three- or four-day intervals with complete medium without ARA-C. The percentages of mononucleate, fusing, and multinucleate cells at various times during myogenesis are shown in Table 1.

^{35}S-Methionine Labeling of Newly Synthesized Proteins

Primary myoblasts were labeled immediately after dissociation from embryonic

muscle. 5 x 10^6 cells were washed twice in methionine-free minimum essential medium (MEM-M), and labeled in suspension for 1 hr in MEM-M containing 50 µCi ^{35}S-methionine (1300 Ci/mMole, Amersham Searle). At the end of the labeling period, cells were pelleted, and washed twice with phosphate-buffered saline (PBS: 0.17 M NaCl, 2 mM KCl, 10 mM Na^+-K^+ phosphate, pH 7.4). The cell pellet was then solubilized in 200 µl IEF sample buffer containing 8 M urea (ultra-pure, Schwartz-Mann), 1% NP-40, 0.5% 2-mercaptoethanol, 5 mM EGTA, and 0.1 mM O-penanthroline. Solubilized samples were then frozen in a dry ice-ethanol bath, and stored at -25°C until use. Cultures grown in petri plates were labeled for 1 hr with 3 ml MEM-M, and scraped from the dish with a rubber policeman. These cells were then pelleted, solubilized, and frozen as above. A cell plating density of 4 x 10^6/100 mm plate was used for time points between 0 and 2 days, and 1 x 10^6/plate for labeling in 3 day and older cultures. For pulse-chase experiments, 6-day myotubes were labeled for 2 hr as above, washed twice with complete medium, and incubated for the indicated chase period in complete medium.

Two-Dimensional Isoelectric Focusing-SDS Gel Electrophoresis

Two-dimensional IEF/SDS-PAGE was performed according to the method of O'Farrell (1975), as modified and described by Hubbard and Lazarides (1979). Sample loads were adjusted to contain 1-2 x 10^6 trichloroacetic acid precipitable counts per minute of ^{35}S-methionine. Gels were stained overnight with Coomassie brilliant blue, destained, and photographed as previously described (Hubbard and Lazarides, 1979). Dried gels were autoradiographed for the times indicated (see figure legends) with Kodak XR-5 X-ray film. For quantitation of ^{35}S-methionine incorporation by liquid scintillation counting, protein spots were cut from duplicate gels and solubilized overnight in a toluene-based scintillation cocktail containing 5% NCS and 0.5%

distilled water. Samples were counted for 5 min each in a Beckman LS-200. Results are reported as the percentage of total (TCA precipitable) protein synthesis.

Preparation and Characterization of Antibodies

The preparation and characterization of antisera to α-actinin, desmin and vimentin have been described elsewhere (Lazarides and Burridge, 1975; Lazarides, 1976, 1978; Lazarides and Hubbard, 1976; Granger and Lazarides, submitted for publication). Immunoautoradiography of IEF/SDS-PAGE gels indicate that there is no detectable cross-reaction between anti-desmin or anti-vimentin, and the non-homologous antigen (Granger and Lazarides, submitted for publication).

Preparation of Rhodamine B Conjugated IgG

Rhodamine B (Sigma) conjugated antibodies to α-actinin and vimentin were prepared essentially according to the method of Cebra and Goldstein (1965). The IgG fraction was prepared from immune sera by ammonium sulfate precipitation (to 37% saturation at 4°C), and DEAE cellulose chromatography in 10 mM phosphate buffer, pH 7.5. The resulting IgG (flowthrough) was concentrated by ultrafiltration and reacted with rhodamine isothiocyanate (25-50 μg/mg protein) in a buffer containing 0.15 M NaCl and 0.1 M sodium carbonate, pH 9.5, for 1 hr. At this time, an additional 1/5 volume of 0.5 M sodium carbonate, pH 9.5, was added, and the reaction continued overnight at 4°C. Unconjugated rhodamine B was removed by chromatography on Sephadex G-25 in 10 mM phosphate, pH 7.5, followed by DEAE chromatography as described by Cebra and Goldstein (1965). 280/560 ratios of the conjugates used were 4.3 for RBITC anti-α-actinin and 1.3 for RBITC-vimentin.

Immunofluorescence

Cells for immunofluorescence were fixed in 3.7% formaldehyde (in PBS) at 37°C

and 95% ethanol as previously described (Lazarides, 1976). Double immunofluorescence was performed using a modification of the indirect:direct staining method described by Hynes and Destree (1978b). Coverslips containing fixed cells were first incubated at 37°C with anti-desmin (45% ammonium sulfate fraction diluted to 1 mg/ml) for 30 min, washed and incubated for 30 min in a 1:30 dilution of fluorescein-conjugated goat anti-rabbit IgG (Miles-Yeda). Unreacted anti-rabbit IgG was blocked for 3-5 hr with a 1:5 dilution of normal rabbit serum. This treatment was found sufficient to eliminate completely any binding of the directly labeled antibodies to the preexisting rabbit IgG-goat anti-rabbit IgG complex. Cells were then washed, and incubated for 30 min with RBITC-conjugated antisera (either anti-α-actinin or anti-vimentin). Washes between antibody incubations consisted of at least three changes of PBS, totalling 30-60 min. Coverslips were mounted in elvanol, and observed immediately with a Leitz phase/fluorescence microscope using a Zeiss 63X oil immersion lens and filter modules H for fluorescein and N2 for rhodamine. Fluorescence results were photographed on Kodak Tri-X panchromatic film at ASA 1600, and developed in Diafine, or on Kodak Ektachrome 200 color slide film. All double-immunofluorescence results were verified by single labeling techniques using either indirect (desmin) or direct (vimentin and α-actinin) fluorescence. Immunofluroescence of colcemid-induced filament caps was performed on Triton-KCl cytoskeletons as previously described (Gard et al., 1979).

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References

Behrendt, H. (1977). Effect of anabolic steroids on rat heart muscle cells. I. Intermediate filaments. Cell Tiss. Res. 180, 303-315.

Bennett, G. S., Fellini, S. A. and Holtzer, H. (1978). Immunofluorescent visualization of 100 Å filaments in different cultured chick embryo cell types. Differentiation 12, 71-82.

Bennett, G. S., Fellini, S. A., Toyama, Y. and Holtzer, H. (1979). Redistribution of intermediate filament subunits during skeletal myogenesis and maturation in vitro. J. Cell Biol. 82, 577-584.

Blose, S. H. and Chacko, S. (1976). Rings of intermediate (100 Å) filament bundles in the perinuclear region of vascular endothelial cells. J. Cell Biol. 70, 459-466.

Brecher, S. (1975). The occurrence and possible role of 80-100 Å filaments in Ptk_1 cells. Exp. Cell Res. 96, 303-310.

Brown, S., Levinson, W. and Spudich, J. A. (1976). Cytoskeletal elements of chick embryo fibroblasts revealed by detergent extraction. J. Supramol. Struct. 5, 119-130.

Campbell, G. R., Uehara, Y., Mark, G. and Burnstock, G. (1971). Fine structure of smooth muscle cells grown in tissue culture. J. Cell Biol. 49, 21-34.

Campbell, G. R., Chamley-Campbell, J., Gröschel-Stewart, U., Small, J. V. and Anderson, P. (1979). Antibody staining of 10-nm (100 Å) filaments in cultured smooth, cardiac, and skeletal muscle. J. Cell. Sci. 37, 303-322.

Cebra, J. J. and Goldstein, G. (1965). Chromatographic purification of tetramethylrhodamine-immune globulin conjugates and their use in the cellular localization of rabbit γ-globulin polypeptide chains. J. Immunol. 95, 230-245.

Cooke, P. (1976). A filamentous cytoskeleton in vertebrate smooth muscle fibers. J. Cell Biol. 68, 539-556.

Croop, J. and Holtzer, H. (1975). Response of myogenic and fibrogenic cells to cytochalasin B and to colcemid. I. Light microscopic observations. J. Cell Biol. 65, 271-285.

Devlin, R. B. and Emerson, C. P., Jr. (1978). Coordinate regulation of contractile protein synthesis during myoblast differentiation. Cell 13, 599-611.

Emerson, C. P., Jr. (1977). Control of myosin synthesis during myoblast differentation. In:Pathogenesis of Human Muscular Dystrophies, L. P. Rowland, ed. (Amsterdam: Excerpta Medica) pp. 799-811.

Emerson, C. P., Jr. and Beckner, S. K. (1975). Activation of myosin synthesis in fusing and mononucleate myoblasts. J. Mol. Biol. 93, 431-447.

Ericksson, A. and Thornell, L.-E. (1979). Intermediate filaments in heart Purkinje fibers. J. Cell Biol. 80, 231-247.

Ferrans, V. J. and Roberts, W. C. (1973). Intermyofibrillar and nuclear-myofibrillar connections in human and canine myocardium. An ultrastructural study. J. Mol. Cell. Cardiology 5, 247-257.

Franke, W. W., Schmid, E., Osborn, M. and Weber, K. (1978). Different intermediate-sized filaments distinguished by immunofluorescence microscopy. Proc. Nat. Acad. Sci. USA 75, 5034-5038.

Franke, W. W., Schmid, E., Breitkreutz, D., Lüder, M., Boukamp, P., Fusenig, N. E., Osborn, M. and Weber, K. (1979). Simultaneous expression of two different types of intermediate-sized filaments in mouse keratinocytes proliferating in vitro. Differentiation 14, 35-50.

Gard, D. L., Bell, P. B. and Lazarides, E. (1979). Coexistence of desmin and the fibroblastic intermediate filament subunit in muscle and non-muscle cells: Identification and comparative peptide analysis. Proc. Nat. Acad. Sci. USA 76, 3894-3898.

Granger, B. L. and Lazarides, E. (1978). The existence of an insoluble Z-disc scaffold in chicken skeletal muscle. Cell 15, 1253-1268.

Granger, B. L. and Lazarides, E. (1979). Desmin and vimentin coexist at the periphery of the myofibril Z-disc. Cell (submitted for publication).

Holtzer, H., Croop, J., Dienstman, S., Ishikawa, H. and Somlyo, A. P. (1975). Effects of cytochalasin B and colcemid on myogenic cultures. Proc. Nat. Acad. Sci. USA 72, 513-517.

Hubbard, B. D. and Lazarides, E. (1979). The co-purification of actin and desmin from chicken smooth muscle and their co-polymerization in vitro to intermediate filaments. J. Cell Biol. 80, 166-182.

Hynes, R. O. and Destree, A. T. (1978a). 10 nm filaments in normal and transformed cells. Cell 13, 151-163.

Hynes, R. O. and Destree, A. T. (1978b). Relationship between fibronectin (LETS protein) and actin. Cell 15, 875-886.

Ishikawa, H., Bischoff, R. and Holtzer, H. (1968). Mitosis and intermediate-sized filaments in developing skeletal muscle. J. Cell Biol. 38, 538-558.

Izant, J. G. and Lazarides, E. (1977). Invariance and heterogeneity in the major structural and regulatory proteins of chick muscle cells revealed by two-dimensional gel electrophoresis. Proc. Nat. Acad. Sci. USA 74, 1450-1454.

Kelly, D. E. (1969). Myofibrillogenesis and Z-band differentiation. Anat. Rec. 163, 403-426.

Konigsberg, I. R., Sollmann, P. A. and Mixter, L. O. (1978). The duration of the terminal G_1 of fusing myoblasts. Dev. Biol. 63, 11-26.

Lazarides, E. (1976). Actin, α-actinin, and tropomyosin interaction in the structural organization of actin filaments in nonmuscle cells. J. Cell Biol. 68, 202-219.

Lazarides, E. (1978). The distribution of desmin (100 Å) filaments in primary cultures of embryonic chick cardiac cells. Exp. Cell Res. 112, 265-273.

Lazarides, E. (1979). Intermediate filaments - mechanical integrators of cellular space. Nature (in press).

Lazarides, E. and Burridge, K. (1975). α-Actinin: Immunofluorescent localization of a muscle structural protein in nonmuscle cells. Cell 6, 289-298.

Lazarides, E. and Hubbard, B. D. (1976). Immunological characterization of the subunit of the 100 Å filaments from muscle cells. Proc. Nat. Acad. Sci. USA 73, 4344-4348.

Lazarides, E. and Weber, K. (1974). Actin antibody: The specific visualization of actin filaments in non-muscle cells. Proc. Nat. Acad. Sci. USA 71, 2268-2272.

Liem, R. K. H., Yen, S.-H., Salomon, G. D. and Shelanski, M. L. (1978). Intermediate filaments in nervous tissues. J. Cell Biol. 79, 637-646.

Moss, M., Asch, B. and Schwartz, R. (1979). Differentiation of actin-containing filaments during chick skeletal myogenesis. Exp. Cell Res. 121, 167-178.

O'Connor, C. M., Balzer, D. R. and Lazarides, E. (1979). Phosphorylation of the subunit proteins of intermediate filaments from chicken muscle and non-muscle cells. Proc. Nat. Acad. Sci. USA 76, 819-823.

O'Farrell, P. H. (1975). High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. 250, 4007-4021.

O'Neill, M. C. and Stockdale, F. E. (1972). A kinetic analysis of myogenesis in vitro. J. Cell Biol. 52, 52-65.

Page, S. G. (1969). Structure and some contractile properties of fast and slow muscles of the chicken. J. Physiol. 205, 131-145.

Patterson, B. and Strohman, R. C. (1972). Myosin synthesis in cultures of differentiating chicken embryo skeletal muscle. Dev. Biol. 29, 113-138.

Rash, J. E., Shay, J. W. and Biesele, J. J. (1970). Preliminary biochemical investigations of the intermediate filaments. J. Ultrastruct. Res. 33, 399-407.

Rash, J. E., Biesele, J. J. and Gey, G. O. (1970). Three classes of filaments in cardiac differentiation. J. Ultrastruct. Res. 33, 408-435.

Rubinstein, N., Chi, J. and Holtzer, H. (1976). Coordinated synthesis and degradation of actin and myosin in a variety of myogenic and non-myogenic cells. Exp. Cell Res. 97, 389-393.

Schachner, M., Hedley-Whyte, E. T., Hsu, D. W., Schoonmaker, G. and Bignami, A. (1977). Ultrastructural localization of glial fibrillary acidic protein in mouse cerebellum by immunoperoxidase labeling. J. Cell Biol. 75, 67-73.

Small, J. V. and Sobieszek, A. (1977). Studies on the function and composition of the 10 nm (100 Å) filaments of vertebrate smooth muscle. J. Cell. Sci. 23, 243-268.

Starger, J. M., Brown, W. E., Goldman, A. E. and Goldman, R. D. (1978). Biochemical and immunological analysis of rapidly purified 10 nm filaments from baby hamster kidney (BHK-21) cells. J. Cell Biol. 78, 93-109.

Sun, T.-T. and Green, H. (1978). Immunofluorescent staining of keratin fibers in cultured cells. Cell 14, 469-476.

Uehara, Y., Campbell, G. R. and Burnstock, G. (1971). Cytoplasmic filaments in developing and adult smooth muscle. J. Cell Biol. 50, 485-497.

Viragh, Sz. and Challice, C. E. (1969). Variations in filamentous and fibrillar organization, and associated sarcolemmal structures, in cells of the normal mammalian heart. J. Ultrastruct. Res. 28, 321-334.

Whalen, R. G., Butler-Browne, G. S. and Gros, F. (1976). Protein synthesis and actin heterogeneity in calf muscle cells in culture. Proc. Nat. Acad. Sci. USA 73, 2018-2022.

Zani, B., Cossu, G., Adamo, S. and Molinaro, M. (1978). Biosynthetic changes of myosin heavy subunit during myogenesis in culture. Differentiation 10, 95-100.

Table 1. Fusion Kinetics of Myoblasts

	24 hr primary (n = 360)	16 hr secondary (II°) (n = 217)	40 hr II° (n = 491)	4D II°[b] (n = 387)	8D II°[c] (n = 363)
Mononucleate	49%	35	15	7	6
Fusing[a]	41	28	7	1	0
Multinucleate	10	36	79	93	94

Nuclei were counted, and the percentage fusion was calculated in cultures of myogenic cells fixed for immunofluorescence at the indicated times.

[a]Aggregates of mononucleate cells which appear to be in the process of fusion.

[b]No ARA-C treatment.

[c]Cells were treated with 10 μM cytosine arabinofuranoside between days 3 and 7, to prevent overgrowth of mononucleate fibroblasts.

Figure Legends

Figure 1. Autoradiographs from Myogenic Cells Labeled at Different Times during Myogenesis

Approximately 1 x 10^6 TCA precipitable CPM of ^{35}S-methionine labeled protein samples were subjected to two-dimensional IEF/SDS-PAGE. Isoelectric focusing was from right (basic) to left (acid) in all gels presented and SDS-PAGE was from top to bottom. Autoradiographs were exposed for 18 hr. Labeling time was for 1 hr. (A) Cells were labeled immediately after dissociation from embryonic thigh. The major protein species synthesized at this time are the tubulins (Tb), β, γ actins (Ac), and vimentin (V). No desmin synthesis is detectable in this autoradiograph (approximate positions of α and β desmin marked by arrows), or in longer exposures of the same gel (not shown). α-Actin is not synthesized in significant amounts in these mononucleate cells. A protein species (star) migrating with the same molecular weight, but slightly more acidic, than α-actin has not been conclusively identified. (B-C) Primary myoblasts were labeled at 6 hr (B) or 18 hr (C) after plating. Synthesis of vimentin (V) continues, and desmin synthesis (arrows) is detectable by 6 hr, and increases significantly between 6 hr and 18 hr. Incorporation of ^{35}S-methionine into α-actin (α) is apparent by 6 hr, and into tropomyosins (Tm) by 18 hr after plating. (D-F) Secondary cultures were labeled at 3 days (D), 7 days (E), or 14 days (F) after subculturing. Desmin synthesis (arrows) has increased between 18 hr and 3 days (compare autoradiographs C and D), and continues through 14 days in culture (F). Vimentin synthesis also continues through 14 days (F); similar results indicate that both desmin and vimentin continue to be synthesized through at least 20 days in culture (not shown).

Figure 2. Two-Dimensional Electrophoresis of Myogenic Cell Proteins

2A shows the stained gel of primary myoblasts 1 hr after dissociation from embryonic thigh (corresponding to the autoradiograph shown in Figure 1A). Tubulin (Tb), vimentin

Figure 2 (continued)

(V), and β,γ actin (Ac) are the major identifiable protein species observed. 2B shows the proteins of 7-day secondary myotubes (corresponding to the autoradiograph in Figure 1E). α and β desmins (α, β) and a third desmin variant (arrow, see text) are apparent. All three actin species are resolved (Ac), as well as α and β tropomyosins (Tm). The series of proteins more acidic (to the left) and with lower molecular weights than vimentin (stars) have been identified as proteolytic fragments of vimentin (see Gard et al., 1979).

Figure 3. Autoradiographs of 6-day Myotubes Pulsed 2 hr with ^{35}S-Methionine Followed by a 24 hr Chase with Unlabeled Methionine

(A) Radioactivity is incorporated only into α and β desmin during the 2 hr labeling period. No label is detectable in the third, acidic desmin variant (arrow). After a 24 hr chase with unlabeled methionine (B), a significant amount of radioactivity is detectable in the acidic variant (arrow). Autoradiographs were exposed for 5 days.

Figure 4. Double Immunofluorescent Localization of Desmin and Vimentin during Myogenesis

Phase contrast, anti-desmin fluorescent, and anti-vimentin fluorescent images of each field are shown. Bar represents 20 μm.

(A) An aggregate of 18 hr secondary myoblasts is shown in the process of fusion. Desmin-specific fluorescence indicates that several of the fusing cells contain desmin, while several bipolar myoblasts (M) and fibroblasts (F) do not stain with anti-desmin. All cells, including myoblasts and fibroblasts, possess filament networks containing vimentin.

(B) A 48 hr secondary myotube stains with both anti-desmin and anti-vimentin. Several nuclei are visible in this cell (N). A mononucleate myoblast (M) is also shown which does not stain with anti-desmin.

Figure 4 (continued)

(C) A 3-day secondary myotube is shown. Phase microscopy reveals phase-dense bundles of filaments (arrows), presumably assembling myofibrils. Fluorescence microscopy reveals a concentration of both desmin and vimentin associated with these bundles (see text).

(D) Striated myofibrils (arrows) are apparent in the phase image of this 5-day secondary myotube. Desmin and vimentin are distributed in a dense network of filaments, lending a solid appearance to the sarcoplasmic staining. No association between the intermediate (desmin and vimentin) filaments and the striated myofibrils is apparent in this cell.

(E) A 7-day myotube contains many well-developed striated myofibrils, with phase-dense Z lines (arrows). Both desmin and vimentin antisera display a transversely striated staining pattern, corresponding to the myofibril Z lines (see text, Figures 8 and 9).

Figure 5. Double Immunofluorescence of a 4-Day Myotube: Anti-Desmin and Anti-Vimentin

Comparison of the images obtained with anti-desmin (A) and anti-vimentin (B) reveals the correspondence in the distributions of these proteins. Arrows denote increases in fluorescent intensity observed in both desmin and vimentin images, corresponding to higher intermediate filament densities associated with actin-filament bundles (see text). Bar equals 10 μm.

Figure 6. Double Immunofluorescence Localization of Desmin and α-Actinin in 3-Day Myotubes

Phase microscopy (A) reveals many phase-dense filament bundles within the sarcoplasm

Figure 6 (continued)

(arrows); however, no cross striations are apparent. α-Actinin specific fluorescence (B) is distributed along these filament bundles, in a punctate pattern similar to that described for non-muscle stress fibers (Lazarides and Burridge, 1975). These α-actinin containing foci appear to coelesce into recognizable Z line structures (right arrowhead), as seen in the filament bundle between the two arrowheads. Desmin fluorescence (C) is distributed in a dense filament array, with little discernible association with the assembling myofibrils. Bar equals 20 μm.

Figure 7. Double Immunofluorescent Localization of Desmin and α-Actinin in 5-Day Myotubes

This 5-day secondary myotube contains many striated myofibrils visible in both phase microscopy (not shown) and with anti-α-actinin immunofluorescence (A). Desmin specific fluorescence (B) is still distributed as a cytoplasmic filament network. Arrowheads denote a concentration of desmin filaments that appear to correspond to a loosely organized myofibril bundle. Desmin-containing striations can faintly be detected amongst this concentration of desmin filaments (small arrows). Bar equals 20 μm.

Figure 8. Localization of Desmin and α-Actinin in 7-Day Myotubes

Immunofluorescence with α-actinin antisera (A) reveals that many myofibrils have been organized into bundles, with sarcomeres (Z lines) in register (arrows). Desmin antisera (B) reveal a transversely striated distribution of desmin in the majority of the cells, with cell processes still displaying dense networks of desmin filaments. Comparison of phase, α-actinin, and desmin images at higher magnification (C-D) reveals the correspondence between the myofibril Z lines and the transverse striations observed with α-actinin and desmin antisera. Bar equals 20 μm in (A) and (B), 10 μm in (C-E).

Figure 9. Localization of α-Actinin and Desmin in 15-Day Myotubes

Antiserum to α-actinin (A) reveals a well-developed system of striated myofibrils, and myofibril bundles. Desmin antiserum (B) displays a desmin distribution almost identical to α-actinin. Little remains of the cytoplasmic desmin filament network observed in younger cells. Bar equals 20 μm.

Figure 10. Immunofluorescence of Colcemid-induced Filament Aggregates in Myotube "Cytoskeletons"

(A-B) 5-day myotubes were incubated for 18 hr in 5 μM colcemid, and extracted with Triton X-100 and KCl (see Materials and Methods). Anti-desmin (A) and anti-vimentin (B) reveal that the distributions of the two intermediate filament proteins in the colcemid-treated cells are indistinguishable.

(C-D) In seven-day myotubes, treated with colcemid, both desmin (C) and vimentin (D) remain associated with the myofibril Z lines. A colcemid-induced filament "cap" in a fibroblastic cell (F) stains strongly with anti-vimentin, but only marginally with anti-desmin (see text). Bar equals 20 μm.

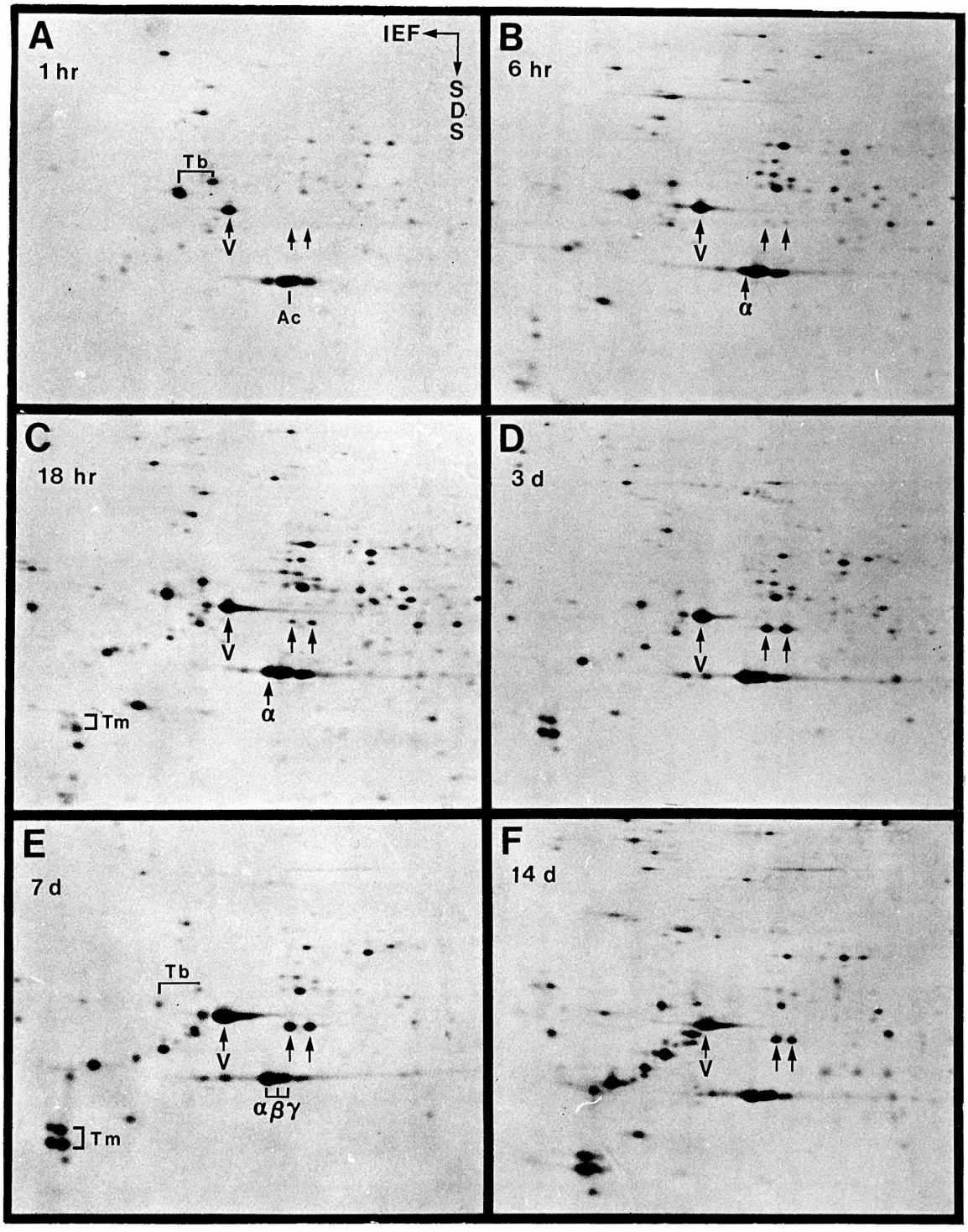
A
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Figure 1

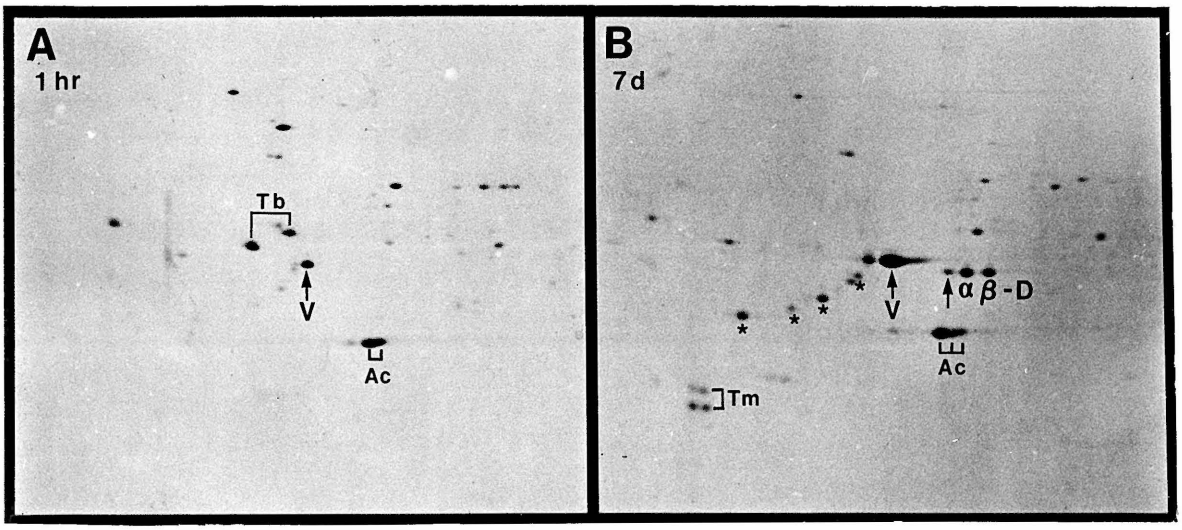


Figure 2

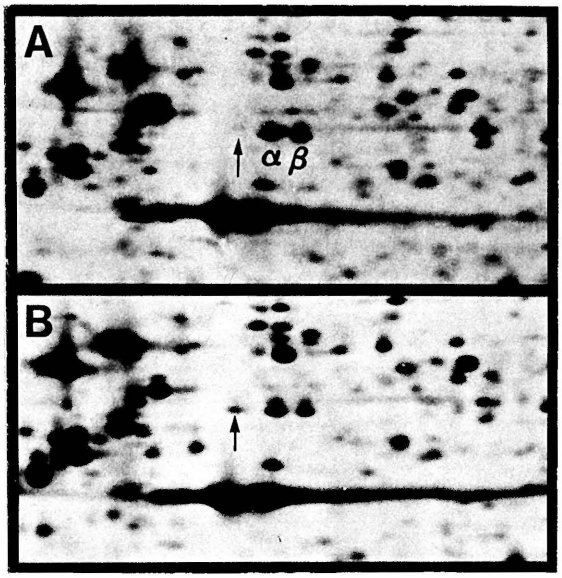


Figure 3

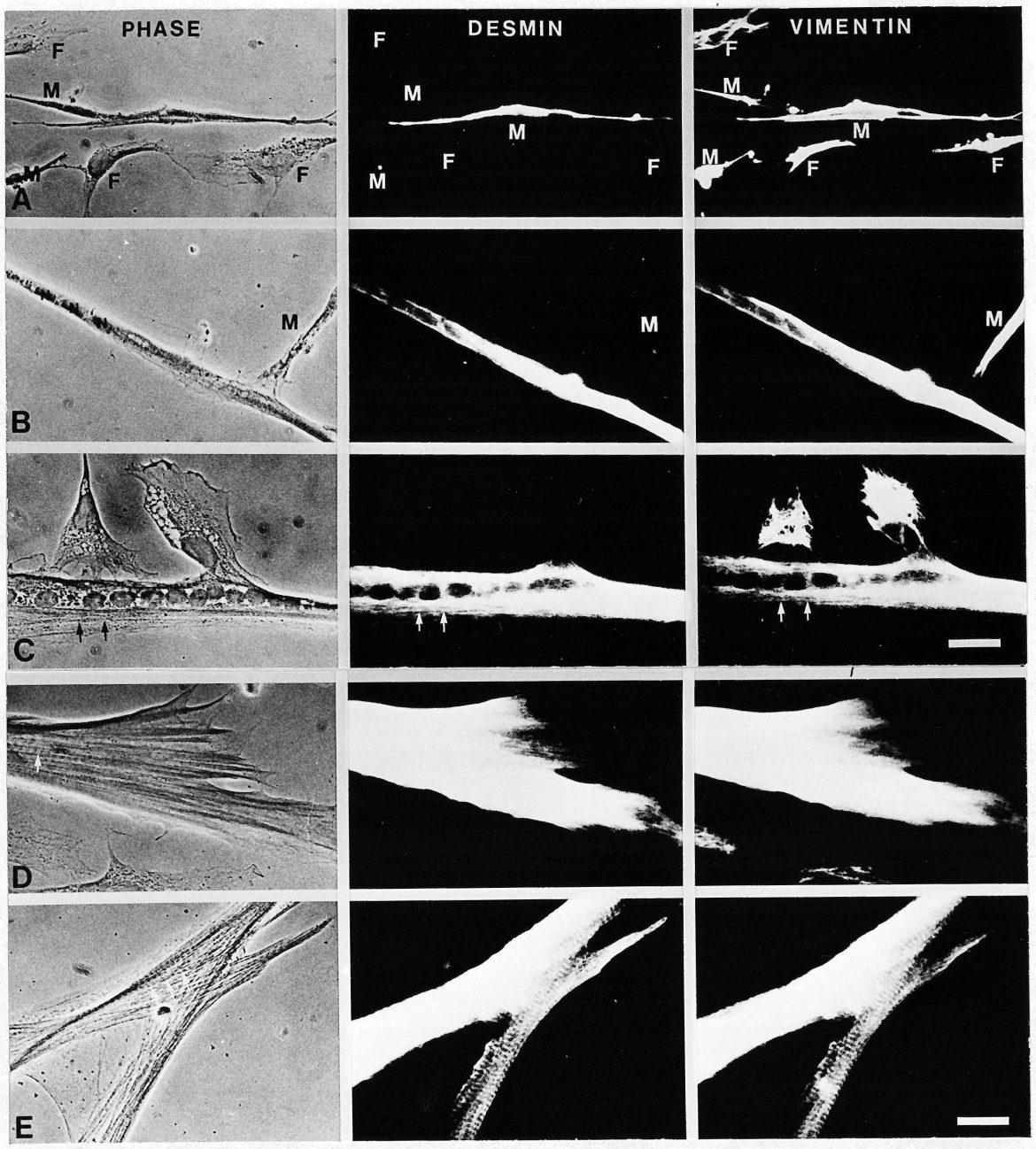


Figure 4

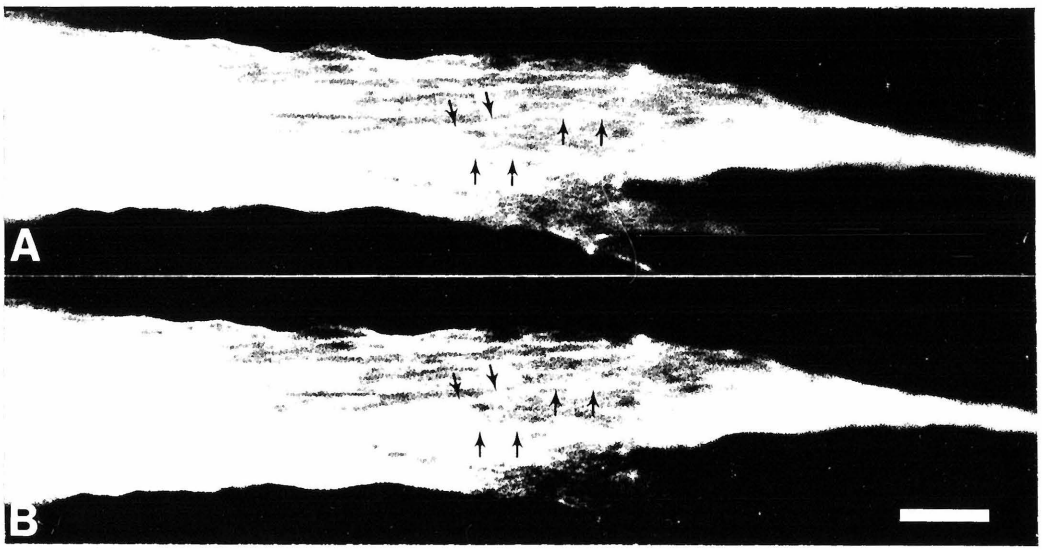
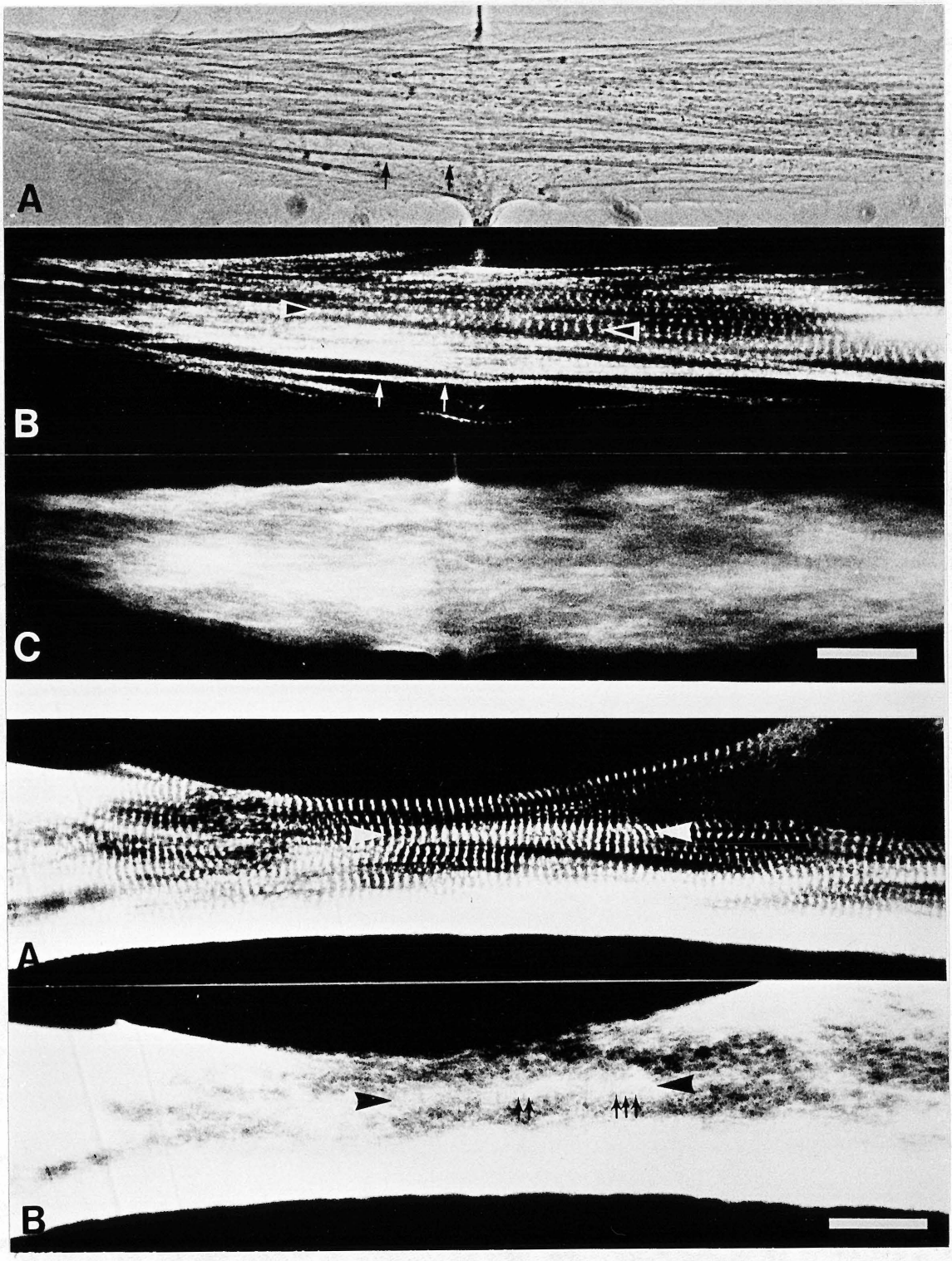
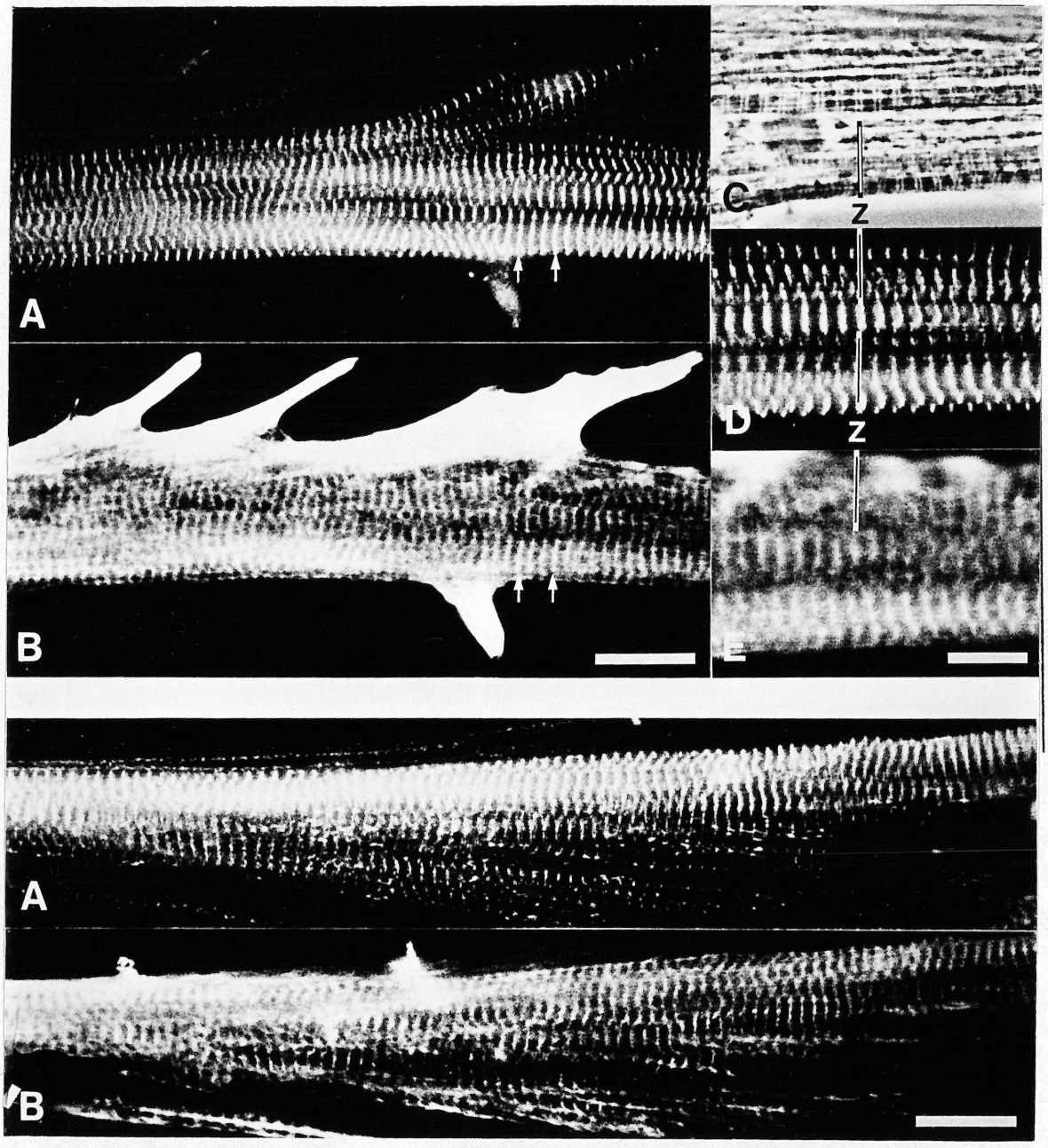


Figure 5



Figures 6 and 7



Figures 8 and 9

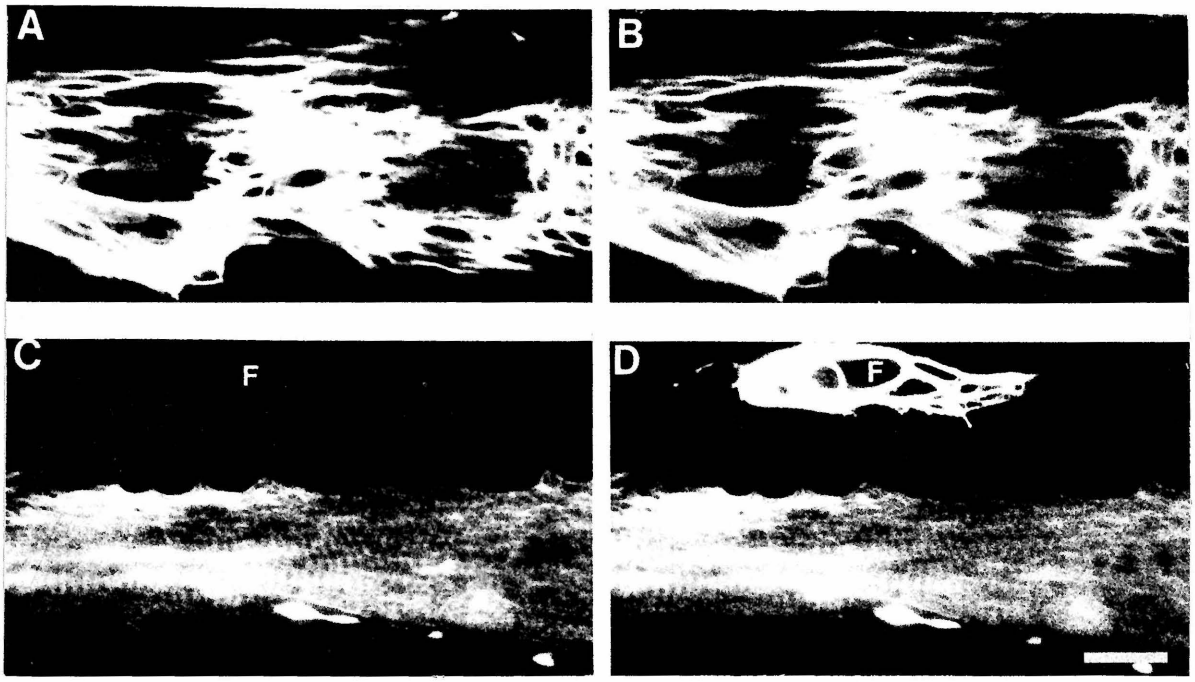


Figure 10

Chapter 4

Phosphorylation of intermediate filament proteins by cAMP-dependent protein kinases

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Preface to Chapter 4

The major portion of work presented in this chapter was carried out by Dr. C. M. O'Connor, with whom I was fortunate to share a lab during her fellowship. My contribution to this work was the comparative analysis of desmin phosphorylated in vivo and in vitro (by cAMP-dependent protein kinase) using the technique of two-dimensional tryptic peptide mapping. My results indicate that desmin is phosphorylated at multiple sites in vivo, and that these sites correspond to those phosphorylated by cAMP-dependent protein kinase in vitro. Partial acid hydrolysis of ^{32}P-desmin peptides revealed that the two major in vivo phosphopeptides contained O-phosphoserine.

Summary

The intermediate filament proteins, desmin and vimentin, are phosphorylated in skeletal muscle cells in vivo (O'Connor et al., 1979). Desmin kinase activities have been purified from mature chicken skeletal muscle and identified as the cAMP-dependent kinases. Chicken skeletal muscle contains two cAMP-dependent protein kinases which are similar to those of other tissues in their subunit composition and chromatographic behavior. The catalytic subunits purified from the two chicken cAMP-dependent kinases phosphorylate both desmin and vimentin in vitro, using cytoskeletal residues prepared from cultured myogenic cells as a substrate. Likewise, the purified catalytic subunits of the rabbit skeletal muscle and bovine heart cAMP-dependent protein kinases phosphorylate desmin and vimentin in vitro. Desmin and vimentin phosphorylation by the rabbit skeletal muscle catalytic subunit is inhibited by the addition of its regulatory subunit. This inhibition is reversed by the presence of cAMP in the reaction mixture.

Tryptic peptide analysis of desmin phosphorylated in vivo shows two major phosphopeptides. Serine is the phosphorylated amino acid in both peptides. The same two peptides are phosphorylated in vitro by the bovine heart catalytic subunit, but additional peptides are also phosphorylated.

Introduction

The intermediate filaments are a heterogeneous class of distinct filament types, each composed of a specific subunit protein or proteins. All of the intermediate filaments have been shown to have phosphorylated subunits (reviewed in Lazarides, 1980). We have previously observed that desmin and vimentin, the subunit proteins of the intermediate filaments found in muscle, are actively phosphorylated in vivo (O'Connor et al., 1979). Using two-dimensional isoelectric focusing/sodium dodecyl sulfate (IEF/SDS) polyacrylamide gel electrophoresis and autoradiography, it was shown that muscle cells have a major unphosphorylated form as well as several phosphorylated isoelectric variants of each protein. Neither desmin nor vimentin is solubilized by extraction of the cells with nonionic detergents at physiological or higher ionic strengths. Instead, they are retained in a cytoskeletal residue composed of networks of 6 nm and 10 nm filaments (O'Connor et al., 1979; Granger and Lazarides, 1979; Brown et al., 1976).

The cytoplasmic regulatory mechanisms which govern the assembly of these structures are obscure. One possible control mechanism is the post-translational modification of the filament-forming proteins or of their associated proteins. Since phosphorylation-dephosphorylation reactions are involved in many biological control phenomena (Krebs and Beavo, 1979), it is possible that the assembly of desmin and vimentin into cellular structures might be regulated by a similar phosphorylation event. A first step towards defining a regulatory mechanism is to identify the kinase activities in muscle which use desmin and vimentin as substrates. In this paper we present several kinds of evidence that the kinases which phosphorylate desmin and vimentin in vitro are the cAMP-dependent kinases. The desmin kinase activities from chicken skeletal muscle co-purify with the cAMP-dependent kinases from the same tissue. Furthermore, the catalytic subunits of the cAMP-dependent

kinases purified from several sources phosphorylate desmin and vimentin in vitro in a cAMP-dependent manner at sites which are the same as those phosphorylated in vivo.

Results

Co-chromatography of Desmin Kinase and cAMP-Dependent Kinases

Desmin kinase activity can be extracted from both smooth (gizzard) and skeletal (thigh or breast) chicken muscle by homogenizing the tissue in neutral solutions of low ionic strength in the absence of detergent, suggesting that a soluble enzyme(s) is responsible for the activity. All of the activity is absorbed onto DEAE-cellulose and elutes from the resin with 20-200 mM NaCl. The pattern of desmin kinase elution resembles that of the cAMP-dependent kinases, as assayed in the same fractions by both histone kinase and cAMP binding activity. Two peaks of cAMP-dependent kinase activity are eluted from DEAE at 20 mM and 140 mM NaCl (Figure 1). These two peaks correspond to the Type I and Type II kinases reported in the literature (Hofmann et al., 1975).

Although the first peak of histone kinase activity shows no cAMP-dependence when assayed directly from the column, cAMP dependence with either histone or desmin as substrate (Figure 2) can be demonstrated after concentrating the enzyme with ammonium sulfate. Figure 2 shows the kinetics of desmin phosphorylation by the Type I enzyme purified on DEAE. The reaction is clearly cAMP-dependent.

The activity of the Type II enzyme is always much lower than that of the Type I enzyme with either desmin or histone as substrate. This may be accounted for in part by the presence of endogenous substrates in the Type II preparation. In addition, a soluble heat and acid-stable inhibitory activity elutes from DEAE at a similar salt concentration (unpublished observations). These factors appear to be removed by subsequent chromatographic steps (see below).

Co-chromatography of desmin kinase and the cAMP-dependent kinase activities was also observed and demonstrated on hydroxyapatite, gel filtration (Sephacryl S-200) and Cibacron Blue columns (not shown).

Isolation of Kinase Subunits

In most tissues that have been examined, two peaks of cAMP-dependent kinase activity can be resolved by DEAE-cellulose chromatography (Hofmann et al., 1975). The distinct chromatographic properties of the two kinases result from differences in the properties of their cAMP binding regulatory subunits, while the catalytic subunits are identical or very similar (Hofmann et al., 1975; Bechtel et al., 1977). The regulatory subunits can be purified with affinity resins of cAMP analogs (Dills et al., 1975, 1976). These resins bind the regulatory subunits with high affinity, thereby dissociating the kinase holoenzymes.

The regulatory subunits of the two chicken muscle cAMP-dependent protein kinases were isolated by affinity chromatography of the DEAE-purified enzymes on $N\text{-}H_2(CH_2)_6$-cAMP coupled to Sepharose 4B as described by Dills et al. (1976). The purified regulatory subunits typically have a binding capacity of 5-10 pmoles cAMP bound per µg of protein, or approximately 0.25-0.50 pmoles cAMP bound per pmole protein. Since the proteins are greater than 90% electrophoretically homogeneous (Figure 3), the lack of full binding capacity (expected to be 1 pmole cAMP bound per pmole protein with this measuring technique, see Builder et al., 1980) is probably due to the incomplete removal of cAMP used to elute the proteins from the affinity resin. The regulatory subunits isolated by affinity chromatography co-migrate with soluble acidic proteins which are specifically photo-affinity labeled with ^3H-cAMP (unpublished data) using the technique described by Antonoff and Ferguson (1974).

Figures 3a and 3b show the stained two-dimensional gels of the affinity purified regulatory subunits. The Type I regulatory subunits (Figure 3a) consist of several proteins with a molecular weight of 50,000 and isoelectric points ranging from 5.4 to 5.65. The Type II regulatory subunits (Figure 3b) migrate as two bands of molecular weights 52,000 and 54,000 and isoelectric points ranging from 5.1 to 5.2. Similar electrophoretic patterns have been observed for regulatory subunits isolated from a variety of sources (Rangel-Aldao et al., 1979; Steinberg and Coffino, 1979). In the latter cases, the regulatory subunits are comprised of variants which differ in their degree of phosphorylation. The molecular weights and isoelectric points reported here were determined using cytoskeletal proteins as markers (O'Connor et al., 1979; Hubbard and Lazarides, 1979), as shown in Figure 3c. Although the Type I regulatory subunit and desmin migrate to similar positions on these gels, we have established by one-dimensional peptide mapping (Cleveland et al., 1977) that they are distinct proteins (unpublished observations).

Negligible cAMP binding activity is found in the components of the sample which do not bind to the cAMP affinity resin. A cAMP-independent histone kinase activity is found in this unbound material, as expected for the dissociated catalytic subunits of cAMP-dependent kinases. The catalytic subunit activity was purified from both the Type I and Type II unbound fractions by chromatography on Sephadex C-50, a resin reported to bind the catalytic subunits of cAMP-dependent kinases (Bechtel et al., 1977). In both cases, a single peak of histone kinase activity elutes between 100 and 200 mM potassium phosphate (Figure 4). Table I shows the purification data for the two catalytic subunits. Although the recovery from the C-50 column is very low, the enzymes have been purified over a thousand-fold each. The loss in enzyme activity suffered on the C-50 columns is probably due to dilution of the enzyme during purification, since the kinase activity of the catalytic-subunit

can be irreversibly lost by dilution of the protein during purification (Beavo et al., 1974).

Desmin and Vimentin Phosphorylation by Purified Catalytic Subunits

The ability of the catalytic subunits(s), purified as above, to phosphorylate desmin and vimentin was tested using cytoskeletal residues as substrates. Figure 5a shows a two-dimensional IEF/SDS polyacrylamide gel of the cytoskeletal residue proteins. These cytoskeletal preparations contain no detectable kinase activity, since the addition of [γ-^{32}P] ATP to the kinase reaction mixture causes no phosphate incorporation (Figure 5b) in the absence of added kinase activities. However, when either the Type I or Type II catalytic subunit is added to the mixtures, phosphate is incorporated into the more acidic variants of desmin and vimentin, but not into β-desmin or the major vimentin variant (Figure 5c). A similar pattern of incorporation occurs when either the catalytic subunit isolated from rabbit skeletal muscle (Figure 6) or a highly purified commercial preparation of bovine heart cAMP-dependent protein kinase catalytic subunit is used in the reaction mixtures (Figure 5d). These same variants of desmin and vimentin which we now observe to incorporate phosphate in vitro have been previously shown to be phosphorylated in vivo (O'Connor et al., 1979). In contrast, phosphate is not incorporated into either of the tropomyosins in vitro (Figures 5c and 5d), even though they are both phosphorylated in vivo (O'Connor et al., 1979).

Figures 5c and 5d show several phosphoproteins which do not comigrate with major cytoskeletal proteins. These may represent minor cytoskeletal proteins, contaminants in the enzyme preparations, and/or breakdown products of desmin and vimentin (Granger and Lazarides, 1979). One interesting example is the phosphoprotein indicated by the arrow in Figures 4c and 4d which migrates at the same molecular weight and is slightly more acidic than α-actin. This protein, which

is probably a trace component of cytoskeletons, is readily apparent in other samples of muscle tissue which are more enriched in actin. Recent work has suggested that this protein may be an actin species, since limited proteolytic digestion of it yields the same family of peptides as does similar treatment of the α-actin spot (B. L. Granger and E. Lazarides, unpublished observations; Steinberg, 1980).

Inhibition of Desmin and Vimentin Phosphorylation by the Regulatory Subunit and Its Reversal by cAMP

Phosphorylation reactions by the catalytic subunits of the cAMP-dependent kinases are inhibited by the kinase regulatory subunit in the absence of cAMP (Hofmann, 1980). We tested the effect of the regulatory subunit on desmin and vimentin phosphorylation in vitro using the rabbit skeletal muscle catalytic and regulatory subunits isolated as described by Hofmann (1980). The rabbit enzymes were used instead of the chicken enzymes for this experiment because our yield of chicken enzymes was so consistently low. As shown in Figure 6a, the rabbit catalytic subunit also phosphorylates the acidic variants of desmin and vimentin. When the regulatory subunit is added in excess (w/w) over the catalytic subunit, phosphorylation of desmin is completely inhibited (Figure 6b). This inhibition is substantially reversed, however, if the regulatory subunit has been previously complexed with cAMP (Figure 6c). It is interesting that phosphorylation of several proteins indicated by the arrows in Figure 6 is not inhibited by the regulatory subunit. Presumably these reactions are catalyzed by a cAMP-independent kinase which has remained associated with these particular cytoskeletons, since it also appears when no exogenous kinase is added to the cytoskeletons (Figure 5d). In addition, a small fraction of the vimentin phosphorylation is cAMP-independent. Most of the vimentin phosphorylation, however,

is cAMP-dependent.

Peptide Mapping of Desmin Phosphorylated In Vivo and In Vitro

The authenticity of desmin phosphorylation in vitro was determined by comparing the two-dimensional tryptic peptide maps of desmin phosphorylated in vivo and in vitro. In vivo ^{32}P-labeled desmin was prepared by labeling myogenic cultures with ^{32}P-inorganic phosphate as described in Experimental Procedures. For the experiment shown in Figure 7, all of the desmin isoelectric variants were pooled before the digestion. It is clear that two major phosphopeptides are present in in vivo ^{32}P-labeled desmin (Figure 7a). Comparing the maps on Figures 7a, 7b and 7c, it is clear that the cAMP-dependent kinases phosphorylate the same two sites as are phosphorylated in vivo, but that they phosphorylate several additional sites as well. At the present time, it is unclear if these additional sites are also phosphorylated in vivo (see Discussion) or if this is an artifact of the nonphysiological conditions used to label desmin in vitro. Since the substrate desmin is assembled into filaments, it should also be considered that the access of the kinases to various sites may be limited.

Characterization of the Desmin Phosphorylation Sites

The phosphate incorporated into in vivo ^{32}P-labeled desmin shows the characteristics of a phosphoester bond in that it is stable at neutral or acidic pH, but is rapidly lost at basic pHs. The identity of the phosphorylated amino acid in the major tryptic phosphopeptides was established by high voltage electrophoresis of the partially hydrolyzed ^{32}P-desmin on cellulose thin layer plates. Samples eluted from the tryptic maps were hydrolyzed at 110°C in 6 N HCl for 2 hr, since complete digestion of the protein also hydrolyzes the phosphoester bond (Bylund and Huang,

1976). The phosphorylated amino acids derived from both major phosphopeptides comigrated with the phosphoserine marker and were clearly resolved from phosphothreonine and phosphotyrosine (Figure 8).

Discussion

A limited amount of evidence from in vivo experiments has suggested a link between cAMP concentrations and cytoskeletal assembly. Recently, Steinberg et al. (1979) demonstrated that vimentin phosphorylation increased in the presence of agents expected to increase intracellular cAMP levels. At the same time, vimentin phosphorylation in a mutant cell line which was kinase-deficient did not change in response to these agents. The kinase deficiency was not accompanied by a change in cell morphology from the parent phenotype which is transformed and nonadhesive. On the other hand, mutant sublines of CHO cells with deficiencies in kinase activities show abnormal assembly of their cytoskeletons (Evain et al., 1979), suggesting that cAMP-dependent protein phosphorylation is involved in cytoskeletal assembly in nontransformed cells. Since all of these experiments utilized whole cells, the data, though suggestive, are only correlative in nature. In this paper we present direct evidence that the intermediate filament proteins, desmin and vimentin, can serve as substrates for cAMP-dependent kinases in vitro.

Two fractions of desmin-kinase activity from chicken muscle have been resolved by chromatography on DEAE-cellulose, eluting between 20 and 140 mM NaCl. Both fractions also contain histone kinase and cAMP binding activities. Based upon these properties, we suggest that the desmin-kinase activities correspond to the Type I and Type II protein kinases which appear to be present in virtually all tissues (Hofmann et al., 1975, 1977). The kinases we describe appear to have

a subunit structure similar to other more thoroughly characterized kinases in that they contain cAMP binding and kinase activities on distinct subunits which can be separated chromatographically (Dills et al., 1975, 1976; Bechtel et al., 1977; Hofmann, 1980). The Type I and Type II regulatory subunits have been isolated by cAMP affinity chromatography and characterized by two-dimensional IEF/SDS polyacrylamide gel electrophoresis. Each is composed of several proteins differing in isoelectric point, and for the Type II kinase, in molecular weight as well. The catalytic subunits, which do not bind to the affinity resin, have been purified by chromatography on carboxymethyl Sephadex. They show similar chromatographic profiles and activities when assayed with either histone or desmin as substrate. We have not attempted to determine if the two catalytic subunits are the same protein, as has been reported in other tissues (Hofmann et al., 1975; Bechtel et al., 1977).

Several lines of evidence, in addition to the chromatographic data, suggest that these kinase activities correspond to the cyclic AMP dependent kinases. First, phosphorylation of cytoskeletal proteins by the holoenzyme forms purified on DEAE columns is cyclic AMP-dependent. In addition, using kinase isolated from rabbit muscle (which provides a much higher yield than chicken muscle), we have shown that desmin and vimentin phosphorylation by the isolated catalytic subunit is inhibited by an excess of regulatory subunit isolated from the same tissue. This inhibition can be reversed by the addition of cAMP. Catalytic subunits isolated from chicken skeletal, rabbit skeletal, or bovine heart muscle all produce nearly identical phosphorylation of cytoskeletal proteins.

Finally, we have used two-dimensional tryptic peptide mapping to compare the products of in vitro phosphorylation and in vivo phosphorylation of desmin. This analysis confirmed the ability of isolated bovine catalytic subunit to phosphorylate

the two major phosphorylation sites observed in desmin in vivo. In addition to these sites, however, several other phosphopeptides were detected in in vitro labeled desmin. The physiological significance of these additional sites is uncertain. We have observed up to four different phosphorylated desmin variants in vivo (O'Connor et al., 1979) which are especially prevalent when care is taken to reduce phosphatase activity (unpublished observation). On occasion, minor peptides are apparent in tryptic maps of in vivo-labeled desmin, which may correspond to the in vitro-labeled sites. If these additional sites are in fact more labile, their attached phosphate may be lost during prepartion of in vivo ^{32}P-desmin. With a lower fractional occupancy with phosphate, these additional sites might then serve as more effective substrates for phosphorylation in vitro. Also, we cannot rule out the possibility that these additional phosphopeptides are artifacts of the nonphysiological conditions of the phosphorylation reaction in vitro.

The definite relationship between the major desmin isoelectric variants is an important question that remains unanswered. It is possible that β-desmin is the unphosphorylated precursor of α-desmin, which in turn is further phosphorylated to the more acidic variants observed in vivo and in vitro. In this respect, ^{35}S-methionine pulse-chase experiments show a significant lag in the time of appearance of the most acidic desmin variants relative to α- and β-desmin (D. Gard and E. Lazarides, unpublished observations). Alternatively, the different desmin variants may represent discrete gene products, and the precursor pools for the phosphorylation reactions may be either too small to be detected or unresolvable on our gels.

The most significant unanswered question with respect to intermediate filament protein phosphorylation is its function. The postulated roles for desmin and vimentin are purely structural. While evidence cited above has suggested a connection between cytoskeletal morphology and the phosphorylation of cytoskeletal components,

we have been unable to detect a predominance of either phosphorylated or unphosphorylated variants (of either desmin or vimentin) during any stage of structural differentiation or in any particular subcellular fraction of muscle cells. An equilibrium between phosphorylated and unphosphorylated variants may be established early in myogenesis since the concentration of cAMP-dependent kinases in embryonic muscle remains fairly constant from the time of desmin appearance (day 13 of embryogenesis) (La Peuch et al., 1979). Alternately, the phosphorylation of intermediate filaments may have more subtle effects on cytoskeletal organization. Perhaps the function of desmin and vimentin phosphorylation will be more clearly established in a cell-free system where the assembly of desmin and vimentin into filaments and the affects of phosphorylation on assembly can be studied.

Experimental Procedures

Materials

Calf thymus histones (Type II), protein kinase catalytic subunit, ATP and cAMP were purchased from Sigma Chemical Co. Phosphoserine, phosphothreonine and Coomassie Brilliant Blue R were also from Sigma. TPCK-Trypsin was purchased from Worthington, Inc. Aquasol-2 was from New England Nuclear. 6-Chloropurine riboside-3', 5'-cyclic monophosphate was obtained from Boehringer Mannheim. DEAE-cellulose (DE-52) was purchased from Whatman; Sephadex C-50 and Sepharose 4B were from Pharmacia. Carrier-free [γ-^{32}P] ATP was purchased from ICN, and carrier-free [^{32}P] H_3PO_4 was purchased from Amersham. Cyclic [^{3}H]-cAMP (42.5 Ci/mmol) was from New England Nuclear. Thin layer chromatography plates (13255 Cellulose) were obtained from Eastman Kodak Co. Ampholines were obtained from LKB.

Purification of Kinase Holoenzymes

Breast muscle was dissected from mature female chickens immediately after asphyxiation of the animals with CO_2. All subsequent operations were carried out at 4°C. All solutions contained 15 mM 2-mercaptoethanol and 10 μM PMSF. The chilled muscle was passed through the coarse disk of a meat grinder and then homogenized in 2.5 volumes of 5 mM EDTA, pH 7.5, for 1 min in a Waring blender at top speed. The homogenate was spun at 10,000 g for 15 min, and the supernatant was filtered through glass wool. The pH of the supernatant was adjusted to 6.8 with 1 N KOH and the conductivity to 0.6 mmhos (Radiometer Type CDC 114 electrode) with cold distilled water. Fifty grams of DEAE-cellulose pre-equilibrated in Buffer A (5 mM potassium phosphate, 2 mM EDTA, pH 6.8) were added to the supernatant for each 100 g of starting material, and the mixture was stirred for 30 min. The resin was collected, washed on a Buchner funnel with 3 volumes of Buffer A and packed into a column. The column was eluted with 8 volumes of a linear gradient of 0-400 mM NaCl in Buffer A.

Purification of Enzyme Subunits

The Type I or Type II kinases purified by DEAE-cellulose chromatography were precipitated by adding ammonium sulfate to a final concentration of 0.35 g/cc, resuspended at concentrations of 10-20 mg/ml and dialyzed against Buffer A overnight. The dialyzed fractions were stirred for 4 hr at 4°C with an affinity resin consisting of N^6-(6-aminohexyl)-cAMP synthesized as described by Dills et al. (1975, 1976) which had been coupled to Sepharaose 4B (March et al., 1974). To prepare regulatory subunits the resin was eluted batchwise at room temperature as described by Dills et al. (1975). To prepare catalytic subunits, the unbound fractions were passed through a column of DEAE-cellulose with a capacity one-fourth that of the original

column and the eluant was stirred immediately with Sephadex C-50 (0.25 g/kg starting material) pre-equilibrated in Buffer A. The column was eluted with 5 volumes of a linear gradient of 5-200 mM potassium phosphate, pH 6.7, containing 0.1 mM EDTA.

The catalytic and regulatory subunits of the cAMP-dependent kinase from rabbit skeletal muscle were purified as described by Hofmann et al. (1980).

Kinase Substrate Preparation

Smooth muscle desmin was isolated from the KI-insoluble proteins of chicken gizzard by three cycles of solubilization in 1 N CH_3CO_2H and precipitation at pH 4 as described previously (Hubbard and Lazarides, 1979). This preparation, which is 90% desmin, contains actin as the major contaminant. Cytoskeletal residues were prepared by extracting primary cultures of chicken embryonic thigh cells with 0.6 M KCl and 1% Triton X-100 as described previously (O'Connor et al., 1979).

Enzyme Assays

Column fractions were assayed by adding a 15 µl aliquot to 100 µl of an incubation mixture containing 20 mM HEPES, 0.3 mM EGTA, 10 mM NaF, 10 mM $MgAc_2$, 2.5 µM [γ-^{32}P] ATP (500-2000 cpm/pmol) and substrate proteins. In some cases, 5 µM cAMP was also present in the assay mixture. Reactions were carried out for 30 min at 30°C. Incorporation of ^{32}P into histone was measured as described by Corbin and Reimann (1974). The concentration of histone in the assay was 0.5 mg/ml. To assay desmin kinase activity, 5 µg of smooth muscle desmin (1.9 mg/ml in 1 mM HCl) was included in the reaction mixture. The reaction was terminated by the addition of 1 ml of distilled water; desmin, which is insoluble at neutral pH (Hubbard and Lazarides, 1979), was then pelleted by centrifugation at 2000 g for 5 min, drained, and resuspended in SDS sample buffer. SDS polyacrylamide

gel electrophoresis was performed as described (Hubbard and Lazarides, 1979) until the tracking dye ran off the gel. The gels were stained with Coomassie Blue, dried and autoradiographed with intensifying screens as previously described (O'Connor et al., 1979).

To assay cytoskeletal protein phosphorylation, cytoskeletal residues from $\sim 3 \times 10^6$ cells (approximately 25 µg protein) were included in 200 µl of reaction mixture to which was added 25 µl of kinase. The mixture was incubated 15 min at 30°C with occasional agitation. The insoluble residue was resuspended and electrophoresed on two-dimensional IEF/SDS polyacrylamide gels (Hubbard and Lazarides, 1979).

Cyclic AMP binding activity was assayed essentially as described by Rangel-Aldao et al. (1979).

Tryptic Peptide Analysis of In Vivo- and In Vitro-labeled ^{32}P-desmin

Primary cultures of embryonic ckicken muscle cells (Gard et al., 1979) were labeled for 24 hr in a phosphate-free medium supplemented with 1% horse serum and 1% chick embryo extract. Tracer $^{32}PO_4$ was added to a final concentration of 80-100 µCi/ml. Following the incubation, cultures were washed twice with cold PBS, scraped from the petri dishes and pelleted. Cell pellets were solubilized in 10 volumes of 8 M urea, 2% NP-40, and 0.5% 2-mercaptoethanol prior to electrophoresis on two-dimensional IEF/SDS polyacrylamide gels (Hubbard and Lazarides, 1979).

In vitro-labeled desmin was prepared by incubating the cytoskeletal residues from 20×10^6 cells with 500 units of commerical bovine heart catalytic subunit. Incubation conditions were the same as those described in Enzyme Assays except that the ATP concentration was 20 µM and the specific activity was 12,5000 cpm/pmole.

^{32}P-labeled desmin in polyacrylamide gel slices was prepared for two-dimensional tryptic peptide mapping following a modification of the procedure of Elder et al. (1977). Gel slices containing radioactive protein were washed extensively (48 hrs in two changes of 1 liter each) in 10% ethanol, dehydrated in 95% ethanol, and vacuum dried overnight. Dried gel slices were rehydrated in 50 mM ammonium bicarbonate containing 0.1 mg/ml trypsin TPCK (Worthington). Approximately 6 µl of trypsin in bicarbonate buffer was added per 1 mm^3 gel slice. Digestion was carried out at 37°C for 4 hrs, at which time a second trypsin aliquot was added in 1 ml of bicarbonate buffer. Digestion and peptide elution was allowed to continue for 24 hrs at 37°C. The supernatant was then collected and lyophilized.

Approximately 2000-4000 cpm of the eluted material was spotted on cellulose thin-layer plates. Plates were then electrophoresed at 800 V for 45 min in acetic acid: formic acid: water (11.4:10:379) pH 1.9. After drying in a 60°C oven for at least two hours, plates were chromatographed in butanol: acetic acid: pyridine: water (60:12:40:48) for the second dimension. The location of phosphopeptides was determined by autoradiography (O'Connor et al., 1979).

Phosphoamino Acid Identification

To identify the phosphorylated amino acid, the ^{32}P-labeled phosphopeptides were eluted from the tryptic maps of in vivo-labeled desmin with 100 mM ammonium bicarbonate, lyophilized, dissolved in 6 N NCl and hydrolyzed for 2 hr at 110°C (Bylund and Huang, 1976). The hydrolysate was lyophilized and resuspended in water containing marker phosphoserine and phosphothreonine. This sample was electrophoresed for 2 hr at700 V on cellulose thin-layer plates (20 cm x 20 cm) in formic acid: acetic acid: water (10:11.4:379), pH 1.9. Marker spots were visualized with 1% ninhydrin. [^{32}P] amino acids and peptides were identified by autoradiography.

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References

Antonoff, R. S., and Ferguson, J. J. (1974). Photoaffinity labeling with cyclic nucleotides. J. Biol. Chem. 245, 3319-3321.

Beavo, J. A., Bechtel, P. J., and Krebs, E. G. (1974). Preparation of homogeneous cyclic AMP-dependent protein kinase(s) and its subunits from rabbit skeletal muscle. Methods Enzymol. 38, 299-308.

Bechtel, P. J., Beavo, J. A. and Krebs, E. G. (1977). Purification and characterization of catalytic subunit of skeletal muscle adenosine 3':5-monophosphate-dependent protein kinase. J. Biol. Chem. 252, 2691-2697.

Brown, S., Levinson, W., and Spudich, J. A. (1976). Cytoskeletal elements of chick embryo fibroblasts revealed by detergent extraction. J. Supramol. Struct. 5, 119-130.

Builder, S. E., Beavo, J. A., and Krebs, E. G. (1980). Stoichiometry of cAMP and 1,N^6-etheno-cAMP binding to protein kinase. J. Biol. Chem. 255, 2350-2354.

Bylund, D. B., and Huang, T.-S. (1976). Decomposition of phosphoserine and phosphothreonine during acid hydrolysis. Anal. Biochem. 73, 477-485.

Cleveland, D. W., Fischer, S. G., Kirschner, M. W., and Laemmli, U. K. (1977). Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. J. Biol. Chem. 252, 1102-1106.

Corbin, J. D., and Reimann, E. M. (1974). Assay of cyclic AMP-dependent protein kinases. Methods Enzymol. 38, 287-290.

Dills, W. L., Beavo, J. A., Bechtel, P. J., and Krebs, E. G. (1975). Purification of rabbit skeletal muscle protein kinase regulatory subunit using cyclic adenosine-3', 5'-monophosphate affinity chromatography. Biochem. Biophys. Res. Commun. 62, 70-77.

Dills, W. L., Beavo, J. A., Bechtel, P. J., Myers, K. R., Sakai, L. J., and Krebs, E. G. (1976). Binding of adenosine 3', 5'-monophosphate-dependent protein kinase regulatory subunit to immobilized cyclic nucleotide derivatives. Biochemistry 15, 3724-3731.

Elder, J. H., Pickett, R. A., Hampton, J., and Lerner, R. A. (1977). Radioiodination of proteins in single polyacrylamide gel slices. J. Biol. Chem. 252, 6510-6515.

Evain, D., Gottesman, M., Pastan, I., and Anderson, W. B. (1979). A mutation affecting the catalytic subunit of cyclic AMP-dependent protein kinase in CHO cells. J. Biol. Chem 254, 6931-6937.

Gard, D. L., Bell, P. B., and Lazarides, E. (1979). Coexistence of desmin and the fibroblastic intermediate filament subunit in muscle and nonmuscle cells: Identification and comparative peptide analysis. Proc. Natl. Acad. Sci. U.S.A. 76, 3894-3898.

Granger, B. L., and Lazarides, E. L. (1979). Desmin and vimentin coexist at the periphery of the myofibril Z disc. Cell 18, 1053-1063.

Hofmann, F. (1980). Apparent constants for the interaction of regulatory and catalytic subunit of cAMP-dependent protein kinase I and II. J. Biol. Chem. 255, 1559-1564.

Hofmann, F., Beavo, J. A., Bechtel, P. J., and Krebs, E. G. (1975). Comparison of adenosine 3':5'-monophosphate-dependent protein kinases from rabbit skeletal and bovine heart muscle. J. Biol. Chem. 250, 7795-7801.

Hofmann, F., Bechtel, P. J., and Krebs, E. G. (1977). Concentrations of cyclic AMP-dependent protein kinase subunits in various tissues. J. Biol. Chem. 252, 1441-1447.

Hubbard, B. D., and Lazarides, E. (1979). Copurification of actin and desmin from chicken smooth muscle and their copolymerization in vitro to intermediate filaments. J. Cell Biol. 80, 166-182.

Hunter, T., and Garrels, J. I. (1977). Characterization of the mRNAs for α-, β-, and γ-actin. Cell 12, 767-781.

Krebs, E. G. and Beavo, J. A. (1979). Phosphorylation-dephosphorylation of enzymes. Ann. Rev. Biochem. 48, 923-959.

Lazarides, E. (1980). Intermediate filaments as mechanical integrators of cellular space. Nature 283, 249-256.

La Peuch, C. J., Ferraz, C., Walsh, M. P., Demaille, J. G., and Fischer, E. H. (1979). Calcium and cyclic nucleotide dependent regulatory mechanisms during development of chick embryo skeletal muscle. Biochemistry 18, 5267-5273.

March, S. C., Parikh, I., and Cuatrecasas, P. (1974). A simplified method for cyanogen bromide activation of agarose for affinity chromatography. Anal. Biochem. 60, 149-152.

O'Connor, C. M., Balzer, D. R., and Lazarides, E. (1979). Phosphorylation of subunit proteins of intermediate filaments from chicken muscle and nonmuscle cells. Proc. Natl. Acad. Sci. U.S.A. 76, 819-823.

Rangel-Aldao, R., Kupiec, J. W., and Rosen, O. M. (1979). Resolution of the phosphorylated and dephosphorylated cAMP-binding proteins of cardiac muscle by affinity labeling and two-dimensional electrophoresis. J. Biol. Chem. 254, 2499-2508.

Steinberg, R. A., and Coffino, P. (1979). Two-dimensional gel analysis of cyclic AMP effects in cultured S49 mouse lymphoma cells: Protein modifications, inductions and repressions. Cell 18, 719-733.

Figure Legends

Figure 1. DEAE-Cellulose Chromatography of Skeletal Muscle Kinase Activities

Muscle kinases were absorbed to and eluted from DEAE-cellulose as described in Experimental Procedures. Top, fractions were assayed for histone kinase activity in the absence (O-O-O) and presence (●-●-●) or 5 μM cAMP and for [^{3}H] cyclic AMP binding activity (Δ - Δ - Δ). Bottom, fractions from the same column were assayed for desmin kinase activity. The position of desmin (D) on the autoradiogram is indicated.

Figure 2. Kinetics of Desmin Phosphorylation in the Absence and Presence of 5 μM Cyclic AMP

The first peak of kinase activity eluting from DEAE-cellulose was concentrated by ammonium sulfate precipitation and dialyzed against 10 mM potassium phosphate, 0.1 mM EDTA, 15 mM 2-mercaptoethanol overnight. The pooled fractions were assayed for desmin kinase activity in the absence (left) and presence (right) of cAMP. Autoradiogram shows four time points corresponding to 5, 10, 15 and 20 min. The position of desmin (D) on the autoradiogram is indicated.

Figure 3. Two-Dimensional IEF/SDS Polyacrylamide Gel Electrophoresis of the Type I and Type II Regulatory Subunits Isolated by cAMP-Affinity Chromatography

(A) Regulatory subunit of the Type I kinase (RI). (B) Regulatory subunit of the Type II kinase (R2). (C) Co-electrophoresis of R1 and R2 with cytoskeletal proteins (CSK) prepared as described in Experimental Procedures. The positions of vimentin (V), α- and β-desmin (D), R1, R2, actin (Ac) and the tropomyosins (Tm) are indicated. The acidic end of the isoelectric gradient is on the left.

Figure 4. Chromatography of Protein Kinase Catalytic Subunits on Sephadex C-50.

The Type I and Type II protein kinases purified by DEAE-cellulose chromatography were passed through a cAMP affinity resin. The histone kinase activities which did not bind to the cAMP affinity resin were absorbed to and eluted from Sephadex C-50 as described in Experimental Procedures. Fractions were assayed for histone kinase activity. (O-O-O) Type I kinase; (●-●-●) Type II kinase.

Figure 5. Phosphorylation In Vitro of Cytoskeletal Proteins

Cytoskeletal protein phosphorylation by preparations of the cAMP-dependent protein kinase catalytic subunits was assayed as described in Exprimental Procedures. (A) Coomassie Blue-stained gel of the cytoskeletal proteins. (B) Autoradiogram of proteins incubated with [γ^{32}P] ATP, but without added kinase. (C) Autoradiogram of cytoskeletal proteins incubated with 8 μg of the chicken skeletal muscle Type II catalytic subunit. (D) Autoradiogram of cytoskeletal proteins incubated with 5 μg of commercial bovine heart cAMP-dependent protein kinase catalytic subunit. Autoradiograms were exposed for 18 h. The positions of the major isoelectric variants of vimentin (V), α- and β-desmin (D), a third more acidic desmin variant, actin (Ac), and tropomyosin (Tm) are indicated. Additional spots previously observed to be phosphorylated in vivo, and presumed to represent isoelectric variants of vimentin, are indicated by dotted lines. The arrow denotes the position of a previously unreported ^{32}P-labeled protein which may be a phosphorylated actin species (see Results).

Figure 6. Cyclic AMP Dependence of Cytoskeletal Protein Phosphorylation In Vitro

Cytoskeletal protein phosphorylation by the rabbit skeletal muscle cAMP-dependent protein kinase catalytic subunit and reconstituted holoenzyme was assayed in the absence and presence of cAMP as described in Experimental Procedures. The autoradiograms were exposed for 3 days.

(A) Cytoskeletal proteins were incubated with 3 μg of rabbit catalytic subunit.

(B) As in (A) plus 10 μg of rabbit regulatory subunit.

(C) As in (B) plus 1 μM cAMP.

(D) Cytoskeletal proteins were incubated with [α-^{32}P]ATP, but with no enzymes or cAMP.

Figure 7. Tryptic Peptide Maps of ^{32}P-Desmin Labeled In Vivo and In Vitro

Tryptic peptide maps were prepared as described in Experimental Procedures. The principal phosphopeptides of in vivo-labeled desmin are indicated as A and B. The positions of the origin (O) and of inorganic phosphate (P_i) are indicated.

(A) Tryptic peptides of in vivo labeled desmin.

(B) Tryptic peptides of a mixture of (A) and (C)

(C) Tryptic peptides from in vitro-labeled desmin.

A product of incomplete proteolysis (*) of in vivo-labeled desmin is seen in (B). Though not seen in this preparation of in vivo desmin, it is often observed in other desmin samples.

Figure 8. Identification of the Phosphorylated Amino Acids of Desmin Labeled In Vitro

^{32}P-labeled desmin was hydrolyzed and electrophoresed with marker phosphoserine and phosphothreonene as described in Experimental Procedures. The position of the origin (O), phosphoserine (pS), phosphothreonine (pT) and inorganic phosphate

(P_i) are indicated. Phosphotyrosine migrates with phosphothreonine in this system. The positions of the positive and negative poles are indicated. The autoradiograms were exposed for 2 weeks.

The asterisk (*) denotes the position of unhydrolyzed peptides. Positions of phosphoamino acid markers were determined by ninhydrin staining.

(Top) Hydrolysis products of phosphopeptide A in Figure 7.

(Bottom) Hydrolysis products of phosphopeptide B in Figure 7.

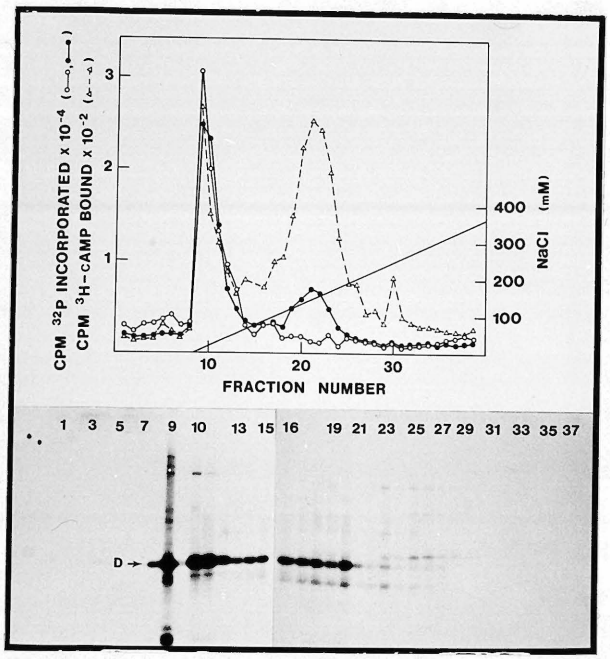

Figure 1

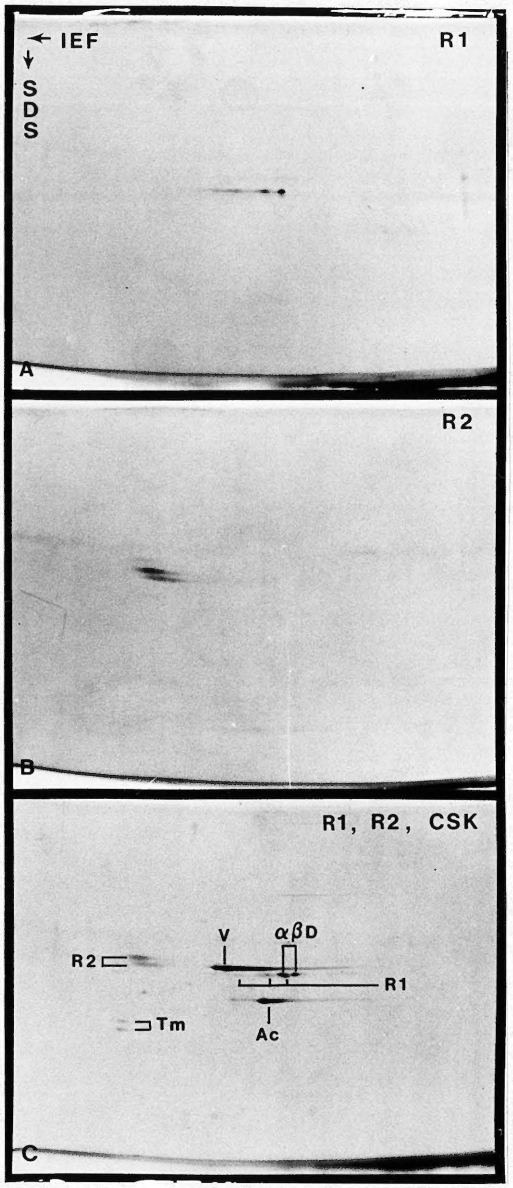

Figure 3

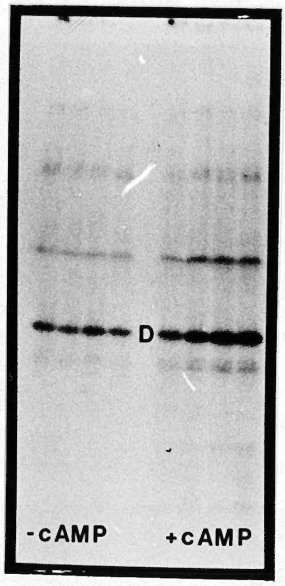

Figure 2

Table 1. Purification of cAMP-Dependent Protein Kinase Catalytic Subunits

	Volume (ml)	Protein (mg)	Total Activity (pmole/min)	Specific Activity (pmole/min/mg)	Recovery (%)	Purification (fold)
Extract	2050	24,600				
Type I kinase						
DE52-I	540	1,620	8,000	4.94	100 (23)[a]	1 (30.6)[a]
CM50-I	2.0	0.41	129	314	1.6	63.7
Type II kinase						
DE52-II	400	320	5,800	18.1	100 (11)	1 (81)
CM50-II	1.5	0.11	120	1090	2.1	60.2

Cyclic AMP-dependent histone kinase activity was measured as described in Experimental Procedures.
[a] Cyclic AMP-dependent kinase activity could not be reliably measured in homogenate fractions due to many interferences in the homogenates (Hofmann et al., 1977). The recovery and purification values in parentheses correspond to the recovery and purification of cyclic AMP binding activities from the homogenate. Recovery and purification of kinase activity should be similar, since the enzyme has not been dissociated by cAMP.

Table 1

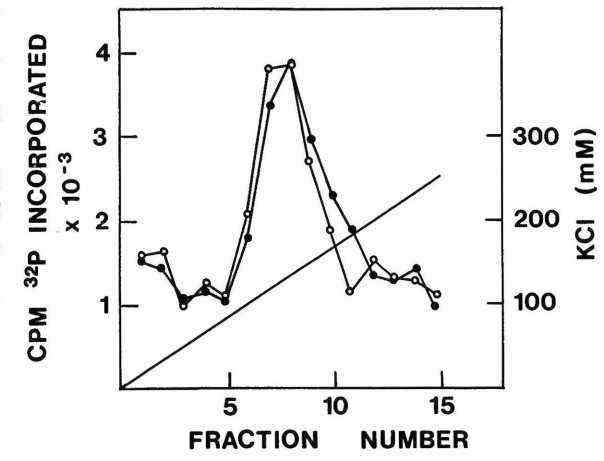

Figure 4

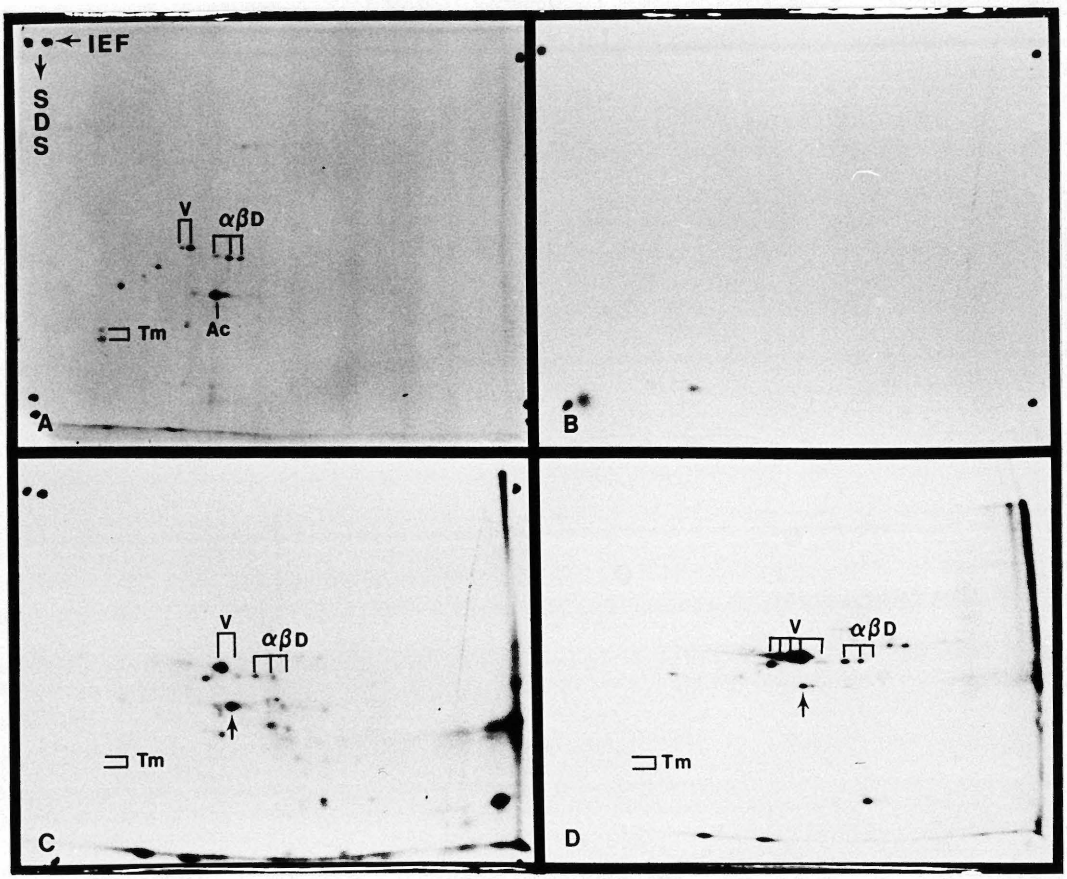

Figure 5

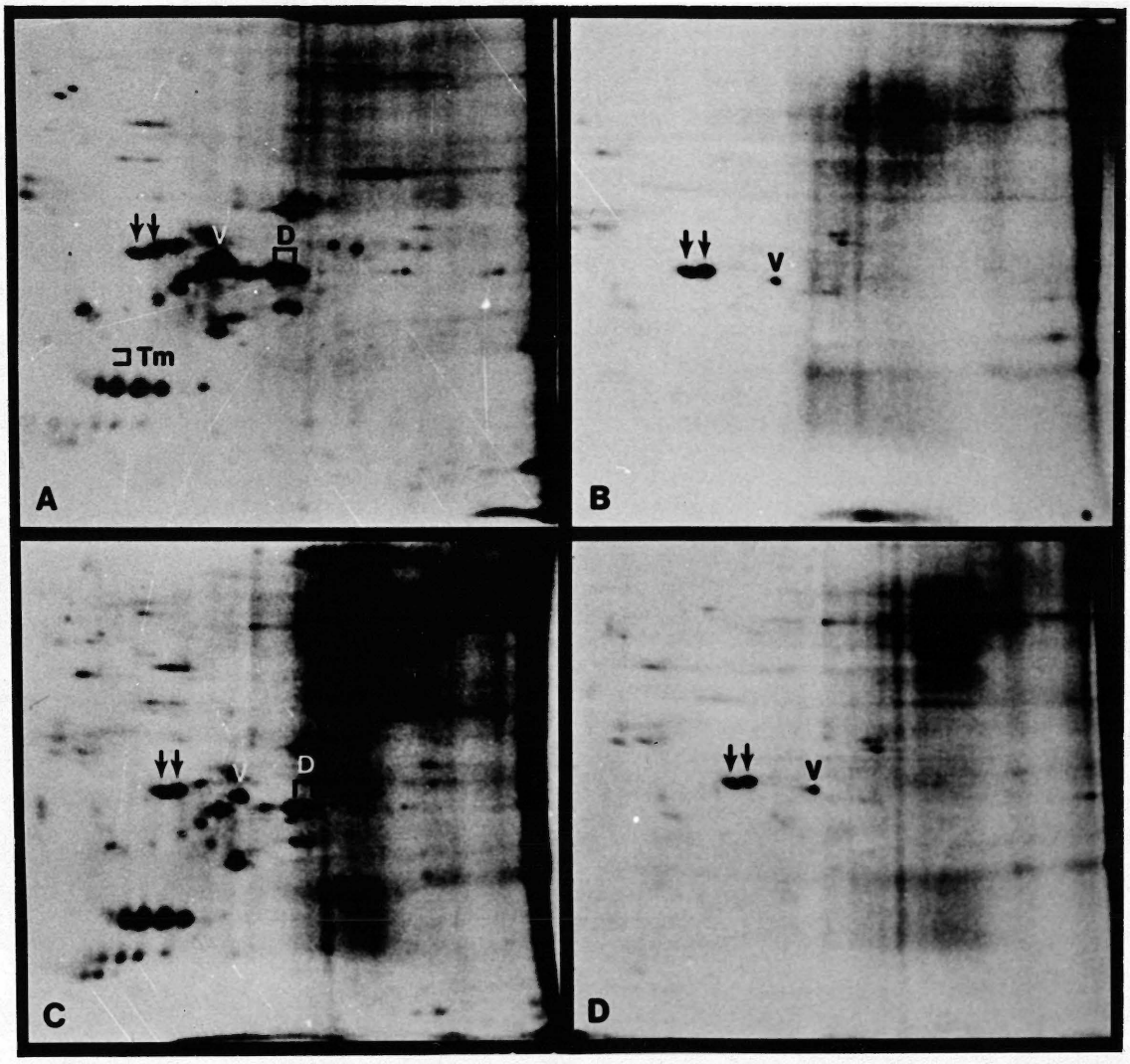

Figure 6

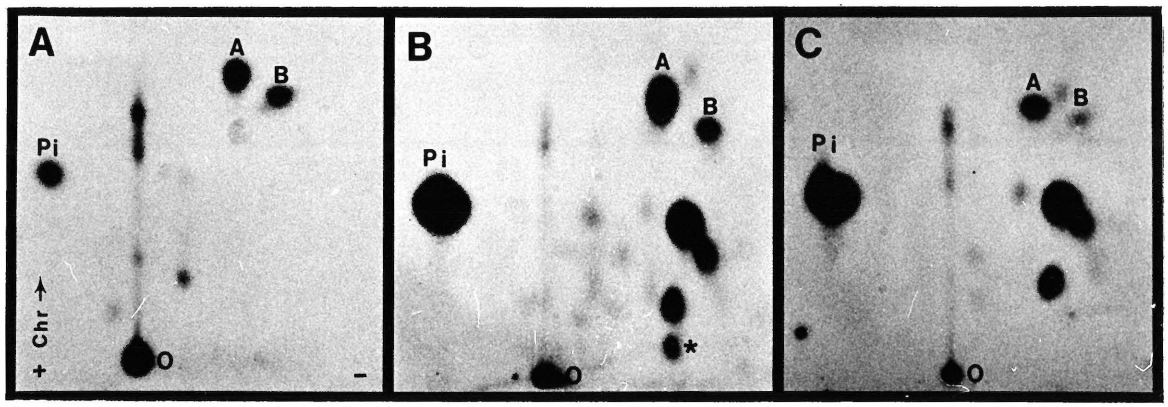

Figure 7

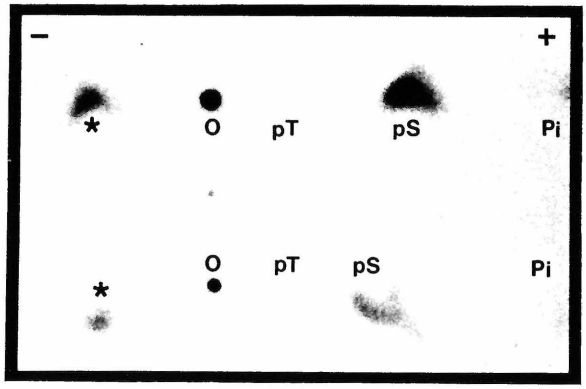

Figure 8

Chapter 5

Phosphorylation of the intermediate filament proteins, desmin and vimentin, in muscle cells

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Preface to Chapter 5

This chapter represents an amalgam of several distinct approaches to the role of intermediate filaments, and phosphorylation of intermediate filament proteins, presenting an overview of our results and current hypotheses. My contributions to this work were two-fold: first, the immunofluorescence results presented were reprinted (with permission) from Gard and Lazarides (1980) which is presented as Chapter 3 of this thesis; I was also responsible for a more detailed study of the phosphorylation sites of desmin and vimentin by two-dimensional tryptic peptide mapping. Briefly, my results indicated that both α'- and α-desmin were multiply phosphorylated, exhibiting qualitatively identical peptide maps. This result suggested that the generation of acidic desmin species from β-desmin did not occur by simple sequential addition of phosphate residues. I also found that vimentin exhibited a complex peptide map, presumably as a result of incomplete proteolysis as well as multiple-site phosphorylation. At least one vimentin phosphopeptide was observed to comigrate with a major phosphopeptide from desmin, suggesting some homology between the phosphorylation sites of these proteins.

The cellular cytoskeleton provides the mechanical forces necessary for the regulation of cell shape and cell motility (reviewed in Goldman et al. 1976). This cytoskeleton is composed of intricate arrays of submicroscopic filaments which fall into several classes based upon their morphology and subunit composition. The best documented of these are the actin-containing microfilaments, which form the stress fibers of nonmuscle cells and which act in concert with myosin filaments to generate contractile force in muscle cells. Extensive networks of microtubules have also been described in many cell types. Current evidence implicates the microtubules in the movement of chromosomes at mitosis and many other motile phenomena.

A third class of filaments, the intermediate filaments, appear to serve a structural function in virtually all cell types (reviewed in Lazarides 1980). The designation intermediate filaments actually applies to a heterogeneous class of different filament types whose diameters range from 7 to 12 nm. Examples of these are the neurofilaments, glial filaments, fibroblastic intermediate filaments and smooth muscle intermediate filaments, each of which is composed of a biochemically distinct subunit protein or proteins. Although different cell types may be enriched in a particular type of intermediate filament, it is not unusual to find more than one filament class in the cytoplasm of an individual cell.

The principal subunit of the intermediate filaments in smooth muscle cells has been identified as desmin (Lazarides and Hubbard 1976; Small and Sobieszek 1977) and that of fibroblasts and other mesenchyme-derived cells as vimentin (Franke et al. 1978). The intermediate filaments of chicken skeletal muscle cells are composed of both desmin and vimentin (Granger and Lazarides 1979), although it is not clear if desmin and vimentin are present in the same filaments. Using monospecific antibodies to desmin and vimentin, we have shown that these proteins

first appear as a dense network of filaments in the myotube sarcoplasm (Gard and Lazarides 1980). Later in myogenesis, they become associated with Z-line structures; this association is maintained in mature skeletal muscle as well (Granger and Lazarides 1979). These Z-line structures, which appear to anchor the actin filaments of each sarcomere, are composed of the perfectly aligned Z-discs of many individual myofibrils in the muscle. In a cross-section view of muscle at the Z-line it can be shown that both desmin and vimentin form a collar-like structure around the periphery of each Z-disc (Granger and Lazarides 1979). Adjacent Z-disc collars in turn form a honeycomb-like network with apparent elastic properties. Therefore, it was suggested that this network of desmin and vimentin was responsible for maintaining the precise register of myofibrils in the muscle cell.

The factors which regulate the disposition of intermediate filaments in the sarcoplasm and their final incorporation into the Z-disc are obscure. One common feature of several types of intermediate filament proteins, however, is the presence of covalently bound phosphate (Lazarides 1980). Since phosphorylation-dephosphorylation reactions govern the activation of many enzymes (Krebs and Beavo 1979), we investigated the phosphorylation of desmin and vimentin in developing chicken skeletal muscle cells as a first step towards defining the regulatory mechanism for filament assembly or function. In the course of the experiments described here, we have identified several phosphorylated variants of both desmin and vimentin which are found as components of the cytoskeletons of cultured embryonic muscle cells. Peptide analysis of phosphorylated desmin reveals at least two major phosphorylation sites, and comparative analysis of desmin and vimentin phosphopeptides suggest some homology between the phosphorylation sites of these two proteins. Finally, we have identified kinase activities which phosphorylate desmin and vimentin in vitro as the cAMP-dependent protein kinases.

Experimental Procedures

Muscle cell cultures were prepared from the thigh muscle of 10-day chicken embryos as described by Gard and Lazarides (1980). Myogenic cell cultures were labeled with ^{32}P-inorganic phosphate as described by O'Connor et al. (1979). Cytoskeletons were prepared by extraction of the myogenic cell cultures with 0.6 M KCl and 1% Triton X-100 as described (O'Connor et al., 1979).

Immunofluorescent localization studies using desmin (Lazarides and Hubbard, 1976) and vimentin (Granger and Lazarides, 1979) monospecific antibodies were performed as described (Gard and Lazarides, 1980).

Two-dimensional isoelectric focusing/sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed as described by Hubbard and Lazarides (1979). Autoradiography was performed as described (O'Connor et al., 1979).

Tryptic peptide analysis was performed using the modification of the procedure of Elder et al. (1977) described by O'Connor et al. (1980).

Cyclic AMP-dependent protein kinases were isolated from chicken skeletal muscle as described by O'Connor et al. (1980). The catalytic and regulatory subunits of the rabbit skeletal muscle cAMP-dependent kinase were isolated as described by Hofmann (1980). Cytoskeletal protein phosphorylation by the isolated kinases using ^{32}P-γ-ATP was performed as described (O'Connor et al., 1980).

Chicken skeletal muscle and smooth muscle RNA was extracted from tissue which had been pulverized in liquid N_2 by the technique described by Chirgwin et al. (1979). The poly A(+) fraction was prepared from total RNA as described by Hunter and Garrels (1977). RNA was translated in a commercial rabbit reticulocyte cell-free translation system (New England Nuclear Co.) according to the manufacturer's instructions. The translation products were separated by two-dimensional gel electrophoresis.

RESULTS

Phosphorylation of Desmin and Vimentin in Myogenic Cells

Using cell cultures derived from the thigh muscle of 10 to 12-day embryonic chicks, the process of skeletal muscle development can be studied in vitro (Gard and Lazarides, 1980). The myoblast cells derived from the thigh tissue cease dividing after 1 to 2 cell divisions and fuse to form multinucleate myotubes. The young myotubes subsequently differentiate in culture, until they assume the mature sarcomere organization of adult muscle. Immunofluorescent localization studies using monospecific antisera to desmin or vimentin (Fig. 1) have established that in immature myotubes (3-5 days after plating) these proteins are found in a densely packed network of filaments which fills the sarcoplasm. Between the 5th and 7th days of culture under our conditions, there is a gradual transition from a filamentous to a Z line staining pattern. In mature myotubes, and in adult myofibrils, desmin and vimentin are components of the myofibril Z line.

When the proteins of the developing myotubes are analyzed by two-dimensional electrophoresis (O'Connor et al., 1979), it is apparent that both desmin and vimentin are present in the cells, and that several isoelectric variants of each protein can be distinguished (Fig. 2a). The two major isoelectric variants of desmin have been previously identified as α- and β-desmin (Izant and Lazarides, 1977). As the myotubes mature (these are 4-day myotubes), the proportion of desmin to the total cell protein will increase (Gard and Lazarides, 1980). The isoelectric variants of vimentin are not clearly resolved on the stained gel. When the cultures are incubated with ^{32}P-inorganic phosphate for 3 to 4 hr prior to separation of the proteins, phosphate is incorporated into several isoelectric variants of both desmin and vimentin (Fig. 2b). In the case of desmin, α-desmin and several more acidic isoelectric variants incorporate phosphate, while β-desmin does not become

labeled. Due to the immaturity of the myotubes used for Figure 2b, this is difficult to distinguish from the background. Upon enrichment for desmin (Figs. 2c and 2d), however, up to four phosphorylated variants are apparent. Similarly, up to seven acidic variants of vimentin incorporate phosphate, but the major, most basic vimentin isoelectric variant does not become labeled (Figs. 2b, 2d).

To determine if desmin and vimentin phosphorylation could be correlated with their assembly into cytoskeletal structures, ^{32}P-labeled myotube cultures were extracted with 1% Triton X-100 and 0.6 M KCl (O'Connor et al., 1979). This treatment has been previously shown to extract most of the cellular protein, leaving an insoluble residue consisting of intermediate filaments and residual non-filamentous actin (Brown et al., 1976). Two-dimensional electrophoresis and autoradiography of the residue proteins (Figs. 2c and 2d) indicates that both the non-phosphorylated and phosphorylated isoelectric variants of desmin and vimentin are present in cytoskeletal structures.

The experiment of Figure 2 has been repeated on cultures in various stages of differentiation, from 3 to 14 days after fusion (Gard and Lazarides, 1980). The molar ratio of the various phosphorylated to nonphosphorylated desmin and vimentin variants is always similar. Phosphorylated variants do not predominate at any particular stage of differentiation.

Characterization of Phosphorylation Sites

The phosphorylation sites of several desmin variants and vimentin have been compared by two-dimensional tryptic peptide analysis of $^{32}PO_4$-labeled proteins isolated from cultured myotubes by IEF/SDS-PAGE (O'Connor et al., 1980). Figure 3 shows that α-desmin isolated in this manner contains two major tryptic phosphopeptides. The phosphorylated amino acid in both peptides has been identified as phosphoserine

(O'Connor et al., 1980). Comparing the peptide maps of α-desmin and the next more acidic desmin variant (termed α'-desmin), it is clear that the same two phosphorylated peptides are present in both α- and α'-desmin. Since our electrophoresis system is capable of separating two desmin variants which differ only in one phosphate residue (J. Falke, pers. comm.), α- and α'-desmin may be mono- and diphosphorylated species of the same protein. Alternatively, the difference in the isoelectric points of the two variants may be caused by another form of charge modification.

Phosphopeptide maps of total desmin occasionally show several minor phosphopeptides in addition to the two discussed above (Fig. 4). The nature of these minor peptides is unclear at this time. It is possible that these peptides are more rapidly hydrolyzed during isolation or the sites are less frequently phosphorylated in vivo, making their detection more difficult. These additional sites are consistent with the observation of up to four phosphorylated desmin variants in vivo (Fig. 2). On the other hand, some of these peptides may represent products of incomplete proteolysis during the mapping procedure.

The tryptic maps of total ^{32}P-vimentin (isolated from the same gels as those used for ^{32}P-desmin) are often more complex than those of desmin. This complexity is not unexpected, since we have observed up to seven phosphorylated variants of vimentin in vivo (Fig. 2), but it has proven difficult to consistently obtain the same pattern of phosphopeptides. We have already alluded to the problems of differential phosphorylation in vivo, lability during the isolation and mapping procedures, and incomplete proteolysis which might produce such inconsistencies. Figure 5 shows the most common digestion pattern that we obtain. Several major phosphopeptides as well as several minor phosphopeptides are present in all preparations of ^{32}P-vimentin. Direct comparison of the tryptic phosphopeptides of α-desmin and vimentin by coelectrophoresis reveals that at least one of the major phosphopeptides

of vimentin comigrates with the major phosphopeptide of desmin. This suggests the existence of some sequence homology between the phosphorylation site(s) of these two intermediate filament proteins.

Desmin and Vimentin Phosphorylation In Vitro by the cAMP-Dependent Protein Kinases

Biosynthetic labeling studies of myogenic cultures suggested that regulation of desmin phosphorylation in vivo occurs through the net activity of protein kinases and protein phosphatases (C. O'Connor and D. Gard, unpublished observations). Previous experiments from many laboratories had established that the activation-deactivation of several muscle kinases occurred in response to the metabolic condition of the cell (Krebs and Beavo, 1979). Therefore, we isolated desmin kinase activity from chicken skeletal muscle (O'Connor et al., 1980) as a first step towards defining the controlling factor for intermediate filament assembly and/or function in muscle.

Desmin kinase activity is completely extracted from adult skeletal muscle tissue at low ionic strength in the absence of detergent. Using smooth muscle desmin as a substrate, desmin kinase co-elutes from DEAE-cellulose, hydroxyapatite and gel filtration columns with the two cAMP-dependent kinases from the same tissue. The similarity of the elution profiles for the desmin kinases and the cAMP-dependent kinases suggests that a single enzyme catalyzes both phosphorylation reactions.

The cAMP-dependent kinases have been characterized in a large number of tissues (Krebs and Beavo, 1979). In all cases, they are composed of a catalytic subunit which catalyzes the phosphotransferase reaction and a regulatory subunit which binds cAMP. We therefore tested the ability of desmin and vimentin in cytoskeletal residues to serve as a substrate for the purified catalytic subunit of the cAMP-dependent protein kinase (O'Connor et al., 1980). Figure 6 shows the phosphorylation of cytoskeletal residue proteins by the Type II chicken skeletal

muscle and the bovine heart catalytic subunits. While the cytoskeletons themselves have no endogeneous kinase activity, both catalytic subunits phosphorylate several isoelectric variants of desmin and vimentin. These same desmin and vimentin variants are phosphorylated in vivo. In addition to desmin and vimentin, the catalytic subunits phosphorylate a minor fraction of the total actin (Steinberg, 1980). However, they do not phosphorylate either α- or β-tropomyosin. This inability of the kinases to phosphorylate the tropomyosins in vitro supports the specificity of desmin and vimentin phosphorylation in vitro, since the specific activity of α- and β-tropomyosin labeled in vivo is comparable to those of desmin and vimentin (O'Connor et al., 1979).

A reaction catalyzed by the catalytic subunit of the cAMP-dependent kinases should be inhibited by an excess of the regulatory subunit in the absence of cAMP (Hofmann, 1980). The cAMP dependence of the phosphorylation reaction in vitro was tested using the rabbit skeletal muscle regulatory and catalytic subunits (O'Connor et al., 1980). Figure 7 shows that desmin phosphorylation by the catalytic subunit is completely inhibited by an excess of regulatory subunit from the same tissue and is largely reversed by the presence of 1 μM cAMP in the reaction mixture. The majority of the vimentin phosphorylation in Figure 7 shows a similar cAMP dependence, but a small fraction appears to be cAMP-independent. This latter cAMP-independent kinase activity, which is unaffected by either the regulatory subunit or cAMP, is a contaminant of this particular cytoskeletal preparation (Fig. 7d).

To determine if the phosphorylation of desmin by the catalytic subunits in vitro occurs at the same sites as in vivo, the tryptic peptides of in vitro labeled ^{32}P-desmin were compared with those of in vivo-labeled desmin digested under the same conditions (O'Connor et al., 1980). Figure 8 shows the tryptic peptides produced from in vitro-labeled desmin. Co-electrophoresis with similarly treated

in vivo-labeled desmin (O'Connor et al., 1980) demonstrated that the two major in vivo phosphopeptides are also phosphorylated in vitro, but that phosphorylation occurs at several additional sites in vitro. It is unclear at this time if these additional sites have physiological significance (see above).

Cell-Free Translation of Desmin

The precise natures of the different isoelectric variants of desmin remain unknown. All of the desmin variants might be the translation products of a single gene which had been subject to numerous post-translational modifications, such as the observed phosphorylation. On the other hand, they might represent the translation products of several genes which had also been modified in the muscle cytoplasm. Consequently, we have studied the de novo synthesis and modification of desmin in a cell-free translation system derived from rabbit reticulocytes using the poly(A)$^+$ mRNA from either myotube cultures, mature skeletal muscle or mature smooth muscle as a template. In all cases, the major desmin species which is synthesized co-migrates with β-desmin from myotube cytoskeletons. The synthesis of α-desmin can also be detected, especially at higher concentrations of desmin mRNA, e.g., when smooth rather than skeletal muscle RNA was used as a template (Fig. 9a). The more acidic variants of desmin are detected only rarely, again when the content of desmin mRNA in the primer is high. The actual ratio of newly synthesized β-desmin to α-desmin is always much higher in the cell-free system than that which we observe in vivo.

The appearance of α-desmin in the cell-free system might be explained by the post-translational phosphorylation of β-desmin in the lysate. Previous reports have established that kinase activity, including cAMP-dependent kinase activity, is present in these lysates (Levin et al., 1979). To determine if the observed α-desmin could be derived from β-desmin by such kinase activity, we added the

bovine heart cyclic AMP-dependent catalytic subunit to a translation mixture immediately after the addition of nonradioactive methionine to the same mixture. The results shown in Figure 9 indicate that the addition of kinase increases the prevalence of α-desmin relative to β-desmin over the control value. In addition, several more acidic desmin variants are discernible. It appears, therefore, that at least a fraction of the more acidic desmin variants are derived from β-desmin by phosphorylation. We cannot exclude an alternative possibility, however, that the α-desmin which we detect in the cell-free system is actually a mixture of a nonphosphorylated species and a phosphorylated species of desmin. In this case, the two species would have to be translation products of different mRNA molecules. However, our attempts to separate an α-desmin mRNA from β-desmin mRNA have been uniformly negative. It has also been reported that myotube RNA is translated only into β-desmin in the wheat germ cell-free translation system (Devlin and Emerson, 1979), which may have less modifying activities than the reticulocyte system. Based on these data, we conclude that β-desmin is the primary translation product in muscle cells and that α-desmin and the more acidic variants are derived from β-desmin by post-translational phosphorylation.

DISCUSSION

Desmin and vimentin, the intermediate filament proteins found in chicken skeletal muscle, are the substrates for multiple phosphorylation reactions in vivo (O'Connor et al., 1979). When muscle cell cultures are incubated with $^{32}PO_4$ in vivo, as many as four isoelectric variants of desmin and seven variants of vimentin incorporate phosphate. The total number of phosphorylated variants may vary, depending upon the method of sample preparation. This suggests that the phosphatase activity of muscle permits only a minimal estimate of desmin phosphorylation in vivo.

Two-dimensional tryptic mapping of in vivo-labeled $^{32}PO_4$-desmin consistently reveals two major phosphopeptides (O'Connor et al., 1980), as well as variable amounts of several minor species. The tryptic maps of $^{32}PO_4$-labeled vimentin are always more complex than those of desmin (see Results above). In all cases, however, at least one of the major phosphopeptides seen in vimentin digests comigrates with the major phosphopeptide observed in ^{32}P-desmin. These data are consistent with earlier reports of sequence homology detected by one-dimensional peptide mapping of ^{32}P-labeled desmin and vimentin (Gard et al., 1979). The existence of homologous phosphorylation sites in desmin and vimentin is interesting in light of the differences in their biochemical and antigenic properties (Gard et al., 1979; Granger and Lazarides, 1980). One possible explanation for this observation is the conservation of the phosphorylation site sequences during the divergence of desmin and vimentin from an ancestral intermediate filament protein. Such a conservation in the phosphorylation sites of the two molecules might imply the necessity of phosphorylation for intermediate filment function. Alternatively, this homology may be the result of evolutionary convergence of the two proteins. These questions might be answered by a more complete comparison of the protein sequences of desmin and vimentin as well as by comparison of the phosphopeptides with those present in other phosphorylated intermediate filament proteins (keratins, neurofilaments, etc.).

In the case of desmin we have attempted to clarify the relationships between its several phosphorylated variants and its single unphosphorylated variant. Using a cell-free translation system, we have determined that the primary desmin translation product of muscle mRNA is β-desmin, and that this may be converted into α-desmin (and the more acidic variants) by the catalytic subunit of the cAMP-dependent kinases which phosphorylates desmin in vitro (see below). This suggests a simple

model in which β-desmin is modified by the addition of a single phosphate moiety, becoming α-desmin. Through further phosphorylation, α-desmin is then converted into the more acidic variants, with each variant possessing a discrete number of phosphates per polypeptide.

This model is complicated, however, by the presence of two major tryptic phosphopeptides in α-desmin and by the near identity of the phosphopeptide maps of α- and α'-desmin. One possible interpretation of these results is that in the initial phosphorylation reaction (the β-desmin to α-desmin transition), phosphate is transferred with a similar frequency to either site on the desmin polypeptide. The nature of the minor phosphorylation sites on desmin is less clear. While the large number of phosphorylated desmin variants suggests their existence, their sporadic appearance in digests of total ^{32}P-desmin implies either a greater lability or a lower occupancy with phosphate in vivo. Thus, the true extent of desmin phosphorylation in vivo is difficult to evaluate.

The complex nature of phosphorylation-dephosphorylation systems in vivo hinders the identification of the kinase(s) responsible for phosphorylating a given substrate. To circumvent this problem, we have used an in vitro system to characterize the kinases which can phosphorylate desmin. Using smooth muscle desmin as a substrate, we have isolated two fractions of desmin kinase activity from chicken skeletal muscle which we have subsequently identified as the cAMP-dependent protein kinases (O'Connor et al., 1980). As expected for cyclic AMP-dependent reactions, both desmin and vimentin phosphorylation are also catalyzed by the catalytic subunits of the cAMP-dependent kinases isolated from several sources. The in vitro phosphorylation reaction is at least partially authentic in that the bovine heart catalytic subunit phosphorylates the two major sites on desmin which are always observed to be phosphorylated in vivo. Several additional peptides

are phosphorylated in vitro as well, but their physiological significance is unclear at the present time.

Direct demonstration of desmin phosphorylation by the cAMP-dependent kinases in vivo has not yet been accomplished. In an intact cell, an increase in the cAMP concentration perturbs the steady-state level of activity for a whole spectrum of enzymatic systems (Krebs and Beavo, 1979), making it difficult to distinguish between direct and indirect affects of the cAMP-dependent kinases. With these reservations in mind, however, it should be noted that Steinberg and Coffino (1979) have reported that vimentin phosphorylation in intact lymphoma cells increases when the cAMP content of the cells increases. A similar increase in vimentin phosphorylation in response to cAMP is not observed in mutant cells which are deficient in cAMP-dependent kinase activity.

One of the most significant problems remaining with regard to desmin and vimentin phosphorylation is the effect of this phosphorylation upon intermediate filament assembly and function. The only roles which have been proposed for intermediate filaments in muscle are structural ones. During development of skeletal muscle cells, intermediate filament proteins (desmin and vimentin) appear first in a filamentous network and later associate with myofibril Z lines (Gard and Lazarides, 1980). The results reported here show that the catalytic subunit of the cAMP-dependent kinases phosphorylates both soluble and filamentous desmin in vitro, but we have not been able to correlate protein phosphorylation with any particular stage of myofibril assembly in vivo. Considering the complexity and asynchrony of morphogenesis in vivo, a cell-free system for intermediate filament assembly may be more appropriate for deciphering the function of intermediate filament phosphorylation.

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REFERENCES

Brown, S., W. Levinson, and J. A. Spudich. 1976. Cytoskeletal elements of chick embryo fibroblasts revealed by detergent extraction. J. Supramol. Struct. 5:119.

Chirgwin, J. M., A. E. Pryzbyla, R. J. MacDonald, and W. J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 18:5294.

Devlin, R. B. and C. P. Emerson, Jr. 1980. Coordinate accumulation of contractile protein mRNAs during myoblast differentiation. Develop. Biol. 69:202.

Elder, J. H., R. A. Pickett, J. Hampton, and R. A. Lerner. 1977. Radioiodination of proteins in single polyacrylamide gel slices. J. Biol. Chem. 252:6510.

Franke, W. W., E. Schmid, M. Osborn, and K. Weber. 1978. Different intermediate-sized filaments distinguished by immunofluorescence microscopy. Proc. Natl. Acad. Sci. USA 75:5034.

Gard, D. L. and E. Lazarides. 1980. The synthesis and distribution of desmin and vimentin during myogenesis in vitro. Cell 19:263.

Gard, D. L., P. B. Bell, and E. Lazarides. 1979. Coexistence of desmin and the fibroblastic intermediate filament subunit in muscle and nonmuscle cells: Identification and comparative peptide analysis. Proc. Natl. Acad. Sci. USA 76:3894.

Goldman, R., T. Pollard, and J. Rosenbaum, eds. 1976. Cell Motility. Cold Spring Harbor Conferences on Cell Proliferation, Vol. 3. Cold Spring Harbor Laboratory, New York.

Granger, B. L. and E. Lazarides. 1979. Desmin and vimentin coexist at the periphery of the myofibril Z discs. Cell 8:1053.

Hofmann, F. 1980. Apparent constants for the interaction of regulatory and catalytic subunit of cAMP-dependent protein kinase I and II. J. Biol. Chem. 255:1559.

Hubbard, B. D. and E. Lazarides. 1979. Copurification of actin and desmin from chicken smooth muscle and their copolymerization in vitro to intermediate filaments. J. Cell Biol. 80:166.

Hunter, T. and J. I. Garrels. 1977. Characterization of the mRNAs for α-, β-, and γ-actin. Cell 12:767.

Izant, J. G. and E. Lazarides. 1977. Invariance and heterogeneity in the major structural and regulatory proteins of chick muscle cells revealed by two-dimensional gel electrophoresis. Proc. Natl. Acad. Sci. USA 74:1450.

Krebs, E. G. and J. A. Beavo. 1979. Phosphorylation-dephosphorylation of enzymes. Ann. Rev. Biochem. 48:923.

Lazarides, E. 1980. Intermediate filaments as mechanical integrators of cellular space. Nature 283:249.

Lazarides, E. and B. D. Hubbard. 1976. Immunological characterization of the subunit of the 100 Å filaments from muscle cells. Proc. Natl. Acad. Sci. USA 73:4344.

Levin, D., V. Ernst, and I. M. London. 1979. Effects of the catalytic subunit of cAMP-dependent protein kinase (Type II) from reticulocytes and bovine heart muscle on protein phosphorylation and protein synthesis in reticulocyte lysates. J. Biol. Chem. 254:7935.

O'Connor, C. M., D. R. Balzer, Jr., and E. Lazarides. 1979. Phosphorylation of the subunit proteins of intermediate filaments from chicken muscle and nonmuscle cells. Proc. Natl. Acad. Sci. USA 76:819.

O'Connor, C. M., D. L. Gard, and E. Lazarides. 1980. Phosphorylation of intermediate filament proteins by cAMP-dependent protein kinases. Submitted for publication.

Small, J. V. and A. Sobieszek. 1977. Studies on the function and composition of the 10 nm (100 Å) filaments of vertebrate smooth muscle. J. Cell. Sci. 23:243.

Steinberg, R. A. 1980. Actin nascent chains are substrates for cyclic AMP-dependent phosphorylation in vivo. Proc. Natl. Acad. Sci. USA 77:910.

Steinberg, R. A. and P. Coffino. 1979. Two-dimensional gel analysis of cyclic AMP effects in cultured S49 mouse lymphoma cells: Protein modifications, inductions and repressions. Cell 18:719.

FIGURE LEGENDS

Figure 1. Immunofluorescent localization of desmin in myogenic cell cultures at various times after plating. Reprinted from Gard and Lazarides (1980), with permission.

Figure 2. Phosphorylation of desmin and vimentin in the total proteins of myogenic cell cultures and in the cytoskeletal residues prepared from similar cultures. The Coomasssie Blue-stained gel (A) and corresponding autoradiogram of the total proteins of myogenic cell cultures after labeling with ^{32}P-inorganic phosphate. The Coomassie Blue-stained gel (C) and corresponding autoradiogram (D) of the cytoskeletal proteins which remain insoluble after extraction of myogenic cells with 0.6 M KCl and 1% Triton X-100. The positions of α- and β-desmin (D), vimentin (V), and the isoactins (α, β and γ) are indicated. Stars denote degradation products of vimentin. Reprinted from O'Connor et al. (1979), with permission.

Figure 3. Tryptic peptide analysis of ^{32}P-desmin phosphorylated in vivo. (A) α-desmin; (B) α'-desmin. The positions of the origin (O), inorganic phosphate (P_i), and the major desmin phosphopeptides (A and B) are indicated.

Figure 4. Tryptic peptide analysis of ^{32}P-desmin phosphorylated in vivo, showing the minor phosphopeptides. The positions of the origin (O) and major (A and B) and minor (*) phosphopeptides are indicated.

Figure 5. Tryptic peptide analysis of ^{32}P-vimentin phosphorylated in vivo. The phosphopeptide marked by the star co-migrates with the major phosphopeptide A of similar desmin digests.

Figure 6. Phosphorylation of cytoskeletal residues by the catalytic subunit of the cAMP-dependent protein kinases. Autoradiograms depicting phosphorylation by 8 μg of the chicken skeletal muscle (A) or 5 μg of the bovine heart muscle (B) catalytic subunit. The positions of vimentin (V), tropomyosin (Tm) and α- and β-desmin (D) are indicated. The arrow points to a phosphorylated species presumed to represent a small fraction of the total actin. Reprinted from O'Connor et al. (1980).

Figure 7. Cyclic AMP dependence of cytoskeletal protein phosphorylation in vitro. Cytoskeletal proteins were incubated with 3 μg of the rabbit skeletal muscle catalytic subunit either alone (A) or together with 10 μg of rabbit skeletal regulatory subunit (B). In (C), the reaction was conducted as in (B), except that 1 μM cAMP was added to the reaction mixture. The autoradiogram depicted in (D) shows the cAMP-independent kinase activity detected when no exogenous kinase was incubated with the cytoskeletal proteins. The positions of desmin (D), vimentin (V) and tropomyosin (Tm) are indicated. The arrows point to several unidentified phosphoproteins whose phosphorylation is catalyzed by the cAMP-independent activity. Reprinted from O'Connor et al. (1980).

Figure 8. Tryptic peptide analysis of ^{32}P-desmin phosphorylated in vitro by the bovine heart catalytic subunit. The positions of the origin (O), inorganic phosphate (P_i) and the major peptides (A, B) of in vivo-labeled desmin are indicated. The stars indicate the positions of other major peptides characteristic of in vitro-labeled desmin.

Figure 9. Cell-free translation and post-translational modification of chicken smooth muscle proteins. The autoradiograms depict the ^{32}S-methionine labeled proteins translated from chicken gizzard mRNA in a rabbit reticulocyte cell-free

Figure 9 (continued)

system. After a 10-minute labeling period, an excess of nonradioactive methionine was added to each lysate, followed by the addition of buffer (A) or 20 units of the bovine heart muscle catalytic subunit (B). Incubation was continued for an additional 10 minutes. The positions of vimentin (V) and α- and β-desmin are indicated.

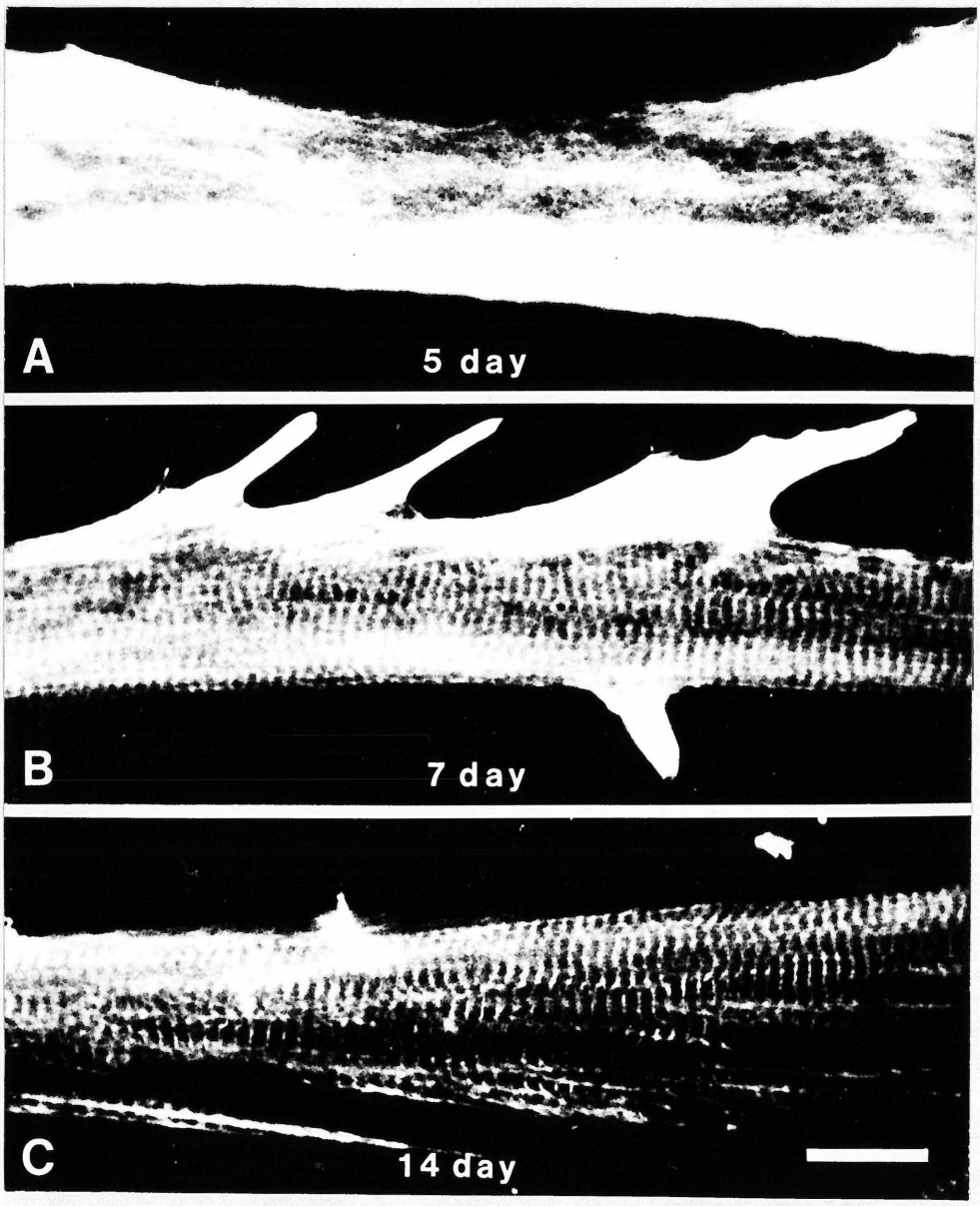
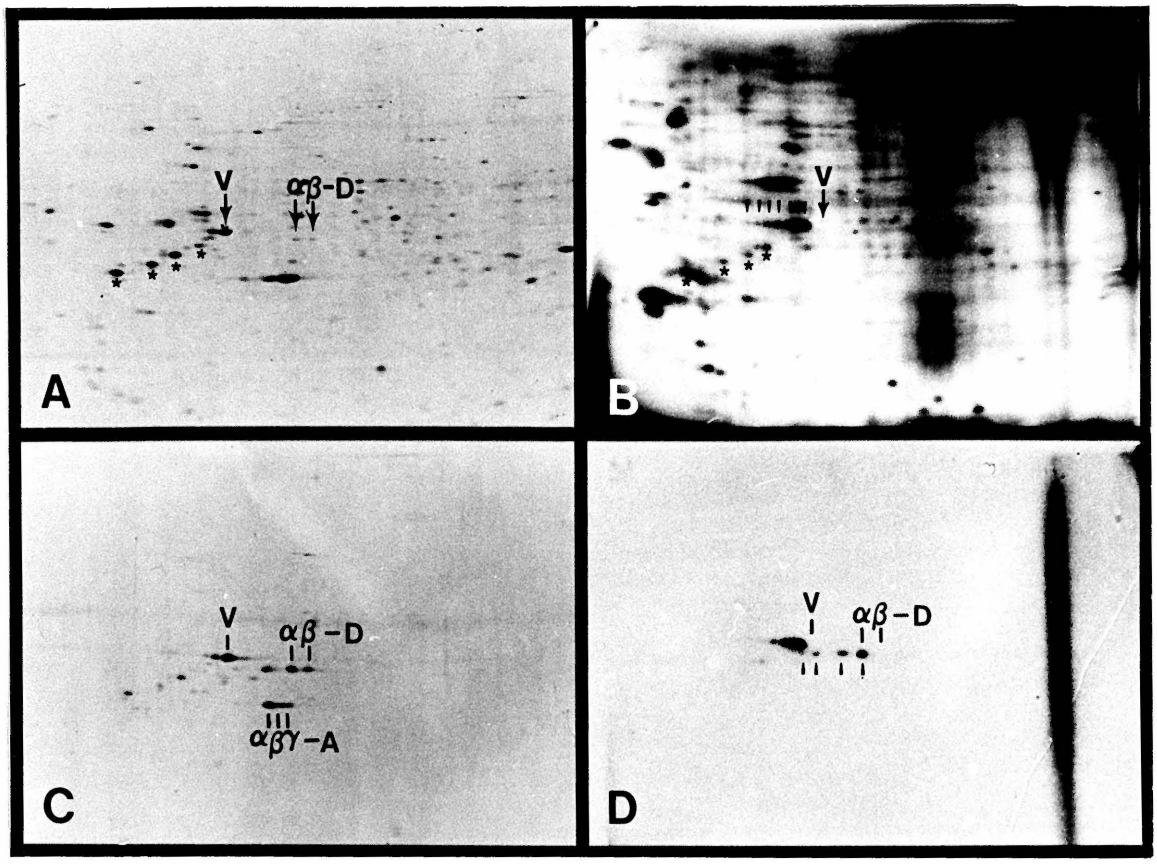


Figure 1



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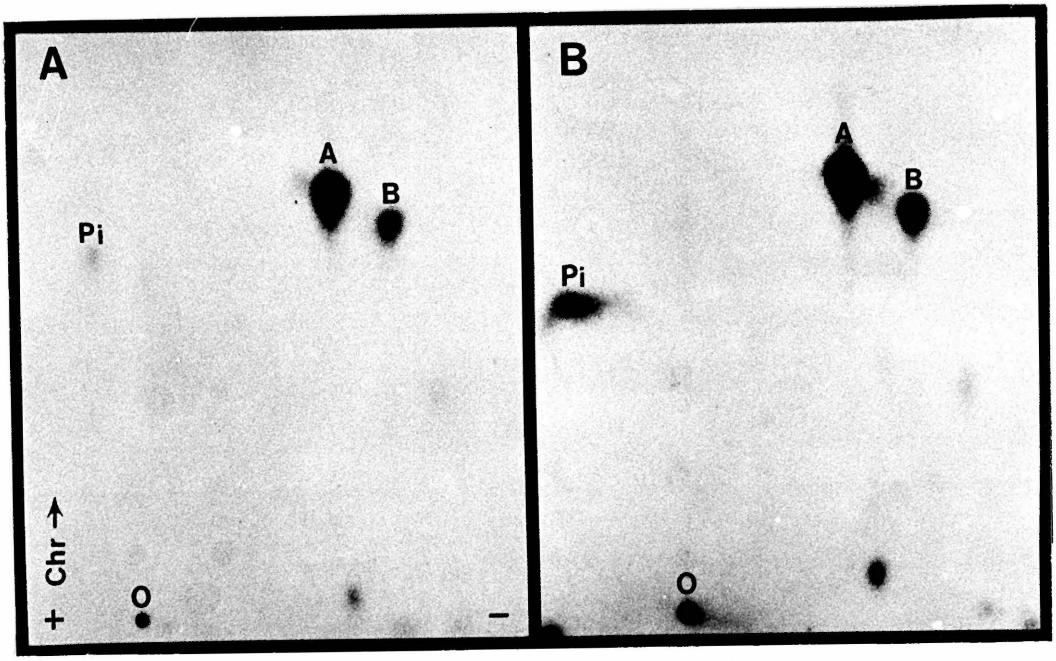

Figure 3

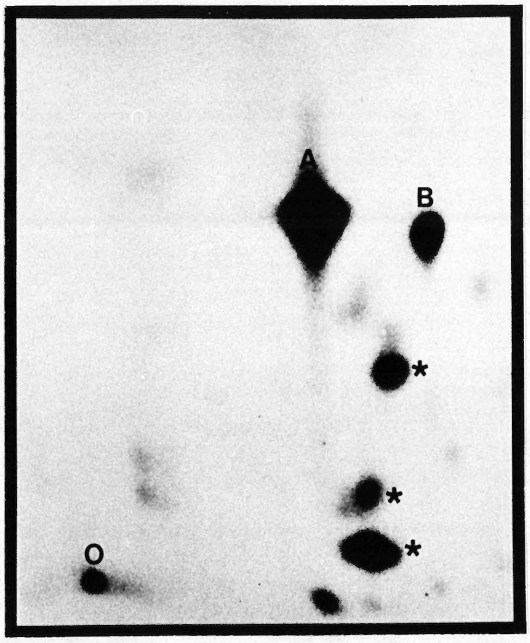

Figure 4

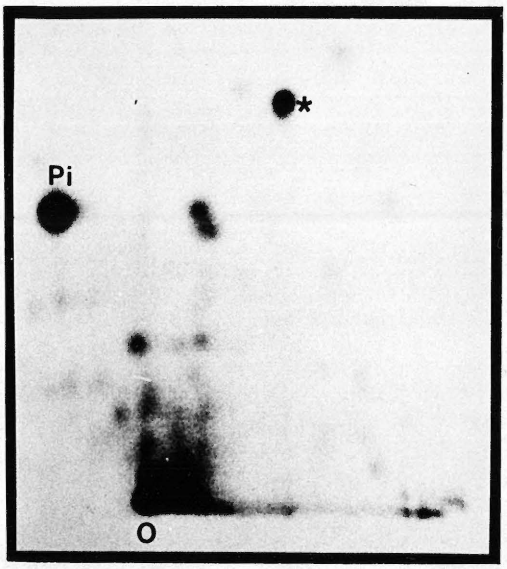

Figure 5

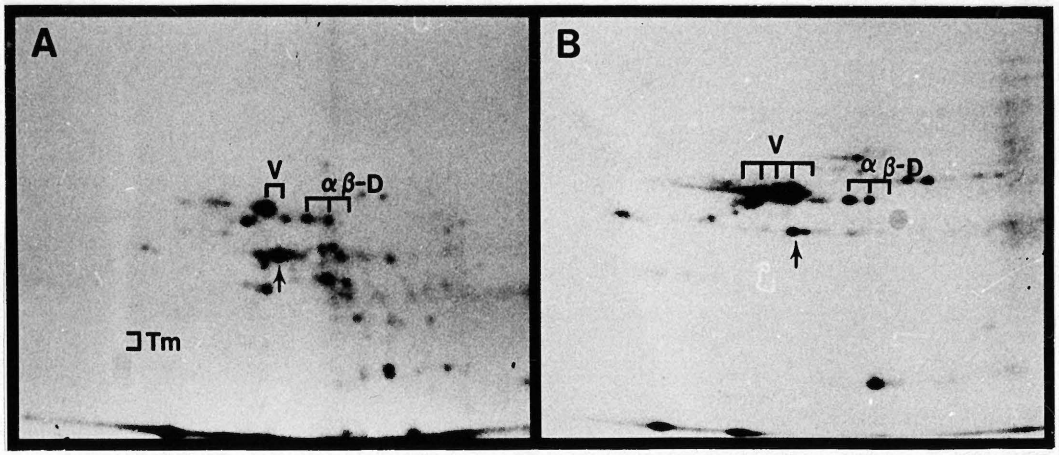

Figure 6

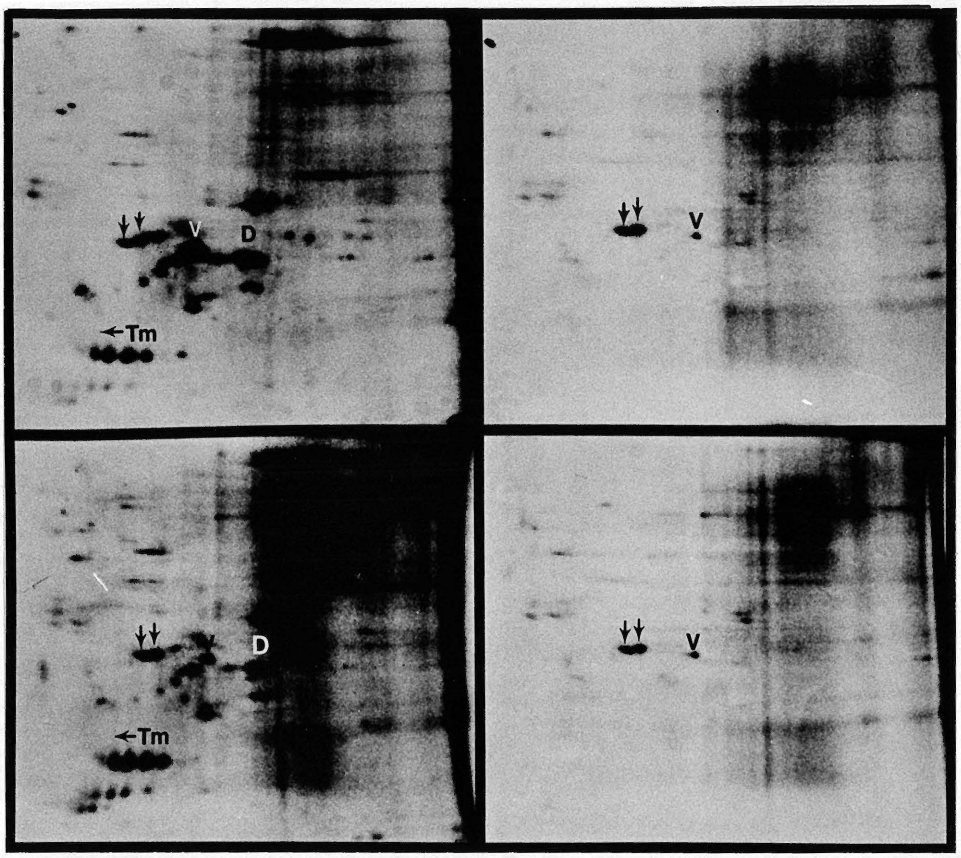

Figure 7

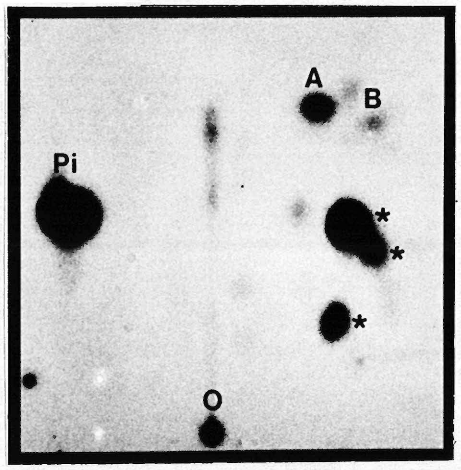

Figure 8

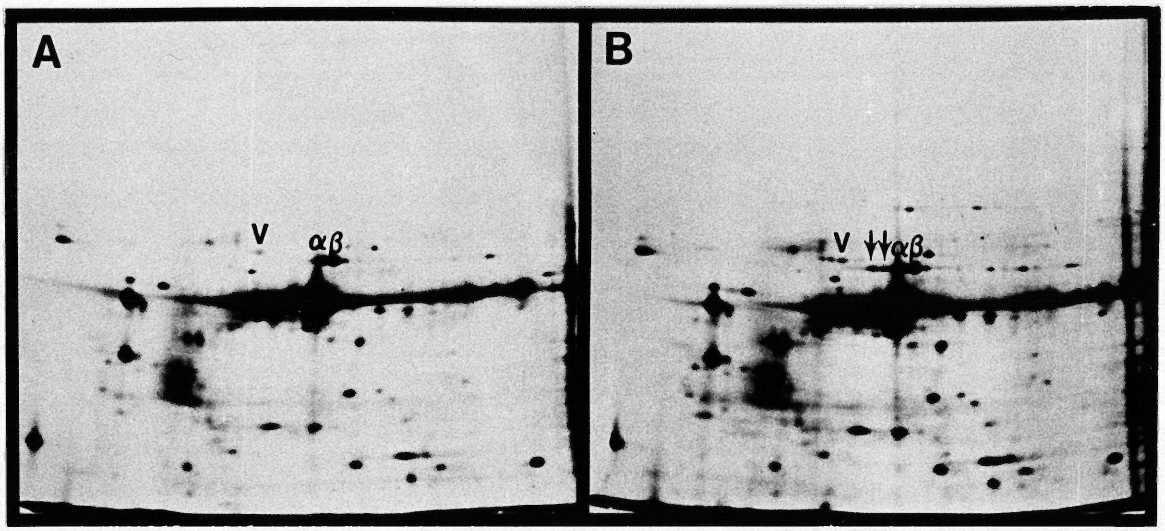

Figure 9

Chapter 6

Cyclic AMP-Modulated Phosphorylation of Intermediate Filament Proteins During Myogenesis In Vitro

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Summary

The intermediate filament proteins desmin and vimentin, and the muscle tropomyosins are the major protein phosphate acceptors in 8-day old myotubes incubated 4 h in medium containing radiolabeled phosphate. Addition of isoproterenol or 8-Bromo-cyclic AMP results in a two- to three-fold increase in incorporation of $^{32}PO_4$ into both desmin and vimentin, while no changes in incorporation into tropomyosin are observed. The 8-BrcAMP or hormonally induced increase in phosphate incorporation into desmin and vimentin is independent of cellular protein synthesis, and 8-BrcAMP does not stimulate phosphate turnover, suggesting that the observed increase in incorporation reflects an actual increase in intermediate filament protein phosphorylation. Though phosphorylation of desmin and vimentin is apparent in myogenic cells at all stages of differentiation, 8-BrcAMP or isoproterenol induced increases in phosphorylation of these proteins are restricted to mature myotubes. Non-myogenic chicken embryo fibroblasts show increased phosphorylation of vimentin when treated with isoproterenol or 8-BrcAMP; however, phosphorylation of desmin and vimentin in BHK-21 cells remains unchanged. Two-dimensional tryptic analysis of desmin phosphorylated in vivo reveals an increase in phosphorylation of at least one major peptide in 8-BrcAMP-treated 8-day myotubes, while no increase was observed in 3 d myotubes treated under identical conditions. Phosphopeptide maps from desmin labeled in vivo are nearly identical to those obtained from myotube desmin phosphorylated in vitro by the bovine heart cAMP-dependent protein kinases. These data strongly suggest that in vivo phosphorylation of the intermediate filament proteins desmin and vimentin is catalyzed by the cAMP-dependent protein kinases.

Introduction

Cytoplasmic filaments with diameters of 9 to 12 nm have been described in a wide variety of cell types from diverse sources. Known collectively as intermediate filaments, they comprise a heterogeneous class of filaments which, though similar in morphology, are composed of one or more biochemically distinct subunit proteins. It is now recognized that a cell may possess one or more subclasses of intermediate filaments whose expression is determined by the tissue of origin and state of differentiation (for review see Lazarides, 1980). Avian embryonic myotubes cultured in vitro possess two intermediate filament (IF) proteins (Fellini et al., 1978; Gard et al., 1979; Bennett et al., 1979; Gard and Lazarides, 1980): desmin, the IF protein first isolated from smooth muscle (Hubbard and Lazarides, 1979; Small and Sobieszek, 1977); and vimentin, which is commonly found in fibroblastic cells (Franke et al., 1978). In immature skeletal myotubes these two IF proteins coexist in an extensive network of cytoplasmic filaments (Bennett et al., 1979; Gard and Lazarides, 1980). Late in myogenesis, however, both filament proteins become associated with the Z lines of myofibril bundles (Gard and Lazarides, 1980), where they form a collar-like lattice surrounding and interlinking Z discs of adjacent myofibrils (Granger and Lazarides, 1979, 1980). The molecular regulatory mechanisms underlying this redistribution of intermediate filaments remain obscure. One common biochemical characteristic of all intermediate filaments is phosphorylation of their subunit proteins (for review see Lazarides, 1980). Protein phosphorylation (and dephosphorylation) is known to be an important regulatory event in many cellular metabolic processes (Krebs and Beavo, 1979), and it is not implausible that a similar mechanism may be involved in the regulation of cellular architecture.

We have previously reported that both desmin and vimentin are phosphorylated in avian embryonic skeletal myotubes cultured in vitro (O'Connor et al., 1979). Both proteins exhibit a single unphosphorylated species, plus several phosphorylated forms

which can be resolved on isoelectric focusing gels due to changes in isoelectric point. Additionally, we have identified protein kinases from chicken skeletal muscle capable of phosphorylating desmin and vimentin in vitro and have shown that they are the cAMP-dependent protein kinases (O'Connor, Gard and Lazarides, 1981; O'Connor et al., 1981). In this report we present evidence that desmin and vimentin are phosphorylated by cAMP-dependent protein kinases in vivo. Incorporation of radiolabeled phosphate into desmin and vimentin can be stimulated several-fold by treatment of 8-day myotubes with β-adrenergic hormones or 8-Bromo-cyclic AMP. This response is independent of protein synthesis, and is not due to increased phosphate turnover, suggesting that it represents an actual increase in protein phosphorylation catalyzed by the cAMP-dependent protein kinases. While phosphorylation of desmin and vimentin is observed throughout myogenesis in vitro, sensitivity to hormonal or cAMP stimulation of phosphorylation is absent or much reduced in myoblasts and immature myotubes. Two-dimensional tryptic analysis of desmin reveals several phosphopeptides, of which one shows increased incorporation of radiolabeled phosphate in 8-BrcAMP treated 8-day myotubes. Additionally, the phosphorylated peptides of desmin isolated from 8-BrcAMP treated myotubes correspond to those phosphorylated by cAMP-dependent protein kinase in vitro. These data suggest that phosphorylation of desmin and vimentin in cultured myotubes is catalyzed by the cAMP-dependent protein kinases, and that changes in intracellular cAMP levels due to hormonal stimulation, or during myogenesis may modulate intermediate filament phosphorylation and regulate intermediate filament function.

Results

Phosphorylation of Desmin and Vimentin in 8-day Myotubes

We have previously shown that the intermediate filament proteins desmin and vimentin are phosphorylated in avian embryonic myotubes cultured in vitro, and that both phosphorylated and nonphosphorylated species of these proteins are components of the Triton-

KCl-insoluble myotube cytoskeleton (O'Connor et al., 1979). We have examined the ^{32}P-phosphoproteins from 8-day myotubes by two-dimensional IEF/SDS-PAGE. The protein species most prominently labeled in myotubes of this age are the muscle tropomyosins, and two proteins we have previously identified as the intermediate filament proteins desmin and vimentin (Fig. 1B). Identification of desmin and vimentin was based upon their electrophoretic mobility, isoelectric point, and their enrichment in Triton-KCl cytoskeletons of myotubes (Gard et al., 1979). Several other less heavily labeled phosphoproteins are also observed, as well as a large amount of radioactive phosphate containing material which does not focus in the IEF gel, remaining near the basic end (top) of the gel. This material may represent ^{32}P-labelled polynucleotides as well as trapped or otherwise poorly-solubilized protein.

Direct comparison of the autoradiogram with the stained gel in Figures 1A and B reveals that (as previously reported) α-desmin is the most heavily labeled desmin species, with a lesser amount of $^{32}PO_4$ incorporation into the more acidic α'-desmin, and no label in β-desmin (O'Connor et al., 1979). Similarly, $^{32}PO_4$ incorporation is greatest into a single acidic variant of vimentin, with slight incorporation into a second, poorly resolved, more acidic variant, and no label in the major most basic vimentin species. These results have suggested that the acidic variants of desmin and vimentin are derived by post-translational phosphorylation of the respective more basic protein species (O'Connor, Gard and Lazarides, 1981).

β-Adrenergic Agonists Increase Incorporation of $^{32}PO_4$ into Intermediate Filament Proteins

Addition of 10^{-6} M isoproterenol during the last 45 min of the $^{32}PO_4$ incubation results in specific increases in the incorporation of $^{32}PO_4$ into IF proteins (figures 1C and D). While no changes in protein composition are observed in gels stained with Coomassie

brilliant blue (compare Figs. 1A, C), autoradiography reveals increased incorporation of $^{32}PO_4$ into both desmin and vimentin. In the case of desmin this is evident as an increase in the incorporation of $^{32}PO_4$ into the more acidic α'-desmin species, as well as into α-desmin. Vimentin shows an overall increase in labeling, as opposed to the specific increase in the more acidic variant as evident with desmin. Other phosphorylated proteins in this pH range, notably the muscle tropomyosins, appear unaffected by hormone treatment. Quantitation by liquid scintillation counting of excised protein spots from gels confirms this observation, allowing the use of the tropomyosins as a convenient internal standard for normalizing differences in gel loading within each experiment (see Table I, footnote 2). Incorporation of phosphate into total desmin or vimentin in cells treated with isoproterenol was increased approximately two-fold (Table I). The isoproterenol-induced increase in $^{32}PO_4$ incorporation into IF proteins is dose-dependent with a maximum response observed at 10^{-6} M isoproterenol (not shown). Similar increases are also observed with 10^{-6} M norepinephrine (see Table I).

8-BrcAMP Stimulates Phosphorylation of Intermediate Filament Proteins

β-adrenergic hormones such as isoproterenol elicit cellular responses through increases in the intracellular levels of cyclic adenosine 3',5' monophosphate (cAMP). We therefore investigated the effects of the cAMP derivative 8-Bromo-cyclic AMP (8-BrcAMP) and the phosphodiesterase inhibitor theophylline on phosphorylation of desmin and vimentin in 8-day myotubes. 8-BrcAMP treatment of 8-day myotubes resulted in a 3-fold increase in phosphorylation of desmin and vimentin (Fig. 2, Table I). This increase was similar to, but slightly greater in magnitude than, the increase observed in isoproterenol treated cells. The response to 8-BrcAMP was apparent as early as 15 min after addition, and peaked within 1 hr. The 8-BrcAMP stimulated increase in phosphorylation was dose-dependent, with maximum effect observed between 10^{-4} and 10^{-3} M. As seen with isoproterenol treatment, the increase in desmin phosphorylation

induced by 8-BrcAMP is characterized by increase in $^{32}PO_4$ incorporation into the acidic α'-desmin species (Fig. 2). In addition a third phosphorylated desmin variant, more acidic than α'-desmin, can be seen in myotubes treated with 8-BrcAMP (arrowhead in Fig. 2B, D). No changes in phosphorylation of tropomyosin are apparent. Theophylline alone (10^{-3} M) had no significant effect on IF protein phosphorylation (Fig. 2 and Table I). Simultaneous inclusion of theophylline and 8-BrcAMP did not enhance the effect observed wtih 8-BrcAMP alone (Figs. 2B, D), consistent with the reported resistance of 8-BrcAMP to cellular phosphodiesterases (Michal et al., 1970; Miller et al., 1973). It has previously been reported that the inhibition of protein synthesis by cycloheximide does not interfere with phosphorylation of desmin and vimentin (O'Connor et al., 1979). We find that the phosphorylation increases in these proteins induced by either isoproterenol or 8-BrcAMP are also independent of protein synthesis. Addition of cycloheximide 1 hr prior to the addition of isoproterenol or 8-BrcAMP plus theophylline had little effect on the ability of these agents to stimulate increased phosphorylation of desmin and vimentin (Table I).

When isoproterenol or 8-BrcAMP are administered together, at concentrations which yield maximal responses when added separately (10^{-6} M isoproterenol, 5×10^{-4} M 8-BrcAMP plus theophylline), no additive effect is detected, yielding a response equivalent to that observed with 8-BrcAMP plus theophylline alone (Table I). This suggests that β-adrenergic agonists and 8-BrcAMP act through similar molecular mechanisms, presumably via an increase in the effective intracellular cAMP level and subsequent activation of the cAMP dependent protein kinase.

Preparation of myotube cytoskeletons by extraction of 8-BrcAMP treated myotubes with 1% Triton X-100 and 0.6 M KCl reveals that the increased phosphorylation of desmin and vimentin evident in whole cell extracts is also reflected in the Triton insoluble cytoskeleton (not shown).

8-BrcGMP treated myotubes showed slight increases in IF protein phosphorylation, which are less than 25% of the response observed in parallel cultures treated with the identical concentration of 8-BrcAMP (not shown).

Several additional protocols for $^{32}PO_4$ labeling and 8-BrcAMP treatment were investigated. Simultaneous addition of 8-BrcAMP and $^{32}PO_4$ to myotubes for either 2 or 8 hr yielded no significant increases in IF protein phosphorylation over control cultures receiving $^{32}PO_4$ alone. Similarly, pretreatment of myotubes with 8-BrcAMP for 2 or 20 hr followed by a 2 hr incubation with $^{32}PO_4$ gave no increase in IF protein phosphorylation. Prelabeling of cells for 15-24 hr with $^{32}PO_4$ results in phosphorylation of desmin and vimentin to high specific activites, with a much higher incorporation of $^{32}PO_4$ into α'-desmin. Subsequent treatment with 5×10^{-4} M 8-BrcAMP plus theophylline yields no observable increase in $^{32}PO_4$ incorporation into these proteins.

Phosphorylation during Myogenesis

Incorporation of radioactive phosphate into both desmin and vimentin is observed during all stages of myogenesis in vitro. However, sensitivity of IF protein phosphorylation to the addition of hormones or cAMP analogues varies during differentiation. In secondary myogenic cultures 6 hr after plating, which consist predominantly of unfused replicating myoblasts, vimentin is the major intermediate filament protein while desmin is a minor protein species restricted to the few fusing myoblasts or young myotubes (Gard and Lazarides, 1980). Though $^{32}PO_4$ incorporation is greatest in vimentin (Fig. 3A,B), a $^{32}PO_4$ labeled species identifiable as α-desmin is observed (Fig. 3A,B, arrowheads). By 24 hr after plating phosphorylated desmin is prevalent, with both α- and α'-desmin identifiable. At later stages (4, 7 or 19 days) desmin represents one of the major cellular phosphoproteins (along with vimentin and tropomyosin). The observed increase in phosphorylated desmin presumably parallels the increase in synthesis of desmin and

its accumulation during myogenesis (Gard and Lazarides, 1980). Several other phosphoproteins are present in altered amounts during myogenesis. For example, ^{32}P-labeled muscle tropomyosin is a very minor component in 6 hr cultures, but increases substantially during myogenesis as the intracellular concentration of tropomyosin increases (Gard and Lazarides, 1980). Conversely, phosphorylation of another prominent cellular protein prevalent in early myoblasts is substantially reduced during the course of differentiation (Fig. 3, asterisks). A phosphoprotein with similar pI and electrophoretic mobility is also observed in non-muscle chick embryo fibroblast cultures and BHK-21 cells (see Fig. 4). The identity of this protein has not been determined.

No changes in phosphorylation of desmin and vimentin are apparent in autoradiograms from isoproterenol or 8-BrcAMP treated myoblasts (6 hr and 24 hr) or early myotubes (4d, Fig. 3E-G, isoproterenol result not shown). This is contrasted with the increased phosphorylation induced by either 8-BrcAMP or isoproterenol seen in 7-8 day old cultured myotubes (shown in Figs. 1 and 2 above) above) and in 19-day old cultured myotubes (Fig. 6H). These data suggest that the increase in IF protein phosphorylation stimulated by hormones or cAMP is restricted to mature myotubes (greater than 7 days in culture).

Phosphorylation in Nonmuscle Cells

Vimentin, the major intermediate filament protein in fibroblastic cells, is phosphorylated in chick embryo fibroblasts. Treatment of these cells with either isoproterenol (not shown) or 8-BrcAMP plus theophylline results in an approximate 2-fold increase in $^{32}PO_4$ incorporation into vimentin (Fig. 4). Cells of the mammalian fibroblastic cell line BHK-21 contain both vimentin and desmin, which are both phosphorylated in vivo (Gard et al., 1979; Steinert et al., 1981). Three phosphorylated isoelectric variants

of mammalian desmin, and one of vimentin are apparent in BHK cells (Fig. 4). Both proteins also exhibit a single, more basic non-phosphorylated species, analogous to that observed in avian muscle. In BHK cells, however, IF proteins surprisingly are not affected by either isoproterenol of 8-BrcAMP at concentrations which give maximal response in chick fibroblasts or myotubes (Fig. 4D).

Tryptic Analysis of Desmin Phosphopeptides

We have previously used two-dimensional tryptic peptide mapping to compare the phosphopeptides from desmin phosphorylated in vivo, or in vitro by bovine heart cAMP dependent protein kinase (O'Connor, Gard and Lazarides, 1981; O'Connor et al., 1981). A sizeable fraction of $^{32}PO_4$ in these earlier preparations migrated as inorganic phosphate, suggesting that some hydrolysis of phosphopeptides had occurred during sample preparation. The use of modified conditions for preparation and digestion of samples for peptide mapping has resulted in substantially less phosphate hydrolysis. Representative peptide maps of $^{32}PO_4$-desmin phosphorylated in vivo from 8-day and 3-day myotubes are shown in Figure 5. Both cultures were incubated with $^{32}PO_4$ for 4 hr, while 8-BrcAMP treated cells received 10^{-3} M 8-BrcAMP plus theophylline during the last hour of the $^{32}PO_4$ incubation.

α-Desmin obtained from $^{32}PO_4$ labeled control myotubes exhibits two or three major ^{32}P-peptides, and several minor species. The major peptide designated "A" in Figure 5 and the minor peptide designated peptide "B" correspond to those peptides previously identified in ^{32}P-desmin phosphorylated in vivo or in vitro (O'Connor, Gard and Lazarides, 1981). The ^{32}P-peptides of α'-desmin from 8d myotubes treated with 8-BrcAMP plus theophylline are qualitatively similar to those of control desmin. The most noticeable difference is a large increase in $^{32}PO_4$ incorporation into peptide labelled "C" (Fig. 5B). An increase in intensity of peptide "D" is also apparent, however

a concomitant decrease in another peptide (asterisk), which might be a partial digestion product is sufficient to account for this increase, but not that of peptide "C". Comparison of α- and α'-desmin from 8-BrcAMP treated cells reveal little qualitative differences; both α and α'-desmin exhibit increases in $^{32}PO_4$ incorporation into peptides "C". and "D". The phosphopeptide maps from 8-BrcAMP treated 8d myotubes are nearly identical to those obtained from myotube desmin phosphorylated in vitro by bovine heart cAMP-dependent protein kinase (O'Connor, Gard and Lazarides, 1981; also Fig. 5F). All major peptides present in vitro can also be identified in in vivo labelled desmin, varying only in intensity.

Phosphopeptide maps of α'-desmin from control or 8-BrcAMP treated 3-day myotube cultures appear qualitatively similar to those from 8d cultures. However, no increase in $^{32}PO_4$ incorporation into peptide "C" or any other peptide is observed in 8-BrcAMP treated 3-day myotubes, consistent with the lack of response of early myotubes to 8-BrcAMP or isoproterenol. As with 8d cells, the phosphopeptide maps of α- and α'-desmin from 3d myotubes appear similar (not shown).

We have not observed any consistent differences between ^{32}P-vimentin peptides from control or 8-BrcAMP treated cells.

Discussion

A growing body of evidence suggests that phosphorylation of intermediate filament proteins may be modulated by cAMP. Phosphorylation of vimentin in S49 cells is enhanced by treatment with β-adrenergic agonists or cAMP analogues, and is deficient in mutant cells lacking functional cAMP-dependent protein kinase (Steinberg et al., 1979). Similarly, C6 glioma-neuroblastoma hybrids show increased phosphorylaton of vimentin when treated with norepinephrine (Browning and Sanders, 1980). We have previously found

that both desmin and vimentin, the intermediate filament proteins found in avian skeletal myotubes, were phosphorylated by cAMP-dependent protein kinase in vitro (O'Connor et al., 1981). In this paper we present evidence that phosphorylation of desmin and vimentin in chicken embryonic skeletal myotubes is stimulated by β-adrenergic hormones or 8-BrcAMP, resulting in increased phosphorylation of a specific desmin tryptic peptides. In addition we report that the response of cultured myogenic cells to hormones or 8-BrcAMP is dependent upon the state of differentiation of these cells.

Desmin and vimentin are two of the major cellular phosphoproteins in 8-day cultures of chick skeletal myotubes. Both proteins exhibit multiple phosphorylated isoelectric variants, as well as a single, more basic species which does not incorporate radiolabeled phosphate either in vivo or in vitro (O'Connor et al., 1979; O'Connor, Gard and Lazarides, 1981; this report). In myotubes incubated for 4 hr in $^{32}PO_4$, α-desmin is the more heavily labeled desmin species, with a lesser amount of label incorporated into the more acidic α'-desmin species. Similarly, a single isoelectric variant of vimentin is labeled to a higher level than more acidic variants. Addition of either isoproterenol or 8-BrcAMP during the last 45 min of the $^{32}PO_4$ incubation results in a 2- to 3-fold stimulation of $^{32}PO_4$ incorporation into both desmin and vimentin. Though the magnitude of the increase in phosphorylation is not large, it is quite consistent, and shows dependence upon the dose of isoproterenol or 8-BrcAMP administered. In the case of desmin there is a relative increase in the proportion of $^{32}PO_4$ incorporated into the more acidic α'-desmin species. The phosphodiesterase inhibitor theophylline has little effect on phosphorylation of IF proteins, and does not significantly enhance the response to 8-BrcAMP, which has been shown to have a high resistance to hydrolysis by phosphodiesterase (Michal et al., 1970; Miller et al., 1973). However, theophylline does potentiate the response of myotubes to another cAMP analogue, dibutyryl cAMP (Gard and Lazarides, unpublished observations) which is more susceptible to enzymatic hydrolysis. Neither

IF protein phosphorylation, nor the phosphorylation increase induced by 8-BrcAMP or isoproterenol is sensitive to the protein synthesis inhibitor cycloheximide, indicating that cAMP induced synthesis of protein substrates and subsequent phosphorylation is not responsible for the observed increase in $^{32}PO_4$ incorporation. This conclusion is also supported by the rapidity of the cellular response to 8-BrcAMP; incorporation of $^{32}PO_4$ into desmin and vimentin is increased nearly 2-fold within 15 min. The possibility that 8-BrcAMP induces an increase in the turnover rate of protein phosphate by activation of a phosphoprotein phosphatase, yielding an apparent increase in IF protein phosphorylation, is rendered unlikely by the lack of increased $^{32}PO_4$ incorporation into desmin and vimentin in myotubes pretreated with 8-BrcAMP. In fact, the lack of increased incorporation (of phosphate into desmin and vimentin) when $^{32}PO_4$ and 8-BrcAMP are added simultaneously suggests that in these experiments maximal cAMP-induced phosphorylation occurs prior to significant incorporation of $^{32}PO_4$ into the intracellular ATP pool. From the above data we have concluded that β-adrenergic hormones, such as isoproterenol, and cAMP derivatives, such as 8-BrcAMP, stimulate actual increases in the phosphorylation of the intermediate filament proteins desmin and vimentin in 8-day myotubes. 8-BrcAMP has been shown to be a potent activator of the cAMP-dependent protein kinases (Miller et al., 1973), suggesting that these enzymes are responsible for the observed increase in phosphorylation. The lack of an additive response when isoproterenol and 8-BrcAMP are added (both at saturating concentrations) to the same culture supports the conclusion that these agents act through a common pathway, via an elevation in the effective intracellular levels of cAMP and subsequent activation of the cAMP-dependent protein kinases.

Under the conditions employed for these experiments no changes in protein phosphorylation aside from those of IF proteins were consistently observed in 8-BrcAMP or isoproterenol treated myotubes. Small changes in phosphate incorporation, changes

in minor protein components, or in components outside the pH range (4.3-6.2) of our gel system might have been missed. In contrast to other reports of cAMP-modulated phosphorylation of actin in S49 mouse lymphoma cells (Steinberg, 1980), we observed no $^{32}PO_4$ incorporation into any actin species.

Phosphorylation of both desmin and vimentin was apparent in all stages of myogenesis examined. However, the sensitivity of phosphorylation to stimulation by 8-BrcAMP or isoproterenol was observed only in mature myotubes (7-8 days after culture). The onset of sensitivity to isoproterenol and 8-BrcAMP appears to coincide chronologically with the redistribution of desmin filaments during myogenesis in vitro (Gard and Lazarides, 1980). The basis for this difference in sensitivity and its relationship to the redistribution of intermediate filaments during myogenesis are not known at this time. Both cAMP levels, and levels of active cAMP-dependent protein kinase have been shown to vary during early myogenesis (Zalin and Montague, 1974). Possibly, in early myoblast or myotube cultures, higher endogenous cAMP levels may result in maximal phosphorylation. Addition of exogenous cAMP could not then stimulate $^{32}PO_4$ incorporation into IF proteins over that due to turnover of previously bound phosphate. It is interesting that the two nonmuscle cell types examined respond differently to 8-BrcAMP and isoproterenol. Increases in phosphorylation of vimentin are observed in CEF cells treated with 8-BrcAMP or isoproterenol, but no increases are seen in similarly treated BHK cells, which contain both desmin and vimentin. This also might reflect differences in endogenous intracellular cAMP levels.

We have previously reported the presence of two major phosphopeptides (corresponding to peptides "A" and "B") in desmin radiolabelled with $^{32}PO_4$ in vivo (O'Connor, Gard and Lazarides, 1981). In this report, modifications to our labelling and tryptic mapping procedures have allowed the identification of an additional peptide (C), whose phosphorylation is modulated by cAMP. In 8-BrcAMP treated 8-day myotubes incorporation

of $^{32}PO_4$ into peptide "C" is substantially increased. The magnitude of this increase appears sufficient to account for the observed increase in desmin phosphorylation stimulated by 8-BrcAMP. No cAMP-induced stimulation of peptide phosphorylation is observed in 3-day myotubes, consistent with the lack of cAMP-stimulated increases in IF protein phosphorylation during ealry myogenesis. The ^{32}P-phosphopeptide maps of desmin from 8-BrcAMP treated or control 8-day myotubes are qualitatively similar to maps of desmin phosphorylated in vitro by the bovine heart cAMP-dependent protein kinase. Though phosphorylation by another kinase with identical specificity cannot be ruled out, this evidence suggests that most desmin phosphopeptides are the result of cAMP-dependent protein kinase catalyzed phosphorylation of desmin. The slight stimulation of IF protein phosphorylation by 8-BrcGMP may indicate additional phosphorylation of these proteins by the cGMP-dependent protein kinase. Alternatively, 8-BrcGMP has been shown to activate cAMP-dependent protein kinases in vitro, and the concentrations of 8-BrcGMP used in our experiments might have resulted in such activation. We have not observed any consistent differences in the vimentin phosphopeptide maps from control or 8-BrcAMP treated myotubes, indicating that the observed increase in $^{32}PO_4$ incorporation into vimentin in treated cells may result from increases in all major phosphopeptides.

The function of intermediate filament phosphorylation has not been conclusively established. No differences in the polymerization properties of BHK cell IF proteins in the phosphorylated or unphosphorylated state have been detected (Steinert et al., 1981). Similarly, phosphorylation has no observable effect on the assembly of desmin and vimentin into Triton-KCl insoluble myotube cytoskeletons (O'Connor et al., 1979); the 8-BrcAMP-stimulated increase in myotube IF protein phosphorylation observed in whole cell extracts is also seen in analyses of Triton cytoskeletons. Preliminary

immunofluorescence evidence suggests that treatment of myotubes with 8-BrcAMP can inhibit the transition from filamentous to Z-line associated IF proteins which occurs in the later stages of myogenesis. It is tempting to speculate that this inhbition is related to the observed increases in IF protein phosphorylation stimulated by 8-BrcAMP.

In summary, we have reported that β-adrenergic agonists such as isoproterenol and cAMP analogues stimulate increases in the phosphorylation of the intermediate filament proteins desmin and vimentin in 8-day myotubes cultured in vitro. Myoblasts show no changes in phosphorylation when exposed to either agonists or cAMP analogues. We have identified specific desmin phosphopeptides whose phosphorylation is increased in 8-BrcAMP treated 8-day myotubes. These data, in conjunction with our previous studies of cAMP-dependent phosphorylation of desmin and vimentin in vitro, suggest that the intermediate filament proteins desmin and vimentin are phosphorylated by the cAMP-dependent protein kinase in vivo, and that changes in intracellular cAMP levels during differentation may modulate IF protein phosphorylation and thereby regulate intermediate filament function.

Experimental Procedures

Cell Cultures

Cultures of avian embryonic myogenic cells were prepared from 10-day chick embryos as previously described (Gard and Lazarides, 1980). Cultures used for experiments within 24 hr of plating were seeded at 5-7 x 10^5 cells per 60 mm collagen coated Petri plate. Other myogenic cultures were seeded at 2-3 x 10^5 cells per 60 mm collagen-coated plate, except as noted. Myotube cultures were treated with 10 or 20 μM cytosine arabinofuranoside on days 4-7 to prevent overgrowth by residual fibroblastic cells. Greater than 75% of cell nuclei were present in multinucleate myotubes in cultures used for these experiments.

Chicken embryo fibroblastic (CEF) cells were obtained as a byproduct of the preparation of myogenic cultures and were subcultured 2-3 times on noncollagenized petri plates to eliminate residual myogenic cells (Gard et al., 1979). Passage 54 BHK-21 cells were obtained from the American Type Tissue Culture Collection and were maintained in Dulbecco's Minimum Essential Medium (GIBCO) supplemented with 10% calf serum. BHK and CEF cultures were used for $^{32}PO_4$-labeling experiments 3-5 days after subculturing, 1-2 days prior to the attainment of a confluent monolayer.

$^{32}PO_4$ Incubations

Duplicate cultures in 60 mm Petri plates were washed twice with 3 ml of phosphate-free MEM, and incubated a total of 4 hr in phosphate-free MEM supplemented with 1% horse serum containing 50 μCi/ml $^{32}PO_4$ (orthophosphate in 0.1 N HCl, New England Nuclear). Hormones or cAMP analogues were added to the indicated final concentrations for 45 min (or indicated time) prior to the end of the 4 hr $^{32}PO_4$ incubation. (±)-Isoproterenol and theophylline were obtained from Sigma. 8-BrcAMP and 8-BrcGMP (sodium salts) were from Boehringer-Mannheim and Sigma. Norepinephrine (DL-Arterenol) was obtained from Calbiochem. Labeled cultures were washed once with cold phosphate-buffered saline (.14 M NaCl, 3 mM KCl, 10 mM NaK phosphate pH 7.2, PBS), scraped from Petri dishes with a rubber policeman, and pelleted at top speed in an IEC tabletop centrifuge (approx. 1000 x g). Cell pellets were then solubilized for 3-5 min at 100°C in 75 μl of sample buffer containing 0.2% SDS, 50 mM Tris HCl pH 6.8, 1% 2-mercaptoethanol, and 15% glycerol. Samples were then prepared immediately for electrophoresis, or frozen at -20°C for later use.

Two Dimensional Electrophoresis

In using the O'Farrell two-dimensional isoelectric-focusing-SDS-polyacrylamide gel electrophoresis (IEF/SDS-PAGE) technique (O'Farrell, 1975) we have found that

vimentin is particularly sensitive to degradation by endogenous proteases when in urea extracts. This proteolysis was resistant to most common protease inhibitors or divalent cation chelating agents, but could be prevented by solubilization in buffers containing 0.2% SDS for 2-5 min at 100°C. Hydrolysis of protein phosphate by phosphatese activity is also minimized by this procedure. These SDS whole cell extracts were then cooled on ice and mixed with equal volumes of saturated urea solution. Duplicate 10 µl aliquots were taken to determine TCA precipitable radioactivity.

1-2 x 10^5 CPM of each sample, approximately 100-150 µl, were electrophoresed on 10 cm O'Farrellisoelectric focusing gels modified by the omission of any nonionic detergent. The second SDS-PAGE dimension was performed as previously described (Hubbard and Lazarides, 1979). Gels were stained, destained overnight, dried and autoradiographed for the indicated times using Kodak XR-A film and duPont Cronex Lightning Plus Intensifying Screens. Though overall focusing of proteins from SDS myotube extracts was good (see Fig. 1), some differences were noted in Coomassie-stained IEF/SDS-PAGe gels of SDS-solubilized samples. Most obvious is the appearance of a prominent 52,000 dalton protein (slightly more basic than vimentin) which is not seen in urea NP-40 extracts of myotubes (inset, Fig. 1c). Whether this species represents an artifactual modification of vimentin, or a physiologically relevant protein has not been determined at this time. Additionally, the tubulin spots are much less conspicuous in SDS extracts, though the basis for this difference is not known. Note, however, that the proteolytic degradation of vimentin is substantially reduced in the SDS extract, allowing a more accurate assessment of changes in phosphorylation of this protein.

For quantitation of $^{32}PO_4$ incorporation into specific proteins, individual gel slices were cut out, digested with NCS tissue solubilizer (Amersham) according to manufacturer's instructions and counted in Aquasol II (New England Nuclear).

Two-dimensional Tryptic Mapping

Two-dimensional tryptic mapping of $^{32}PO_4$-labeled desmin and vimentin was performed essentially as described previously (O'Connor et al., 1981a). 3 or 8-day myotubes (10^6 cells per 100 mm plate) were labeled as above for 4 hr with 150 μCi $^{32}PO_4$ per ml. 8-BrcAMP plus theophylline were added during the last hour of the labeling period. Gels were stained, destained overnight, and the appropriate proteins excised. Gel slices were washed 12-24 hr in 3 x 500 ml changes of 10% ethanol, and 2 hr in 95% ethanol, followed by vacuum drying. Dried gel slices were rehydrated in 200 mM ammonium bicarbonate containing 0.2 mg/ml trypsin TPCK (Worthington). Approximately 10 μl of trypsin in bicarbonate buffer was added per 1 mm^3 dried gel slice. Digestion was carried out for 12 hr at 37°C, followed by addition of an equal aliquot of trypsin in 1 ml of bicarbonate buffer. Digestion and peptide elution were continued at 37°C for 24 hr, followed by lyophilization of the supernatant. Samples were spotted onto cellulose thin layer plates (Eastman 13255), and electrophoresed at 800 V for 45 min in acetic acid: formic acid:water (11.4:10:379), pH 1.9. After drying for 2 hr, plates were chromatographed in butanol:acetic acid:pyridine:water (60:12:40:48) for the second dimension. The locations of phosphopeptides were determined by autoradiography.

Preparation of desmin labeled in vitro by bovine heart cAMP-dependent protein kinase (Sigma) is described by O'Connor, Gard and Lazarides (1981).

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REFERENCES

Bennett, G. S., Fellini, S. A., Toyama, Y., and Holtzer, H. (1979). Redistribution of intermediate filament subunits during skeletal myogenesis and maturation in vitro. J. Cell Biol 82, 577-584.

Browning, E. T., and Sanders, M. M. (1980). Identification of an intermediate filament protein (vimentin) as a norepinephrine dependent phosphoprotein in C-6 glioma cells. J. Cell Biol. 87, 179a.

Cabral, F., and Gottesman, M. M. (1979). Phosphorylation of the 10 nm filament protein from Chinese hamster ovary cells. J. Biol. Chem. 254, 6203-6206.

Evans, R. M., and Fink, L. M. (1981). An alteration in the phosphorylation of vimentin-type intermediate filaments is associated with mitosis in cultured fibroblasts. Fed. Proc. 40, 1547.

Fellini, S. A., Bennett, G. S., Toyawa, Y., and Holtzer, H. (1978). Biochemical and immunological heterogeneity of 100 Åfilament subunits from different chick cell types. Differentiation 12, 59-69.

Franke, W. W., Schmid, E., Osborn, M., and Weber, K. (1978). Different intermediate sized filaments distinguished by immunofluorescence microscopy. Proc. Natl. Acad. Sci. U.S.A. 75, 5039-5038.

Gard, D. L., Bell, P. B., and Lazarides, E. (1979). Coexistence of desmin and fibroblastic intermediate filament subunit in muscle and non-muscle cells: identification and comparative peptide analysis. Proc. Natl. Acad. Sci. U.S.A. 76, 3894-3898.

Gard, D. L., and Lazarides, E. (1980). The synthesis and distribution of desmin and vimentin during myogenesis in vitro. Cell 19, 263-275.

Granger, B., and Lazarides, E. (1978). The existence of an insoluble Z disc scaffold in chicken skeletal muscle. Cell 15, 1253-1268.

Granger, B., and Lazarides, E. (1979). Desmin and vimentin coexist at the periphery of the myofibril Z disc. cell 18, 1053-1063.

Hubbard, B. D., and Lazarides, E. (1979). Copurification of actin and desmin from chicken smooth muscle and their copolymerization in vitro to intermediate filaments. J. Cell Biol. 80, 166-182.

Krebs, E. G., and Beavo, J. A. (1979). Phosphorylation-dephosphorylation of enzymes Ann. Rev. Biochem. 48, 923-959.

Lazarides, E. (1980). Intermediate filaments as mechanical integrators of cellular space. Nature 283, 249-256.

Michal, G., Nelbock, M., and Weiman, G. (1970). Cyclophosphate III. Spaltung verschiedener cyclophosphate durch phosphodiesterase aus Herz und Fettgewebe. Z. Anal. Chem. 252, 189.

Miller, J. P., Boswell, K. H., Muneyama, K., Simon, L. N., Robins, R. K., and Shuman, D. A. (1973). Synthesis and biochemical studies of various 8-substituted derivatives of guanosine 3',5'-cyclic phosphate, inosine 3',5'-cyclic phosphate, and xanthosine 3',5'-cyclic phosphate. Biochem. 12, 5310-5319.

O'Connor, C. M., Asai, D. J., Flytzanis, C. N., and Lazarides, E. (1981). In vitro translation of the intermediate filament proteins desmin and vimentin. Molecular and Cellular Biol. 1, 303-309.

O'Connor, C. M., Balzer, D. R., and Lazarides, E. (1979). Phosphorylation of the subunit proteins of intermediate filaments from chicken muscle and non-muscle cells. Proc. Natl. Acad. Sci. U.S.A. 76, 819-823.

O'Connor, C. M., Gard, D. L., Asai, D. J., and Lazarides, E. (1981). Phosphorylation of the intermediate filament proteins, desmin and vimentin, in muscle cells. In: Protein Phosphorylation, vol. 8, O. M. Rosen and E. G. Krebs, eds. (Cold Spring Harbor Conferences on Cell Proliferation).

O'Connor, C. M., Gard, D. L., and Lazarides, E. (1981). Phosphorylation of intermediate filament proteins by cAMP-dependent protein kinases. Cell 23, 135-143.

O'Farrell, P. H. (1975). High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. 250, 4007-4021.

Simantov, R., Shlolnik, T., and Sachs, L. (1980). Desensitization of enucleated cells to hormones and the role of cytoskeleton in control of normal hormone response. Proc. Natl. Acad. Sci. U.S.A. 77, 4798-4802.

Small, J. V. and Sobieszek, A. (1977). Studies on the function and composition of the 10 nm (100-Å) filaments of vertebrate smooth muscle. J. Cell Science 23, 243-268.

Steinberg, R. A. (1980). Actin nascent chains are substrates for cyclic AMP-dependent phosphorylation in vivo. Proc. Natl. Acad. Sci. U.S.A. 77, 910-914.

Steinberg, R. A., and Coffino, P. (1979). Two-dimensional gel analysis of cyclic AMP effects in cultured S49 mouse lymphoma cells: protein modifications, inductions and repressions. Cell 18, 719-733.

Steinert, P. M., Idler, W. W., Cabral, F., Gottesman, M. M., and Goldman, R. D. In vitro assembly of homopolymer and copolymer filaments from intermediate filament subunits of muscle and fibroblastic cells. Proc. Natl. Acad. Sci. U.S.A. 78, 3692-3696.

Steinert, P. M., Idler, W. W., and Wantz, M. L. (1981). The o-phosphoserine content of intermediate filament subunits. Fed. Proc. 40, 1547.

Zalin, R. J., and Montague, W. (1974). Changes in adenylate cyclase, cyclic AMP, and protein kinase levels in chick myoblasts, and their relation to differentiation. Cell 2, 103-108.

Table I: Response of 7-8 day myotubes to isoproterenol and 8-BrcAMP

Treatment[1]	Phosphorylation increase[2]		No. of Exp.
	Desmin[3]	Vimentin	
10^{-6} M isoproterenol	2.0±0.2	1.8±0.2	3
10^{-6} M norepinephrine	2.1	1.7	1
$5x10^{-4}$ M 8-BrcAMP plus $5x10^{-4}$ M theophylline	2.8±0.2	2.9±0.2	3
10^{-3} M theophylline alone	1.2±0.2	1.3±0.2	2
$5x10^{-4}$ M 8-BrcAMP plus theophylline plus 10^{-6} M isoproterenol	3.1	1.9	1
$5x10^{-4}$ M 8-BrcAMP plus theophylline (pre-treated with cycloheximide[4])	2.9±0.3	2.4±0.5	2
10^{-6} M isoproterenol (pre-treated with cycloheximide[4])	1.9	2.2	1

[1]Myotubes incubated 4 hrs in $^{32}PO_4$, indicated agent(s) added during last 45 min of labeling period.

[2]From eight independent experiments, $^{32}PO_4$ incorporation into tropomyosin represents 0.2% ± 0.09 of total TCA precipitated $^{32}PO_4$ in control myotubes and 0.23% ± 0.09 of total TCA precipitated $^{32}PO_4$ in isoproterenol-treated myotubes. The increase in IF protein phosphorylation was calculated using tropomyosin as an internal standard to normalize gel loading:

$$[(^{32}\text{P-Des})_{exp.}/(^{32}\text{P-TM})_{exp.}] \div (^{32}\text{P-Des})_{control}/(^{32}\text{P-Tm})_{control}]$$

± standard deviation when applicable.

[3]Total desmin (α'- and α-desmin)

[4]Cells pretreated with 100 μg/ml cycloheximide 1 hr before addition of hormone or 8-BrcAMP, sufficient to inhibit greater than 95% of protein synthesis within 30 min.

FIGURE LEGENDS

Figure 1. Two dimensional IEF/SDS-PAGE analysis of phosphorylation of IF proteins in 8-day myotubes.

A,B: Stained gel and corresponding autoradiogram (12 hr exposure) from eight day myotubes labelled with $^{32}PO_4$ (see Methods). Isoelectric focusing is from right to left (basic to acid) in all gels presented. The approximate pH range is shown in (A). SDS-PAGE is from top to bottom. The positions of desmins (α'-, α- and β-D), vimentins (V, arrowhead), tropomyosins (Tm), actins (Ac) and tubulins are shown. The asterisks denote an unidentified protein seen in SDS extracts, but not in urea NP-40 extracts of myotubes (see Methods). The autoradiograph (B) reveals that tropomyosins (Tm), α-desmin, and an acidic vimentin species (arrow) are the major ^{32}P-phosphate acceptors. No $^{32}PO_4$ incorporation into β-desmin or the basic vimentin variant (V) is noted.

C,D: Shows the stained gel and the corresponding autoradiogram (12 hr exposure) of 8-day myotubes labelled with $^{32}PO_4$ and treated 45 min with 10^{-6} M dl-isoproterenol (see Methods). No differences in protein composition are apparent in stained gels of control or isoproterenol-treated myotubes (compare A and C). Autoradiography reveals that $^{32}PO_4$ incorporation into vimentin (V) and desmins (α-, α'-desmin) is significantly increased. The inset in (C) shows a portion of a standard IEF/SDS-PAGE gel of a urea extract of 10-day myotubes for comparison with 1A and C, showing the prominent degradation products (stars) of vimentin (V) (see Methods). The positions of actin (A), desmins (D) and tubulins (Tb) are also shown.

Figure 2. 8-BrcAMP stimulates phosphorylation of desmin and vimentin in 7-day myotubes. Seven-day myotubes were labelled for 4 hr with $^{32}PO_4$, and treated for 45 min with

either 10^{-3} M theophylline, 10^{-3} M 8-BrcAMP, or 10^{-3} M 8-BrcAMP plus 10^{-3} M theophylline.

A control, 4 hr $^{32}PO_4$

B 1 mM 8-BrcAMP

C 1 mM theophylline

D 1 mM 8-BrcAMP plus theophylline

1 mM 8'-Bromo cAMP (b) stimulates phosphorylation of desmin (α-, α'-) and vimentin (V) but not tropomyosin (Tm), which remains at its basal level of phosphorylation. 1 mM theophylline (C) has little effect on phosphorylation, and does not enhance the response to 8-BrcAMP (compare C and D). A third, more acidic phosphorylated species of desmin (arrow) is apparent in 8-BrcAMP-treated cells. (Autoradiographs exposed for 18 hrs.)

Figure 3. Phosphorylation during myogenesis.

Myogenic cultures were labelled for 4 hr in $^{32}PO_4$. 8-BrcAMP and theophylline were added during the last 45 min of the labelling period, to final concentrations of 10^{-3}M in E, F, G and 5×10^{-4} M in H.

A,E Control and 8-BrcAMP-treated 6 hr myoblasts

B,F Control and 8-BrcAMP-treated 24 hr myoblast/myotube

C,G Control and 8-BrcAMP-treated 4-d myotubes

D,H Control and 8-BrcAMP-treated 19-d myotubes

Phosphorylation of desmin (α) and vimentin (V) is apparent in control cultures at all times examined. In 6 hr myoblasts (A) desmin (arrowhead) is a very minor component (see text), however, the amount of phosphorylated desmin and muscle tropomyosins (Tm) increase dramatically during myogenesis. A phosphoprotein prominent in early myogenic cultures (asterisks) is lost in mature myotubes (19-d; see text). 8-BrcAMP

(E-H) stimulated phosphorylation increases are restricted to mature myotubes. No response is observed in myoblasts (E,F) or 4-d myotubes (G), but is found in myotubes older than 7-d (previous figures) and continues through at least 19-d in culture (H). (A, B, E, F autoradiographs exposed 48 hrs; C, D, G, H exposed 24 hrs.)

Figure 4. Phosphorylation in non-muscle cells.

A,B. Control and 8-BrcAMP-treated chick embryo fibroblasts (fourth passage).

C,D. Control and 8-BrcAMP-treated BHK-21 cells (passage 69)

Phosphorylation of vimentin (V) in CEF cells is enhanced by treatment with $5x10^{-4}$ M 8-BrcAMP (B) or isoproterenol (not shown). No changes in phosphorylation of desmin (arrows, D) or vimentin (V) are observed in BHK cells treated with 8-BrcAMP. The asterisks denote a phosphoprotein similar to one found in early myogenic cultures (Fig. 3), but not mature myotubes (see text). (Autoradiographs exposed 24 hrs.)

Figure 5. Two-dimensional tryptic maps of ^{32}P-desmin from 8-day and 3-day myotubes. Myotubes were labelled with $^{32}PO_4$ for 4 hr. 10^{-3} M 8-BrcAMP plus theophylline was added to the indicated cultures for the last hour of the labelling period. Proteins were isolated by IEF/SDS-PAGE and peptide mapped as described in Methods. Peptides A-D are labeled, as is the origin (O). The directions of electrophoresis and chromatography are indicated in A. The prominent peptide denoted by an asterisk may represent a partial proteolysis product (see text).

A α'-desmin from control 8-day myotubes

B α'-desmin from 8-day myotubes treated with 8-BrcAMP plus theophylline

C α-desmin from 8-BrcAMP-treated 8-day myotubes

D α'-desmin from control 3-day myotubes

E α'-desmin from 3-day myotubes treated with 8-BrcAMP plus theophylline

F Total myotube desmin phosphorylated in vitro by bovine heart cAMP-dependent protein kinase (O'Connor et al., 1981, see text).

Results from one representative experiment are shown. Incorporation of $^{32}PO_4$ into specific phosphopeptide from 8-d myotubes was determined by scintillation counting, and is shown below(in CPM minus background):

Phosphopeptide	α'-Desmin from 8d control myotubes	α'-Desmin from 8d 8-BrcAMP myotubes	α-Desmin from 8d 8-BrcAMP myotubes
A	38 (1.0)[+]	28 (1.0)	37 (1.0)
B	4 (0.1)	4 (0.1)	5 (0.1)
C	52 (1.4)	209 (7.5)	200 (5.4)
D	14 (0.4)	41 (1.5)	21 (0.6)
*	27 (0.7)	7 (0.3)	7 (0.2)
Total	135 (3.6)	289 (10.4)	270 (7.3)

[+]The number in parenthesis shows the relative incorporation with peptide A normalized to 1.0.

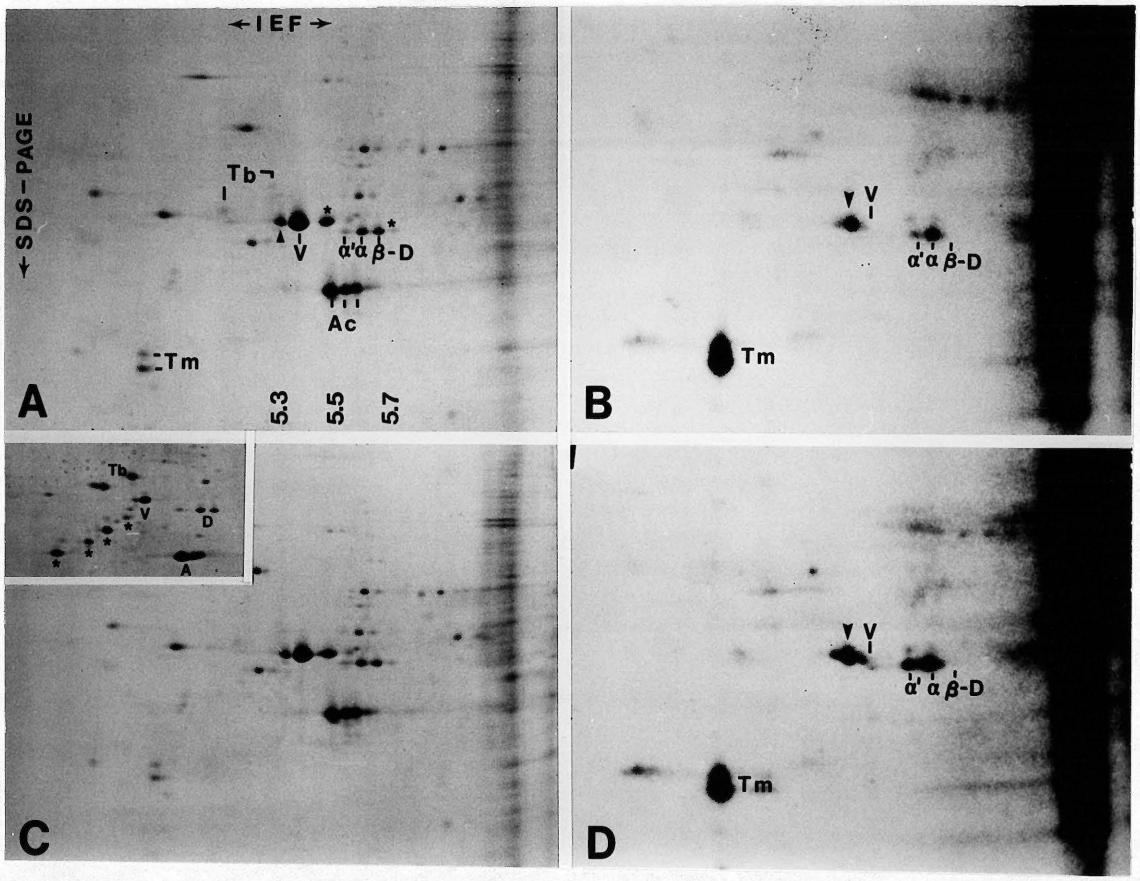

Figure 1

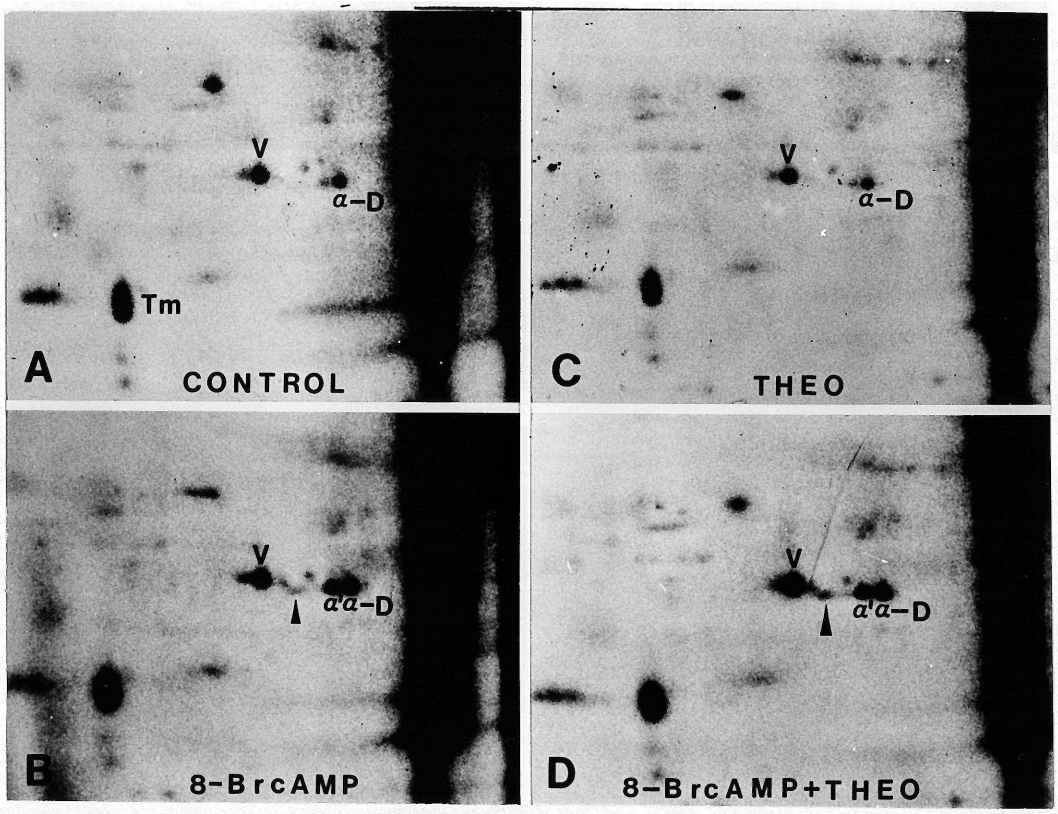

Figure 2

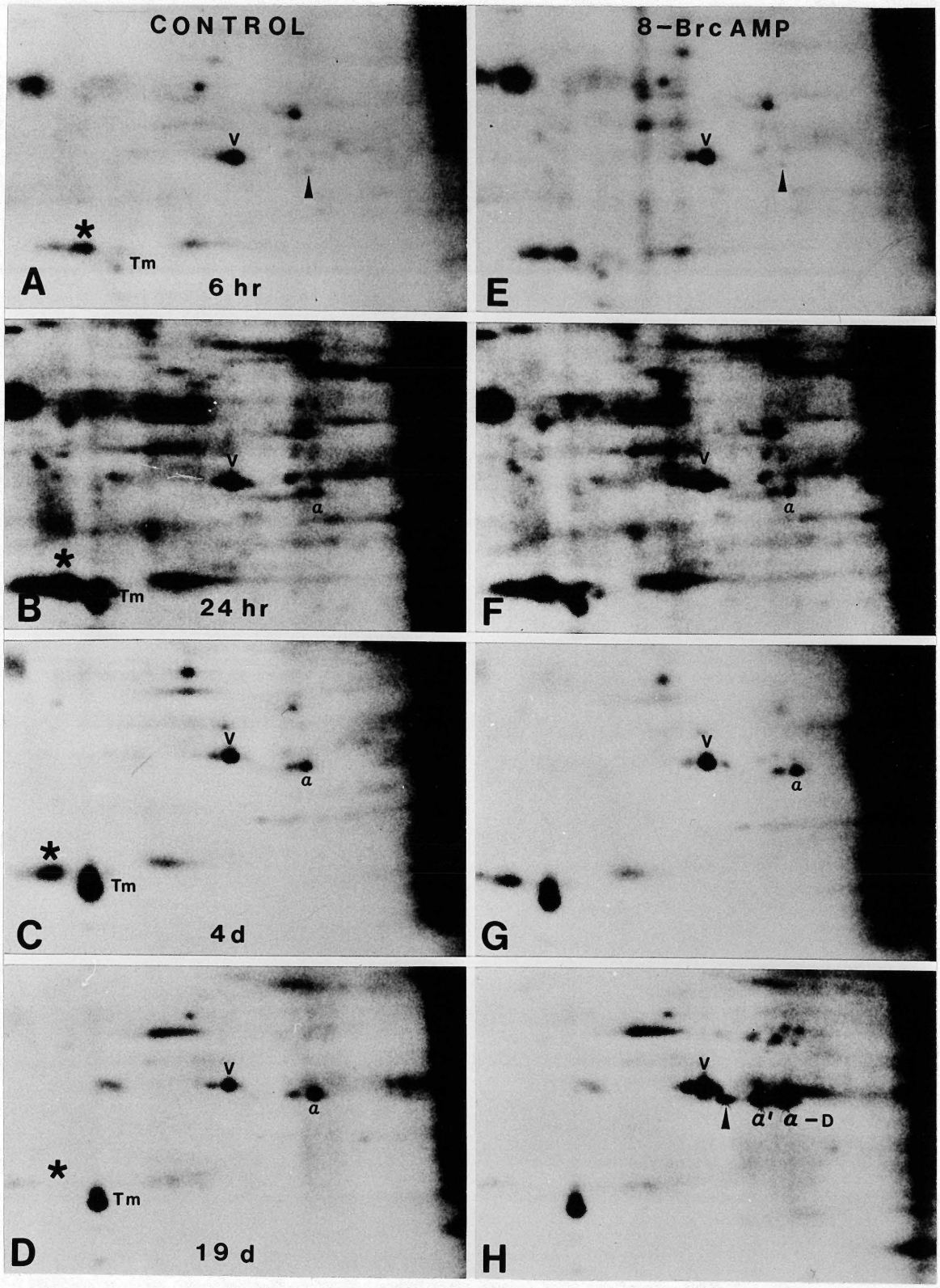

Figure 3

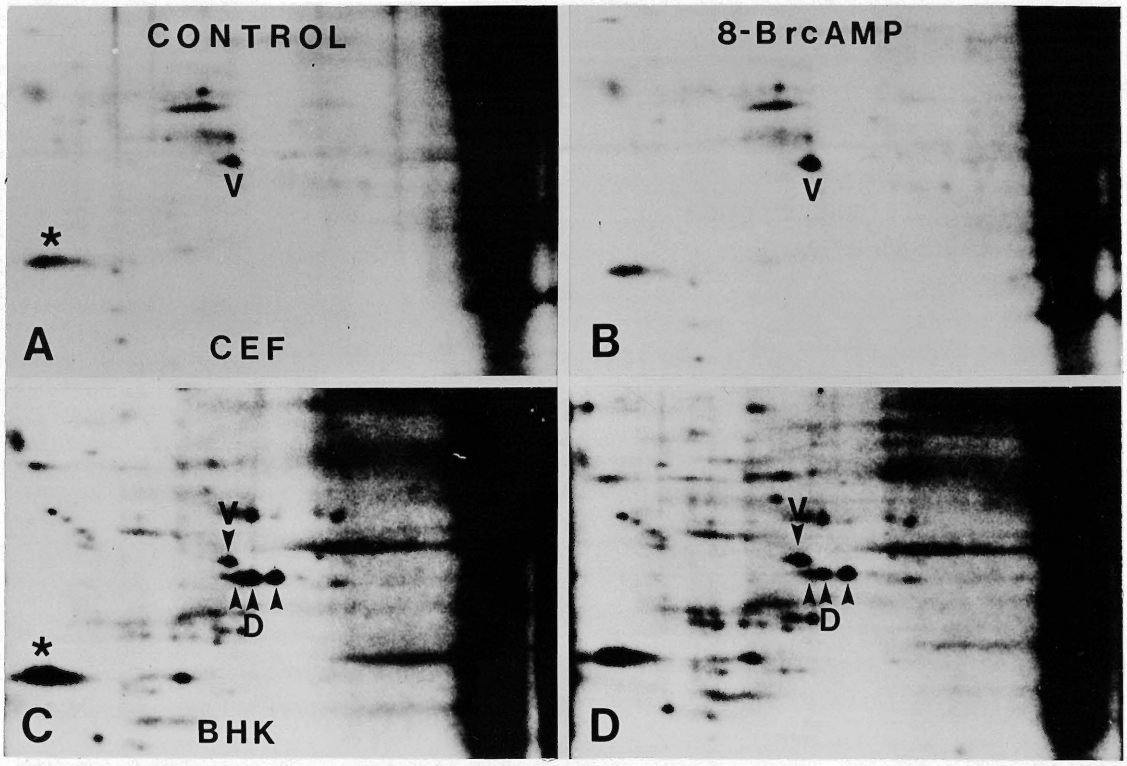

Figure 4

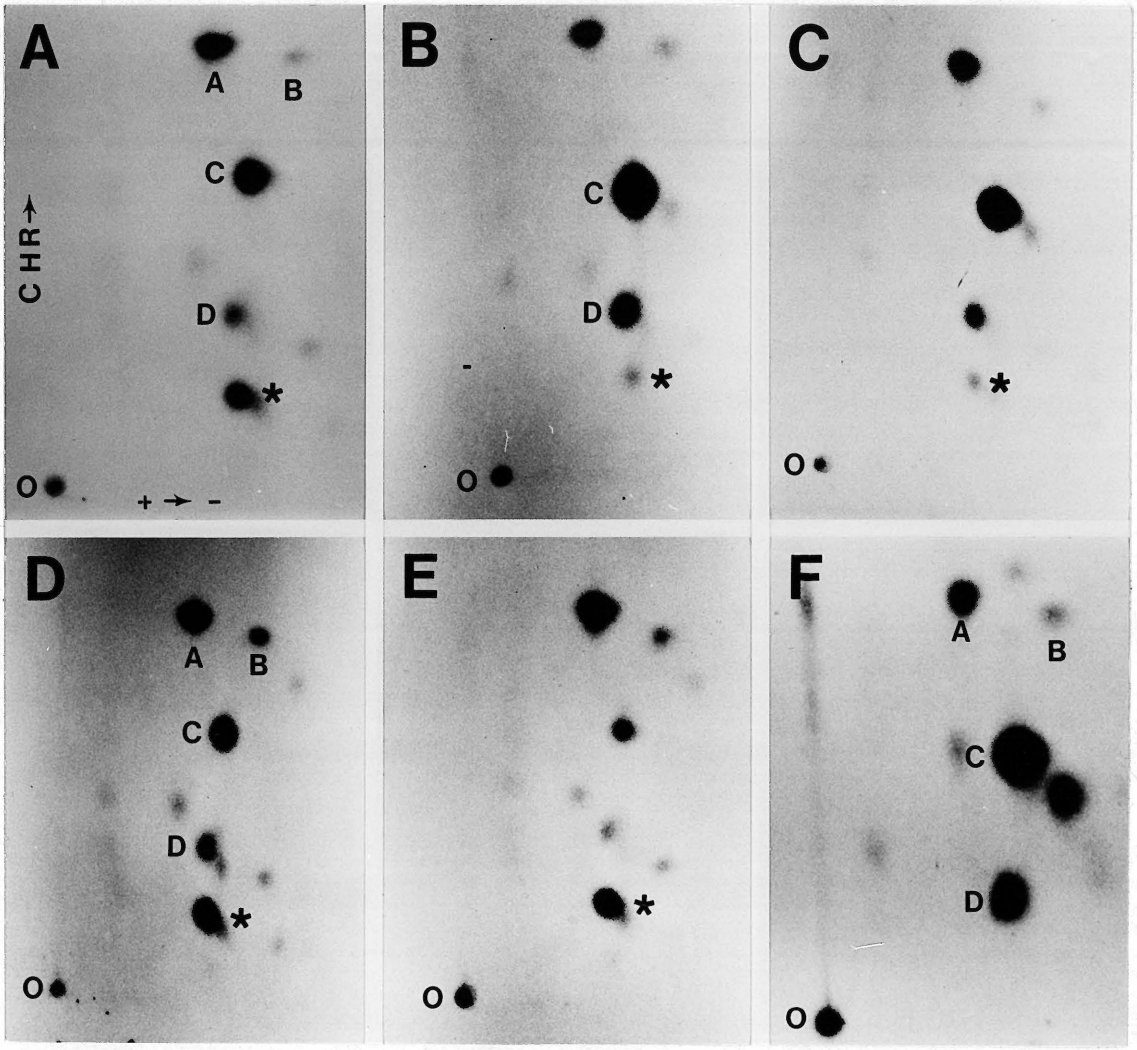

Figure 5

Chapter 7

Preliminary investigations of the effect of 8-Bromo cAMP and isoproterenol on intermediate filament organization during myogenesis in vitro

Introduction

Chicken embryonic skeletal myotubes differentiating in vitro contain two major intermediate filament subunit proteins, desmin and vimentin (Fellini et al., 1978; Gard et al., 1979). During the early stages of myogenesis, these proteins are found to comprise a dense filamentous network distributed throughout the sarcoplasm (Bennett et al., 1979; Gard and Lazarides, 1980), presumably corresponding to the numerous intermediate filaments observed in ultrastructural studies of developing muscle (Ishikawa et al., 1968). Late in myogenesis there is a redistribution of this filament network and both desmin and vimentin become associated with Z-lines of assembling myofibril bundles (Gard and Lazarides, 1980). In adult muscle, both desmin and vimentin are found to comprise a collar-like lattice which surrounds and links Z-discs of adjacent myofibrils (Granger and Lazarides, 1978, 1979).

The molecular basis for the regulation of intermediate filament distribution is currently unknown. Both desmin and vimentin have been shown to be phosphorylated in muscle in vivo, and in myotubes differentiating in vitro (O'Connor et al., 1979). Protein phosphorylation is known to be an important regulatory mechanism in many cellular metabolic processes (Krebs and Beavo, 1979), and may also serve in regulation of cytoskeletal organization. Recently I have shown that phosphorylation of desmin and vimentin in mature myotubes is stimulated 2-3 fold by treatment with the cAMP analogue 8-Bromo cAMP, or the β-adrenergic hormone isoproterenol (Gard and Lazarides, submitted for publication). In this report we demonstrate that while 8-BrcAMP and isoproterenol have no visible effect on intermediate filament distribution in early myotubes (3-4 day), exposure of mature cells to 8-BrcAMP results in a marked decrease in the proportion of cells exhibiting filament redistribution to the Z-lines. The possible correlation between the cAMP effects on filament distribution and phosphorylation are discussed.

Materials and Methods

Cultures of avian embryonic myotubes were prepared as previously described (Gard and Lazarides, 1980). Cells were grown in collagen-coated 60 mm petri plates containing 2-5 collagen-coated coverslips. Cultures were treated with 10 μM cytosine arabinofuranoside (days 4-7) to prevent overgrowth of residual fibroblasts. Cyclic nucleotide derivatives (8-BrcAMP, 8-BrcGMP; Sigma) and isoproterenol (Sigma) were prepared as stock solutions in Earle's balanced salt solution (EBSS) and added to the indicated final concentrations.

Cells were fixed for immunofluorescence as previously described (Gard and Lazarides, 1980). Characterization of antibodies to desmin and vimentin have been presented elsewhere (Granger and Lazarides, 1978, 1979).

The percentage of multinucleate myotubes showing Z-line striations with antibodies to desmin or vimentin in immunofluorescence was determined by counting cells during a grid or random scan of each coverslip. Due to the asynchronous process of myogenesis, most myotubes exhibit both filamentous and Z line IF proteins. These cells were counted as having Z-line-associated proteins. The extensive network of branches formed by many myotubes made it difficult to prevent multiple counts of a single cell. The actual percentage of cells showing striations may thus be larger than our estimates.

Results and Discussion

Treatment of 4-day myotubes with 10^{-3} M 8-BrcAMP or 10^{-5} M isoproterenol had no effect on intermediate filament distribution (see Fig. 2 A-B). In these cells, antisera to desmin and vimentin reveal a dense filamentous network which fills the sarcoplasm. Occasional longitudinal increases in filament density are seen, which may represent the initial association of intermediate filaments with assembling myofibrils (see Gard and Lazarides, 1980). The lack of effect of 8-BrcAMP or isoproterenol on 4-day myotube IF distributions is consistent with the lack of cAMP

stimulated increases in IF protein phosphorylation in cells of this age (Gard and Lazarides, submitted for publication, Chapter 6 of this thesis).

By day 6, approximately 20% of the untreated multinucleate myotubes exhibit a striated pattern in immunofluorescence with desmin or vimentin antisera. Pretreatment of cultures for 20-24 hr with 8-BrcAMP or isoproterenol prior to fixation on day 6 results in a marked decrease in the number of cells with fluorescent striations (see Figure 1). No other obvious changes in the distribution of intermediate filaments are observed in treated cells. In general, those myotubes in 8-BrcAMP-treated cultures which do exhibit desmin- or vimentin-containing striations appear similar to striated myotubes in control cultures (Figure 2C-D).

As myotube differentiation proceeds, the percentage of myotubes in control cultures which exhibit striations with antibodies to intermediate filaments increases, reaching 40-50% by days 8 and 9. Eight-day cultures treated with 10^{-3} M 8-BrcAMP for 24 hours prior to fixation show a marked reduction in cells with Z-line striations, while treatment with 10^{-3} M 8-BrcGMP had no visible effect (data not shown). No conclusions can be drawn at this time regarding the effect of 8-BrcAMP on 9-day or older myotubes, due to inconsistency in the experimental results.

Antisera to α-actinin was used to ascertain the effect of 8-BrcAMP on Z-line assembly. No changes were apparent in the proportion of myotubes exhibiting α-actinin Z-line striations in 6- or 7-day myotube cultures treated with 10^{-3} M 8-BrcAMP (data not shown). This result suggests that 8-BrcAMP acts at a stage subsequent to the assembly of α-actinin-containing Z lines, consistent with the proposed role of intermediate filaments in assembly of myofibril bundles (Gard and Lazarides, 1980).

The 8-BrcAMP-stimulated increase in phosphorylation of desmin and vimentin reaches a peak within 1 hour after administration of 5×10^{-4} M 8-BrcAMP (Gard and Lazarides, submitted for publication). However, this length of treatment has no

noticeable effect on intermediate filament distribution in 7-8 day myotubes. It is possible that the immediate 8-BrcAMP effect on IF protein phosphorylation leads to a more time-consuming rearrangement of intermediate filaments. The data on hand, however, do not adequately address the question of whether 8-BrcAMP can reverse the association of IF proteins with the myofibril Z line, or simply inhibits any new associations. This latter hypothesis could also explain the length of time required for an observable effect (the time required to see 8-BrcAMP effects would be dependent upon the rate at which the redistribution of filaments occurred), as well as explaining the decrease in effectiveness of 8-BrcAMP treatment observed in more mature (9-day) cells. Clearly, further work is necessary to distinguish between these and other possibilities.

Correlation of the inhibition of intermediate filament redistribution by 8-BrcAMP and isoproterenol with the previously reported increase in phosphorylation of desmin and vimentin stimulated by these agents (Gard and Lazarides, submitted for publication) suggests that high levels of cAMP-dependent IF protein phosphorylation are associated with a less differentiated state, when intermediate filaments are dispersed throughout the cytoplasm. This hypothesis is consistent with the age-dependent sensitivity of myotubes to cAMP-stimulated increases in IF protein phosphorylation; maximal cAMP-dependent phosphorylation of IF proteins in immature myotubes would prevent further stimulation of phosphorylation by exogenous cAMP. The onset of sensitivity to exogenous cAMP corresponds in time to the redistribution of intermediate filaments to the Z-line during myotube maturation, suggesting that a decrease in IF protein phosphorylation is associated with this event. Prolonged artificial increases in phosphorylation induced by exogenous cAMP then inhibit or reverse the normal redistribution of filaments during differentiation. Evidence supporting this model is certainly incomplete. Further support could be obtained by measurements of intracellular cAMP levels, active cAMP-dependent kinase levels, and the total levels of IF protein phosphorylation during myogenesis. It is interesting

that this model, which equates high cAMP-dependent phosphorylation with an immature state, is contrary to most reports that cAMP levels increase during differentiation in many cells (for example, Prasad and Kumar, 1974). In this regard increases in intracellular cAMP are noted prior to myoblast fusion, the earliest step in the process of myogenic differentiation (Zalin and Montague, 1974).

The mechanisms by which cAMP-dependent phosphorylation of intermediate filament proteins could control filament distribution are unknown. To date several intermediate filament-associated proteins (as opposed to filament subunits) have been described or identified in muscle (Granger and Lazarides, 1980; Breckler and Lazarides, 1982), and undoubtedly many remain to be found. Phosphorylation of IF proteins may affect interactions of intermediate filaments with other cytoplasmic structures (such as myofibril Z-lines) through a disruption of the binding of these associated proteins.

References

Bennett, G. S., Fellini, S. A., Toyama, Y., and Holtzer, H. (1979) Redistribution of intermediate filament subunits during skeletal myogenesis and maturation in vitro. J. Cell Biol. **82**, 577-584.

Breckler, J. L. and Lazarides, E. (1982) Isolation of desmin and vimentin filaments from avian embryonic skeletal muscle and their association with a new high molecular weight protein. J. Cell Biol., in press.

Fellini, S. A., Bennett, G. S., Toyama, Y., and Holtzer, H. (1978) Biochemical and immunological heterogeneity of 100 Å filament subunits from different chick cell types. Differentiation **12**, 71-82.

Gard, D. L., Bell, P. B., and Lazarides, E. (1979) Coexistence of desmin and fibroblastic intermediate filament subunit in muscle and non-muscle cells: identification and comparative peptide mapping. Proc. Natl. Acad. Sci. U.S.A. **76**, 3894-3898.

Gard, D. L. and Lazarides, E. (1980) The synthesis and distribution of desmin and vimentin during myogenesis in vitro. Cell **19**, 263-275.

Gard, D. L. and Lazarides, E. (1982) cAMP-modulated phosphorylation of intermediate filament proteins during myogenesis in vitro. Submitted for publication.

Granger, B. and Lazarides, E. (1978) The existence of an insoluble Z disc scaffold in chicken skeletal muscle. Cell **15**, 1253-1268.

Granger, B. and Lazarides, E. (1979) Desmin and vimentin coexist at the periphery of the myofibril Z disc. Cell **18**, 1053-1063.

Granger, B. L. and Lazarides, E. (1980) Synemin: a new high molecular weight protein associated with desmin and vimentin filaments in muscle. Cell **22**, 727-738.

Ishikawa, H., Bischoff, R., and Holtzer, H. (1968) Mitosis and intermediate-sized filaments in developing skeletal muscle. J. Cell Biol. **38**, 538-555.

Krebs, E. G. and Beavo, J. A. (1979) Phosphorylation-dephosphorylation of enzymes. Ann. Rev. Biochem. **48**, 923-959.

O'Connor, C. M., Balzer, D. R., and Lazarides, E. (1979) Phosphorylation of the subunit proteins of intermediate filaments from chicken muscle and non-muscle cells. Proc. Natl. Acad. Sci. U.S.A. **76**, 819-823.

Prasad, K. N. and Kumar, S. (1974) Cyclic AMP and the differentiation of neuroblastoma cells in culture. Cold Spring Harbor Conferences on Cell Proliferation, Vol. I, pp. 581-594, B. Clarkson and R. Baserga (eds.), Cold Spring Harbor, New York.

Zalin,R. J. and Montague, W. (1974) Changes in adenylate cyclase, cyclic AMP, and protein kinase levels in chick myoblasts and their relation to differentiation. Cell **2**, 103-108.

Figure 1: Inhibition of IF redistribution to the Z-line during myogenesis by 8-BrcAMP and isoproterenol.

Control myotubes (circles) were fixed for immunofluorescence microscopy on the day indicated. The proportion of cells exhibiting Z-line striations with antisera against desmin or vimentin was determined. At least 100 cells were counted on most coverslips (N = the number of coverslips examined; mean and range are shown for each point). Identical cultures of myotubes were pre-treated for 20-24 hours with 10^{-3} M 8-BrcAMP (triangles) or 10^{-5} M isoproterenol (squares), fixed on the indicated day, and examined as above. Treatment of 5- to 8-day myotubes with these agents results in a marked decrease in the percentage of cells exhibiting Z-line striations on immunofluorescence microscopy. No conclusions can be drawn about the effect of 8-BrcAMP on 9-day myotubes due to the inconsistency of the data.

Figure 2: Distribution of desmin in control and 8-BrcAMP-treated myotubes.

No differences in intermediate filament distribution were apparent between control (A) and 8-BrcAMP-treated four-day myotubes (B) examined by immunofluorescence microscopy with anti-desmin.

The few myotubes from 8-BrcAMP-treated 8-day cultures which exhibit Z-line striations (b) with anti-desmin appear similar to untreated 8-day myotubes (C). Bars are 20 μm.

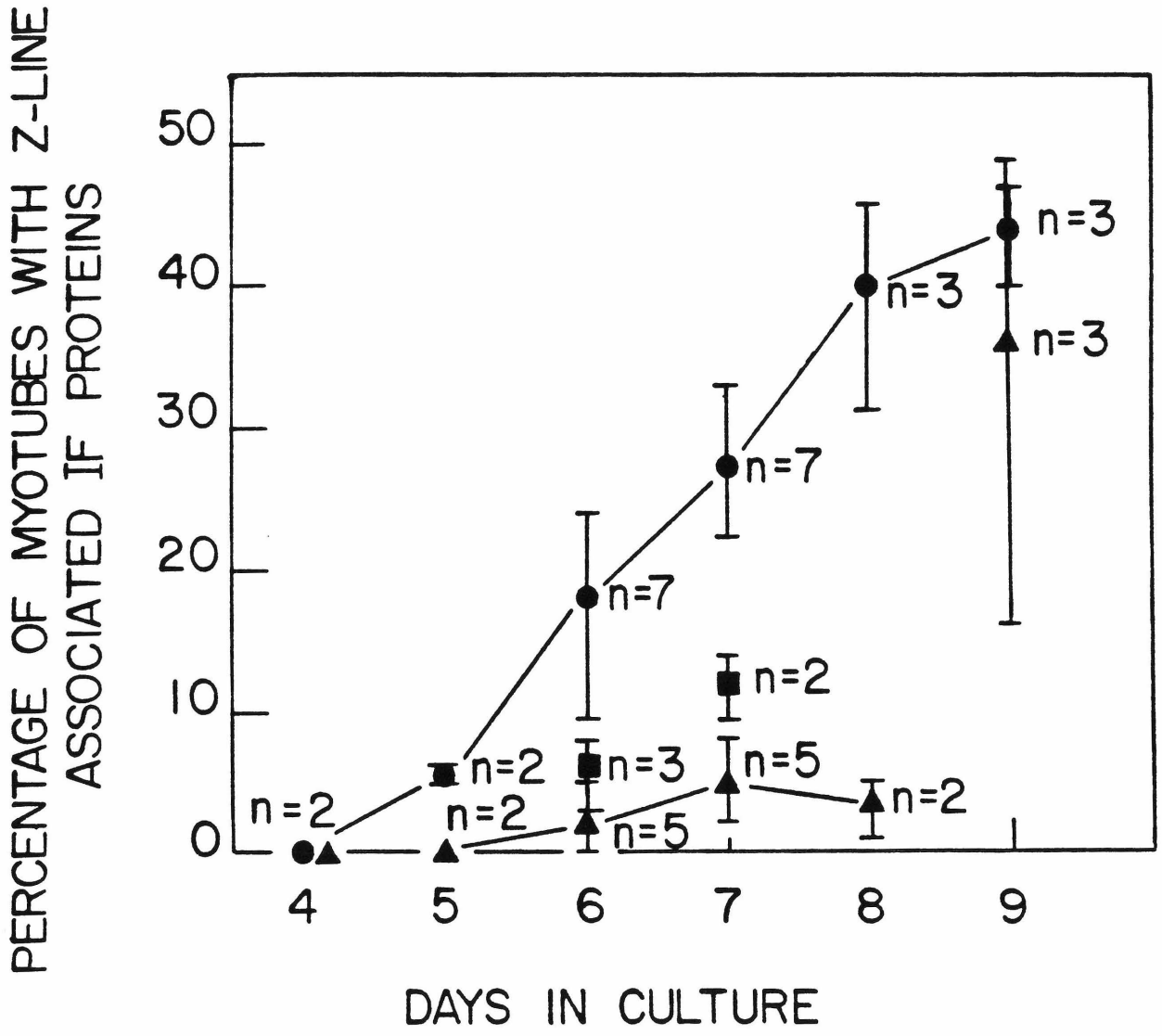
PERCENTAGE OF MYOTUBES WITH Z-LINE ASSOCIATED IF PROTEINS
50
40
30
20
10
0
4
5
6
7
8
9
DAYS IN CULTURE
n=2
n=2
n=7
n=2
n=3
n=5
n=7
n=2
n=5
n=3
n=3
n=2

Figure 1

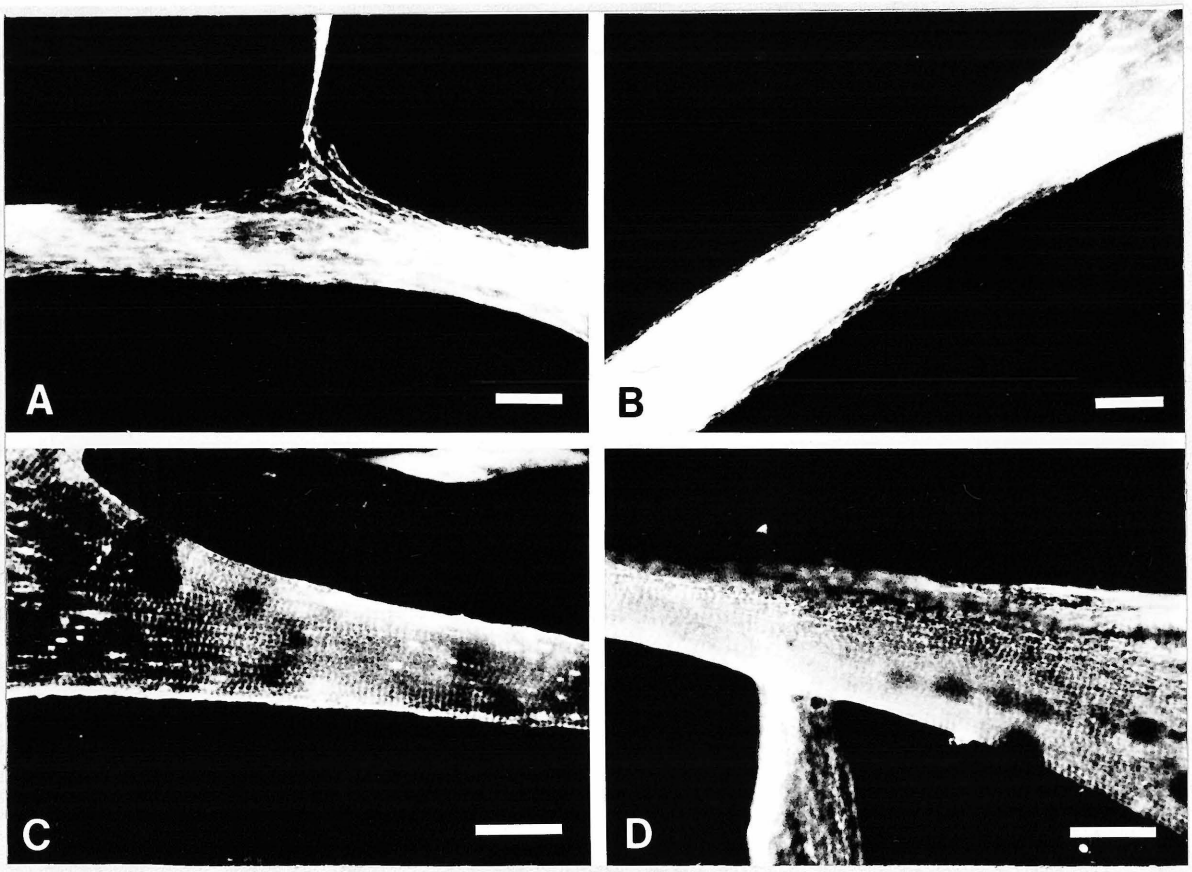

Figure 2

Chapter 8

Summary, general discussion, and conclusions:
Intermediate filaments, phosphorylation, and myogenesis

Skeletal muscle provides one of the most striking examples of cytoplasmic organization found in cell biology. The transverse striations apparent in both light and electron microscopy reflect not only the highly ordered array of actin and myosin filaments which compose the myofibrils, but also the organization of myofibrils into an integrated contractile unit. Each myofibril within a muscle fiber is aligned laterally with adjacent myofibrils, such that Z-lines are all in register. Considerable evidence has accumulated which suggests that intermediate filaments play an integral role in the assembly and maintenance of these highly ordered structures. Based upon observations of the process of myogenesis in vitro, a model is now emerging which describes the role of intermediate filaments during the assembly of myofibrils.

I have found that intermediate filaments in embryonic skeletal myotubes are composed of at least two protein subunits: desmin, the IF protein originally isolated from smooth muscle, and vimentin, the IF protein found in many cells of mesenchymal origin. Immunofluorescence microscopy and metabolic labeling with ^{35}S-methionine indicate that vimentin is the major intermediate filament protein in pre-fusion myoblasts, and that desmin synthesis is restricted to fusing myoblasts and multinucleate myotubes. During the first three days in culture (post-fusion) the rate of desmin synthesis increases more than 10-fold (from an undetectable level to nearly 1% of the total protein synthesis), while synthesis of vimentin increases only by a factor of two. The disparity in the relative increases in synthesis of the two filament proteins results in a significant increase in the ratio of desmin to vimentin in the sarcoplasm. In adult skeletal muscle, desmin is the major IF protein, with only trace amounts of vimentin detectable by electrophoretic and immunological techniques (Granger and Lazarides, 1979; however, see Holtzer et al., 1981).

Immunofluorescence reveals that in early myotubes (3-5 days in culture), desmin and vimentin are found in a dense network of cytoplasmic filaments, which presumably correspond to the numerous intermediate filaments observed in ultrastructural investigations of developing muscle (Ishikawa et al., 1968). Treatment of

these myotubes with the antimitotic drug colcemid results in the formation of cable-like perinuclear aggregates of desmin and vimentin filaments, a characteristic feature of intermediate filaments. Double immunofluorescence microscopy with antisera to desmin and vimentin reveals indistinguishable distributions of the two proteins in both control and colcemid-treated myotubes, suggesting that they may be present in the same heteropolymer filaments. Little association between the intermediate filament network and assembling myofibrils is noted in these immature myotubes. Beginning at 5-7 days, however, I have observed a redistribution of intermediate filaments within the cytoplasm of the cultured myotubes. At this time antisera to desmin and vimentin begin to reveal increased densities of intermediate filaments surrounding myofibrils and transverse striations which correspond to the myofibril Z-lines observed in phase-contrast microscopy. Though this transition in filament distribution is quite asynchronous, both within a population of cells as well as within a single myotube, by days 8-9 a large proportion of myotubes (40-50%) show desmin- and vimentin-containing Z-line striations. The redistribution of intermediate filaments during myogenesis has also been reported by other investigators (Bennett et al., 1979). Once associated with Z-lines, neither desmin nor vimentin can be aggregated by treatment with colcemid into the characteristic filament cables seen in younger myotubes or nonmuscle cells.

I have used double immunofluorescence microscopy to ascertain the correspondence in time between the assembly of α-actinin-containing myofibril Z-lines and the association of intermediate filaments with these structures. Results from these experiments indicate that the association of desmin (and vimentin) with the Z-lines of myofibrils occurs significantly later than the appearance of α-actinin-containing myofibril Z-lines, at a time when individual myofibrils are being organized into bundles. In conjunction with the observations that desmin and vimentin are found at the periphery of each adult myofibril Z-disc, where they form a collar-like lattice which links Z discs from adjacent myofibrils (Granger and Lazarides, 1979, 1979),

these data suggest that the intermediate filament proteins desmin and vimentin are responsible for the integration of individual myofibrils into bundles.

The structures formed by Z-line-associated intermediate filament proteins remain uncertain. Though few intermediate filaments are seen associated with Z-lines in adult skeletal muscle, (Page, 1969; as opposed to the numerous filaments observed in developing muscle, Ishikawa et al., 1968), immunofluorescence microscopy reveals a clear association between desmin, the major intermediate filament protein in adult muscle, and the Z-lines of skeletal myofibrils. In adult cardiac muscle, however, intermediate filaments are more apparent, and are observed to enter upon and surround many myofibril Z-discs (Eriksson and Thornell, 1979; Ferrans and Roberts, 1973). This is even more obvious in muscle from animals treated with certain anabolic steroids (Behrendt, 1977). It seems likely that intermediate filaments are also present in skeletal muscle, but may be obscured by the close packing of skeletal myofibrils (Granger and Lazarides, 1978).

The individual functions of desmin and vimentin during myogenesis in vitro remain unknown. In myotubes these two proteins are found in distributions which are indistinguishable by immunofluorescence microscopy, resulting in the speculation that they may form heteropolymer filaments (Gard and Lazarides, 1980; see Chapter 3). Evidence has been presented which supports the ability of desmin and vimentin to copolymerize in vitro (Steinert et al.,1981); however, the existence of heteropolymers in vivo has not been documented. The recent report of significant homology between the amino acid sequences of desmin and vimentin (Geisler and Weber, 1981), the similarities in amino acid composition of many intermediate filament proteins, (see introduction) and some reports of immunological cross-reactivity (Pruss et al., 1980) all suggest that intermediate filament proteins constitute a multigene family of proteins diverged from a common ancestral gene. Tissue-specific expression of intermediate filament proteins in differentiated cells suggests that divergence in protein structure may also reflect divergence in the functional properties of the

filament types. The copolymerization of desmin and vimentin into heteropolymer filaments in vitro, and the biophysical similarities in structure of keratin filaments and BHK intermediate filaments containing desmin and vimentin suggest that while divergence and specialization of some filament properties may have occurred, the basic structural features governing filament formation are more highly conserved. The switch from expression of vimentin in undifferentiated cells to the tissue-specific intermediate filament proteins in differentiated cells, as documented in muscle and neuronal cells (Gard and Lazarides, 1980; see Chapter 3; Bennett et al., 1981; Tapscott et al., 1981), may then be functionally analogous to the switching of hemoglobin expression during fetal erythropoesis. In muscle, the synthesis of desmin and its copolymerization into a preexisting system of vimentin filaments may alter the properties of the filament network, thereby conferring some function or property necessary during myogenesis.

Recently, it has been found that many cell types cultured in vitro possess more than one intermediate filament subunit. In all cases reported to date such cells contain a filament protein characteristic of their tissue origin (i.e., neurofilament, keratins, glial fibrillary acid protein, or desmin), and vimentin, which is found in many undifferentiated mesenchymal cells and in all cells grown in vitro. The expression of vimentin in cultured cells may represent a state of dedifferentiation necessary for continued growth in vitro.

The molecular regulatory mechanism which governs the redistribution of intermediate filaments during myogenesis has not been fully elucidated. One major clue was provided when it was found that desmin and vimentin are phosphorylated in muscle in vivo and in vitro. Both proteins exhibit multiple phosphorylated variants and single non-phosphorylated species (O'Connor et al., 1979). Using two-dimensional tryptic analysis I found that α'- and α-desmin from cultured myotubes exhibit qualitatively identical ^{32}P-phosphopeptide maps, which indicate that both desmin species are phosphorylated at multiple sites. Substantial evidence suggests that

phosphorylation of desmin and vimentin in myotubes is catalyzed by the cAMP-dependent protein kinases (CDPK). Both desmin and vimentin serve as substrates for cAMP-dependent protein kinase in vitro, resulting in phosphorylation at sites corresponding to those observed in vivo. Recently I have found that incorporation of $^{32}PO_4$ into IF proteins in mature myotubes can be stimulated by brief treatment with 8-BrcAMP (a potent activator of CDPK) or isoproterenol (a β-adrenergic agonist which yields increased intracellular cAMP). This effect is independent of protein synthesis, and is not due to increased phosphate turnover, suggesting that it represents a cAMP-dependent increase in IF protein phosphorylation. Tryptic peptide analysis has revealed that there is a corresponding increase in incorporation of $^{32}PO_4$ into at least one major desmin phosphopeptide.

The function of IF protein phosphorylation in myogenesis (and in general) is less clear. All intermediate filament proteins examined to date, and many other cytoskeletal proteins, are phosphorylated in vivo or have been found to act as protein kinase substrates in vitro (see introduction). Phosphorylation of proteins is a major regulatory mechanism in cellular metabolic processes (Krebs and Beavo, 1979; Greengard, 1978), and it is plausible that it may also function in regulation of cytoskeletal functions. However, phosphorylation does not appear necessary for the assembly of desmin into intermediate filaments in vitro (Steinert et al., 1981), and phosphorylation does not appear to alter the assembly of IF proteins into the Triton-KCl insoluble cytoskeleton in vivo (O'Connor et al., 1979; my unpublished observations). It is interesting to note that though desmin and vimentin phosphorylation is apparent throughout myogenesis, sensitivity to 8-BrcAMP or isoproterenol-induced increases in IF protein phosphorylation is restricted to mature myotubes, coinciding in time to the redistribution of intermediate filaments to the myofibril Z-lines. Preliminary evidence suggests that treatment of 5-8 day myotubes with 8-BrcAMP or isoproterenol (which stimulate IF protein phosphorylation) can inhibit or reverse the normal transition of intermediate filaments to the myofibril Z-lines. Though further

documentation is necessary, this observation could provide evidence of a link between protein phosphorylation and intermediate filament distribution. Based upon these results I propose that early in myogenesis cAMP-dependent phosphorylation of desmin and vimentin is maximal, thereby preventing stimulation of additional phosphorylation in response to exogenous cAMP. At this time desmin and vimentin form a network of cytoplasmic filaments, providing a cytoskeletal framework for early steps in myofibril assembly. Late in myogenesis a decrease in cAMP, and concomitantly in phosphorylation of desmin and vimentin, initiates the condensation of intermediate filaments around the Z-discs of individual myofibrils where they function to link adjacent myofibrils into a cohesive contractile unit. Addition of exogenous cAMP or hormones at this time artificially elevates intracellular cAMP, resulting in increased IF protein phosphorylation and inhibition of filament redistribution. Further proof of this model is necessary, and could be obtained by precise measurements of intracellular cAMP levels, active CDPK levels, and levels of IF protein phosphorylation throughout myogenesis in vitro. Nevertheless, it seems probable that changes in cAMP-dependent phosphorylation of desmin and vimentin are important in the regulation of intermediate filament distribution and function during myogenesis.

References

Behrendt, H. (1977) Effect of anabolic steroids on rat heart muscle cells. 1. Intermediate filaments. Cell Tissue Res. **180,** 303-315.

Bennett, G. S., Fellini, S. A., Toyama, Y., and Holtzer, H. (1979) Redistribution of intermediate filament subunits during skeletal myogenesis and maturation in vitro. J. Cell Biol. **82,** 577-584.

Bennett, G. S., Tapscott, S. J., Kleinbart, F. A., Autin, P. B., and Holtzer, H. (1981) Different proteins associated with 10 nm filaments in cultured chick neurons and non-neuronal cells. Science **212,** 567-569.

Ericksson, A. and Thornell, L.-E. (1979) Intermediate filaments in heart Purkinje fibers. J. Cell Biol. **80,** 231-247.

Ferrans, U. J. and Roberts, W. C. (1973) Intermyofibrillar and nuclear-myofibrillar connections in human and canine myocardium. An ultrastructural study. J. Mol. and Cell. Cardiol. **5,** 247-257.

Gard, D. L. and Lazarides, E. (1980) Synthesis and distribution of desmin and vimentin during myogenesis in vitro. Cell **19,** 263-275.

Geisler, N. and Weber, K. (1981) Comparison of the proteins of two immunologically distinct intermediate-sized filaments by amino acid sequence analysis: Desmin and vimentin. Proc. Natl. Acad. Sci. U.S.A. **78,** 4120-4123.

Granger, B. L. and Lazarides, E. (1978) The existence of an insoluble Z disc scaffold in chicken skeletal muscle. Cell **15,** 1253-1268.

Granger, B. L. and Lazarides, E. (1979) Desmin and vimentin coexist at the periphery of the myofibril Z disc. Cell **18,** 1053-1063.

Greengard, P. (1978) Phosphorylated proteins as physiological effectors. Science **199,** 146-152.

Holtzer, H., Bennett, G. S., Tapscott, S. J., Croop, J. M., Dlugosz, A., and Toyama, Y. (1981) Changes in intermediate-sized filaments during myogenesis and neurogenesis. In International Cell Biology 1980-1981, H. G. Schweiger (ed.), Springer-Verlag, New York.

Ishikawa, H., Bischoff, R., and Holtzer, H. (1968) Mitosis and intermediate-sized filaments in developing skeletal muscle. J. Cell Biol. **38**, 538-558.

Krebs, E. G. and Beavo, J. A. (1979) Phosphorylation-dephosphorylation of enzymes. Ann. Rev. Biochem. **48**, 923-959.

O'Connor, C. M., Balzer, D. R., and Lazarides, E. (1979) Phosphorylation of the subunit proteins of intermediate filaments from chicken muscle and non-muscle cells. Proc. Natl. Acad. Sci. U.S.A. **76**, 819-823.

Page, S. G. (1969) Structure and some contractile properties of fast and slow muscles of the chicken. J. Physiol. **205**, 131-145.

Pruss, R. M., Mirsky, R., Raff, M. C., Anderton, B., and Thorpe, R. (1980) A monoclonal antibody that demonstrates that intermediate filaments share a common antigen. J. Cell Biol. **87**, 178a.

Steinert, P. M., Idler, W. W., Cabral, F., Gottesman, M. M., and Goldman, R. D. (1981) In vitro assembly of homopolymer and copolymer filaments from intermediate filament subunits of muscle and fibroblastic cells. Proc. Natl. Acad. Sci. U.S.A. **78**, 3692-3696.

Tapscott, S. J., Bennett, G. S., and Holtzer, H. (1981) Neuronal precursor cells in the chick neural tube express neurofilament proteins. Nature **292**, 836-838.