FUNCTIONAL AND BIOCHEMICAL STUDIES ON NEURONAL THY-1

Thesis by

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In partial Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

1991

(Defended Feb. 15, 1991)

Acknowledgments

First and foremost, I must thank Paul H. Patterson for being more than a thesis advisor. In the time I have spent in the Patterson laboratory, Paul has been both my teacher, counselor, role model, and friend. Over the course of these studies, he has always treated me as a colleague, and his passionate idealism always proved uplifting in my times of doubt. He has taught me how to do science, and how to have fun doing it. It is one of my fondest desires that we remain great friends and colleagues for years to come.

For providing the very infrastructure through which my science was possible, both Doreen McDowell and William Lease are due my endless thanks for making sure things went smoothly, even when chaos was imminent. They have always been there for me whenever things got crazy, and if I could take them with me in my future endeavours, I would do it without a second thought. That you will continue to be there for the endless parade of graduate students-*cum*-lunatics, will always warm my heart.

Many individuals have taught me specific techniques which were vital to the progress of my research: Josette Carnahan introduced me to neural dissection and cell culture, William Bleisch taught me not to be intimidated by column chromatography, and Zaven Kaprielian was indispensable in assisting me with peptide mapping and immunochemistry. There are many more, among the faculty, staff, postdocs, graduate students, and undergrads who have also brought camaraderie and shared laughs into my life. Some have already moved on, and some I feel I have only just met - all of you I thank and wish the best.

Finally, without the unwavering and implicit support of my family, none of this would have been possible. My parents, K.T. and Prameela Mahanthappa, taught me to set the highest standards for myself and have always encouraged my intellectual and creative efforts. My brothers, Rudresh and Mahesh, have always been there to cheer on their oldest brother even when he looked a little insane. I love you all dearly, and to you I owe the greatest thanks.

Abstract

Thy-1, a cell surface glycoprotein, is one of the smallest members of the immunoglobulin superfamily. It is expressed abundantly in nervous systems of many species and is expressed at highest levels by long projection axons after the major period of neurite outgrowth and synaptogenesis. In this study, I demonstrate that three distinct perturbations that either remove or prevent expression of cell surface Thy-1 result in enhanced neurite outgrowth and initiation of sprouting: (i) Binding by soluble anti-Thy-1 monoclonal antibodies results in increased numbers of neurite-bearing neurons, chromaffin cells, and PC12 cells in culture. Sprouting requires multivalent binding (anti-Thy-1 Fab fragments do not give this effect), and results in shedding of Thy-1 from the cell surface. (ii) Chromaffin and PC12 cells exhibit enhanced sprouting when exposed to phosphotidylinositol-specfic phospholipase C, a treatment that removes cell surface Thy-1. This effect is blocked by anti-Thy-1 Fabs. (iii) When mutagenized and selected for Thy-1-deficiency, 90% of resultant PC12 cell mutants sprout spontaneously. These pertubations suggest that the normal function of Thy-1 is to stabilize neurites and inhibit sprouting, and that removal or prevention of Thy-1 expression disinhibits such mechanisms.

Biochemical analysis reveals that Thy-1 exists in homomultimeric forms *in situ* and in cultured neurons. Neuronal Thy-1 is distributed equally in monomeric, dimeric, and hexameric forms; the latter being composed of monomers and dimers. The dimer does not give rise to monomers when boiled in the presence of disulfide reducing agents and SDS. In PC12 cells, the multimeric forms of Thy-1 predominate, but treatment with agents that cause sprouting make the Thy-1 distribution more neuronal. The homology between Thy-1 and immunoglobulin variable domain allowed peptides to be synthesized that correspond to candidate sites of intermolecular association. These were tested for their effects on neurite outgrowth and Thy-1 multimerization. One peptide induces a decrease in Thy-1 multimerization, as well as enhancing outgrowth. Thus, the stabilization function of Thy-1 may be mediated by

homomultimerization on the cell surface, and its removal from the surface, or dissolution into monomers and dimers, permits outgrowth and sprouting.

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Chapter 1:

INTRODUCTION

Study of the developing nervous system encompasses a wide variety of phenomena from the biochemical and molecular genetic level through the integrative and physiological level. In common with the study of other developing tissues, are questions concerning control of cell proliferation, lineage and fate determination, cell migration, and cell death. Unique to the nervous system, however, is the problem of establishing on the order of thousands of appropriate synaptic connections per neuron among billions of neurons. In the human brain it is estimated that there are approximately 10^{12} neurons and a total of approximately 10^{15} synaptic connections (*117*). Thus, it is easy to see that understanding the mechanisms that underlie the establishment of such connectivity must involve many distinct events: the sprouting of axons and dendrites, neurite outgrowth and growth cone locomotion, target cell recognition, and the subsequent steps of synaptogenesis (*186*). Though each of these phenomena are quite complicated, all of them make use of a variety of adhesion molecules and trophic factors that contribute in varying degrees to the specificity of intercellular interactions in the nervous system.

It is the intention of this study to focus on the initial problem of neurite outgrowth. More specifically, the aim is to better understand the role in neurite outgrowth of cell surface molecules belonging to the immunoglobulin superfamily (IgSF) by studying one member, Thy-1. This chapter provides an overview of the types of molecules known to be involved in neurite outgrowth, a more detailed discussion concerning the IgSF, and finally, a review of the literature concerning Thy-1 in particular.

Molecules involved in neurite outgrowth

Cells committed to neuronal differentiation are faced with the task of establishing synaptic contact in order to carry out the many functions of the nervous system. The first step in the process of establishing neural connectivity is that of neurite outgrowth - the sprouting of axons and dendrites. Individual cell bodies put forth growth cones which make use of

a multitude of cues, both soluble and membrane- or matrix-bound, to link up with their appropriate targets (*186*).

Soluble factors affect neurite outgrowth. Among the soluble factors known to influence neurite outgrowth are trophic factors, tropic factors, neurotransmitters, and proteases. Though nerve growth factor (NGF) is a trophic factor necessary for the survival of certain peripheral (PNS) and central nervous system (CNS) neurons, the tumors from which NGF activity would eventually be characterized were first noticed for their ability to attract dense innervation, even when transplanted to ectopic sites (*136*). When purified NGF was injected intracerebrally into the ventricles of neonatal rats, an aberrant invasion of sympathetic fibers from the PNS into the cerebrospinal axis was observed (*158*). When cultured within semisolid agar matrices containing concentration gradients of NGF, dissociated chick sensory neurons exhibited a preferential orientation and enhanced extension of neurites up the gradient (*135*). Later studies with cultured neurons demonstrated that individual growing axons could be led about the culture dish by a pipet releasing NGF (*92*). Despite all of these findings, it seems unlikely that NGF plays an important role in neurite guidance *in vivo*, since it has now been shown that targets of sensory and sympathetic innervation do not express significant levels of NGF until the onset of innervation, and that neurons do not express NGF receptors until they reach their targets (*47*).

Nevertheless, when co-cultured in collagen gels with presumptive whisker field tissue and control limb bud tissue, the embryonic rat trigeminal ganglion preferentially extends neurites towards its normal target of innervation, the whisker field, even in the presence of a neutralizing anti-NGF antiserum. This occurs when the tissues have been explanted at a stage preceding their contact *in vivo* and thus suggests the presence of a soluble, non-NGF, tropic activity produced by the target tissue (*142*). It has also been demonstrated that growing commisural axons from the embryonic rat dorsolateral spinal cord, which travel ventrally *in vivo* to the floor plate before turning rostrally towards the brain, are drawn towards floor plate explants *in vitro* by a soluble factor

with no known trophic activity (*226*). Thus, trophic factors can act as tropic factors, but a tropic factor need not possess trophic activity.

Neurotransmitters are usually thought of as the molecules that relay signals from neuron to neuron via chemical synapses. In both invertebrate and vertebrate preparations, it has now been shown that neurotransmitters can also affect developing neurites. In the case of the snail, *Helisoma trivolvis*, when a developing, identified neuron, which in the adult is known to receive innervation from a serotonergic neuron, is placed in culture it sprouts many neurites. Strikingly, when any individual growth cone from this cell is exposed to serotonin, the exposed neurite ceases to grow (*101*). *In vivo*, this same neuron shows abnormally extensive neurites if its serotonergic partner was ablated in an earlier embryonic stage (*86*). A similar effect is observed with cultured rat hippocampal pyramidal cells in response to glutamate (*155*). Direct manipulation of neurons by the injection of current pulses, or exposure to calcium ionophores and channel blockers will also affect growth cones similarly (*35*, *154*). It has since been demonstrated that when neurotransmitters affect outgrowth, they affect intracellular calcium levels, and when intracellular calcium concentrations go below or above a given range, outgrowth will cease (*118*). These experiments suggest that the very molecules that will mediate synaptic transmission in the adult are shaping neuronal circuitry during development.

The manner in which proteases affect neurite outgrowth is still a puzzle. The demonstration of metalloprotease secretion by growth cones of cultured neurons can be readily reconciled with the growth cones' need to carve routes through a forest of extracellular matrix since such proteases display collagenase activity (*179*, *180*). Indeed, the presence of metalloprotease inhibitors blocks the ability of sympathetic neurons to penetrate collagen gels on which they are cultured (*180*). The discovery of the secretion of plasminogen activator-type protease activities from growth cones (*123*, *179*), however, has been less easy to understand because natural and synthetic inhibitors of these types of proteases actually

promote neurite outgrowth (*162, 180, 248*). That glia and target tissues of innervation secrete protease inhibitors (*162, 181*), and that the growth cone surface also expresses protease inhbitor (*180*), have suggested that since growth cones must engage in continual cycles of both substrate attachment and detachment, a delicate balance of proteases and their inhibitors in the immediate environment may orchestrate both processes. The presence of exogeneous protease inhibitors may then lead to effects in culture that are superficially paradoxical (*161, 174, 180*). Nevertheless, such experiments make obvious not only a need to understand soluble agents that affect growing neurites, but the equally pressing need to identify and understand molecules that mediate interactions between the growth cones and their relatively immobile substrates.

The extracellular matrix is very complex. As stated above, growth cones must negotiate their way through environments rich in extracellular matrix molecules. Best characterized among these molecules are the collagens, proteoglycans, laminins, and fibronectins. Collagens constitute a large gene family (*23*), and tendon-derived collagen proves to be an adequate substrate for neurons in culture (*100, 147*), but it is likely that it primarily acts as a scaffolding for the assembly of other matrix molecules.

Proteoglycans consist of a core protein to which a variety of sulfated polysaccharides and/or hyaluronate are attached (*105*). Two proteoglycans that have received the most attention in neural development are chondroitin sulfate proteoglycan (ChSPG) and heparan sulfate proteoglycan (HeSPG). The pattern of expression of ChSPG in somites during neural crest cell migration and during the outgrowth of motor neurons from the spinal cord, suggests that it may play a role in guidance by acting as an inhibitor of cellular locomotion (i.e., defining where cells should not go) (*176, 225*). Supporting such a theory is the observation that when the neural tube and pre-migratory neural crest are cultured as explants, crest cell emigration from the explant is drastically reduced by the addition of ChSPG to the cultured medium; this effect appears to be mediated by the

binding of cell surface hyaluron by the ChSPG (*176, 177*). Peripherally, ChSPGs have been localized to the neuromuscular junction (*28*), as well as subsets of neurons and astrocytes in the CNS (*83, 246*). In these cases the function of ChSPG remains unknown.

HeSPG is expressed on the surface of sympathetic neurons and the neuron-like, pheochromocytoma line, PC12 (*152*). It has also been shown that HeSPG greatly enhances neurite outgrowth when used as a culture substrate (*152*). Furthermore, heparin, which is chemically similar to heparan sulfate (*82*), can interfere with the aggregation of dissociated neurons, presumably by competing for HeSPG binding sites that mediate adhesion (*37*). Interestingly, a monoclonal antibody (mAb) selected for its ability to inhibit neurite outgrowth, both *in vivo* and *in vitro* (*153, 203*), is directed against a complex of HeSPG and laminin (*32*). In cryostat sections, this mAb is localized to glial cells of the PNS, the basement membranes of cardiac and skeletal muscle, and areas known to promote axonal regeneration (*32*). It is also interesting to note that pure HeSPG and pure laminin each have distinct effects on neurite extension *in vitro*; the former promotes elongation, while the latter promotes branching (*97*). Thus proteoglycans may play important roles in providing substrates *in vivo* that both promote and inhibit neurite outgrowth.

Laminin was originally characterized as an 800 kD, heterotrimeric, cruciform protein that, together with HeSPG, is one of the earliest extracellular matrix proteins to be detected in the developing embryo (for extensive review on laminin see Ref. *210*). The form of laminin most thoroughly characterized was purified from cultured tumor cells and is composed of an A chain (M_r = 400 kD) that shares homology with a B1 chain (M_r = 210 kD), and a B2 chain (M_r = 200 kD); each of the amino-termini of the three chains form a short arm of the cruciform complex, while the carboxyl-termini come together to form the long arm. An isoform of the B1 chain called s-laminin (S) has been characterized from the extracellular matrix of the neuromuscular junction (*108*), and an isoform of the A chain called merosin (M), has been found to be specifically expressed in the basement membranes of placenta, striated

muscle, and peripheral nerve (*64*). Immunochemistry has demonstrated that all five laminin chains are expressed as subunits of heterotrimeric complexes (A-B1-B2, A-S-B2, M-B1-B2, and M-S-B2), each of which shows tissue restricted distribution (*66*); the name laminin will now be used to discuss the A-B1-B2 form in particular.

Various proteolytic fragments of laminin have been demonstrated to contain distinct sites that mediate association with type IV collagen, heparin/heparan sulfate, neurite outgrowth, and at least two different sites mediating cell attachment (*210*). The carbohydrates linked to laminin appear to be important for cell attachment and neurite promoting activities since specific lectins can block these activities *in vitro* (*52*). The cloning and sequencing of a 35 kD laminin receptor on macrophages reveals identity to a galactose-specific lectin (*242*), and reagents that specifically perturb cell surface galactosyltransferase activity interfere with laminin-mediated motility of cultured cells (*10, 196*). Laminin receptors have also been reported with M_r = 67 kD, 110 kD, and 180 kD, the latter two being involved in neurite outgrowth (*119*). Finally, laminin receptors of the integrin family have also been characterized and will be discussed below.

Fibronectin is found in two forms: as a soluble plasma protein, and as an extracellular matrix protein produced by fibroblasts and epithelial cells (see review in Ref. *245*). The former plays a role in hemostasis and thrombosis, and is produced by hepatocytes. Immunochemical analyses have found that fibronectins are quite polymorphic, and this is due in part to alternative splicing of mRNA precursors (*18*). Fibronectins are composed of two similar subunits, M_r = 220-250 kD, that are linked by disulfide bonds into dimers and higher multimers.

Like laminin, fibronectin has numerous independent domains to which collagen, heparin/heparan sulfate, fibrin, and cell attachment sites have been localized (*245*). Site-directed mutagenesis of the cell binding domain in human fibronectin demonstrated that a second site on the molecule interacts with the cell binding domain synergistically, and that proteins mutant in either of the two sites can complement each other in *trans*, hence suggesting that cell adhesion is dependent on interactions with

multiple fibronectin domains (*171*). Further complicating structure-function analyses of fibronectin is the observation that while CNS and PNS neurons can both extend neurites *in vitro* on a proteolytic fragment that contains the heparin binding site, only PNS neurons can extend processes on a fragment containing the cell attachment site (*192*). Cellular receptors for fibronectin appear to be many, but the receptor for the "cell attachment" site of fibronectin has been demonstrated to be a member of the integrin family.

The integrin family of receptors bind matrix molecules. Integrins are a family of cell surface receptors that bind to a variety of extracellular matrix proteins including collagens, laminins, and fibronectins, as well as to a protein of the complement cascade, and the T cell surface antigen ICAM-1 (reviewed in Ref. *111*). All integrin molecules are heterodimers consisting of an α and β subunit. The α subunits are 130-210 kD and appear to be more polymorphic than the 95-130 kD β subunits. Both have large extracellular domains involved in ligand binding, and relatively small cytoplasmic domains which have been demonstrated to associate with the actin cytoskeleton via talin and vinculin (reviewed in Ref.*24*), thus providing a bridge between the matrix and the cytoplasm. Though many of the ligands of integrins contain the amino acid sequence Arg-Gly-Asp (or RGD), and it has been demonstrated that synthetic peptides containing the RGD sequence will interfere with certain integrin binding interactions (*197*), ICAM-1, the ligand of the integrin LFA-1, does not contain the RGD sequence and thus it is not a necessary feature of integrin ligands (*149*).

Neuronal cells appear to make use of a variety of integrins, all of which contain the β_1 subunit (*189*). The $\alpha_1\beta_1$, $\alpha_3\beta_1$, and $\alpha_6\beta_1$ integrins all bind laminin; $\alpha_1\beta_1$, $\alpha_2\beta_1$, and $\alpha_3\beta_1$ bind collagens; and $\alpha_3\beta_1$ and $\alpha_5\beta_1$ bind fibronectin. Thus it can be seen that certain combinations of α and β subunits, such as $\alpha_3\beta_1$, can bind multiple ligands. That certain matrix proteins can be bound by multiple integrin ligands, however, does not represent direct redundancy on a molecular level. In the case of PC12 cells, it has been shown that $\alpha_1\beta_1$ and $\alpha_3\beta_1$ recognize different neurite

outgrowth-promoting domains of laminin (*227*). Thus single integrins may bind multiple matrix proteins, and single matrix proteins may be bound by multiple integrins, as well as non-integrin receptors. It is not clear at present what purpose this serves, but the simultaneous expression of multiple integrins, as in the case of PC12 cells, might allow cells to negotiate their way through diverse extracellular matrices that incorporate the myriad isoforms and splice variants of proteins such as laminin and fibronectin. That these integrins are indeed critical for process outgrowth to occur on extracellular matrices, and to a far lesser degree on the surfaces of such cells as astrocytes and Schwann cells, has been demonstrated by inhibiting such outgrowth with mAbs directed against either the integrins or their ligands (*189*). Extracellular matrix receptors are not, however, the only types of molecules mediating interactions between neurites and non-diffusible factors. Cell surface molecules also mediate interactions with other cells.

Inhibitory molecules. While the majority of the intermolecular interactions discussed so far have been those that facilitate neurite outgrowth, the possible role of molecules that inhibit outgrowth was introduced in connection with ChSPG. Within the past two to three years, a growing number of cell surface molecules that inhibit neurite outgrowth have begun to be characterized (*175*). The first of these are 35 kD and 250 kD proteins associated with myelin. These proteins are nonpermissive substrates both for fibroblast spreading and neurite growth. Being expressed in CNS white matter, they may be responsible for the inability of CNS neurons to regenerate after injury in adults. MAbs directed against these proteins neutralize their inhibitory activity *in vitro* (*30*). In addition, 48 kD and 55 kD proteins have been characterized in chick somites that cause the collapse of sensory neuron growth cones in culture when applied in liposomes (*48*); an approximately 50 kD protein from embryonic chick brain demonstrates similar growth cone collapsing activity (*188*); and quite interestingly, a 33 kD protein has been isolated from the chick posterior tectum that induces the collapse of temporal retinal growth cones, but not nasal retinal growth cones, and thus may be partly responsible for the

establishment of retinotopic projections to the tectum (*41, 218*). Most recently, it has been found that the extracellular matrix protein, J1/tenascin, is a repulsive substrate for cultured CNS neurons (*69*), though J1/tenascin also supports the adhesion of fibroblasts and endothelial cells through an RGD-dependent integrin (*19*). Thus a given molecule may be permissive for the adhesion and locomotion of some cell types, while being nonpermissive for others.

Cadherins. Up to this point, all of the molecules presented have made use of heterophilic interactions in the promotion of adhesion and neurite outgrowth. The cadherins are a group of homologous proteins that mediate calcium-dependent, homophilic interactions (reviewed in Ref.*224*). In mammals and avians, three cadherins have been found, E-, N-, and P-cadherin; all consist of 723 to 748 amino acids ranging in sequence identity from 43% to 58%. They have a single transmembrane domain with approximately 80% of the protein exposed extracellularly. Transfection of cDNA clones for each of the cadherins into cadherin-deficient cell lines has demonstrated calcium-dependent, homophilic binding, and the use of chimeric constructs has shown that the amino terminal 113 amino acids confer homophilic specificity (*170*). As in the case of the integrins, candidate proteins are being sought that mediate the interaction of the carboxyl termini of cadherins with the cytoskeleton (*224*).

Antibodies directed against N-cadherin significantly inhibit the growth of neurites on the surfaces of Schwann cells and astrocytes *in vitro* (*189*). Furthermore, N-cadherin purified from chick brain is a very adhesive substrate and potent promoter of neurite sprouting for chick ciliary ganglion neurons (*12*). Cells transfected with the various cadherins have provided useful probes for examining the distribution of cadherin activities on dissociated primary cells. The binding of such transfected cells to cultured neuroepithelium of the developing optic tract demonstrates a high level of N-cadherin expression, as does the binding of the probe cells to growing optic axons in culture, thus cadherin-mediated adhesion may be important in optic tract development (*224*). Further

evidence for an important role of N-cadherin *in vivo* comes from forcing ectopic expression of the protein in *Xenopus* embryos by injection of N-cadherin mRNA into one blastomere of 2-cell-stage embryos. Such manipulations resulted in abnormal histogenesis and fusion of cell layers, thus suggesting that strict regulation of cadherins is essential for normal development (*53*, *78*).

Of the best characterized intermolecular interactions making use of substrate- and cell surface-bound proteins in neurite outgrowth, two general types have been presented here. The first of these are heterophilic interactions between members of the integrin family and components of the extracellular matrix. The second type involve calcium-dependent, homophilic interactions between members of the cadherin family. As already mentioned, there are many interactions that are not included among these two types, and deserving of extended discussion is a third category of interactions involving members of the immunoglobulin superfamily (IgSF). These include calcium-independent, homophilic and heterophilic interactions between proteins that themselves are monomeric, homomultimeric, and heteromultimeric.

The immunoglobulin superfamily: a role in neurite outgrowth

The immunoglobulin domain. Immunoglobulins are proteins produced by B lymphocytes that recognize foreign antigens and mediate humoral immunity (*34*). They are heterotetrameric proteins consisting of two identical heavy chains and two identical light chains (Fig. 1.1A). As early as 1966, prior to the availability of crystallographic data on the structure of immunoglobulins, the comparison of amino acid sequences from several Igs revealed a homologous sequence that is repeated twice in Ig light chains, and four times in Ig heavy chains (*102*). This sequence varies in length from 70-110 amino acids and is characterized by a largely invariant disulfide bridge spanning 50-70 residues, as well as several other highly conserved amino acids. It was thus suggested that Igs evolved through the serial duplication of an ancestral gene that encoded one copy

of the homologous sequence, the Ig domain (*102*). As crystal structures for Igs were solved, it became apparent that the repeating Ig domain detected by sequence analysis did indeed correspond to a repeated structural unit in the heavy and light chains . Furthermore, it was found that the homology unit could be broken down into two subgroups, variable (V) domains and constant (C) domains, which are more homologous among themselves than to each other (*63*, *106*, *182*).

From amino- to carboxyl-terminus, Ig heavy chains contain 1 V domain followed by 3 C domains, and light chains contain 1 V domain followed by 1 C domain (Fig. 1.1A). The C domain structure, the simpler of the two, consists of 7 antiparallel β-strands connected by loop sequences, and gives rise to 2 β sheets stabilized by a disulfide bridge (Fig. 1.1B). The β strands consist of alternating hydrophobic and hydrophilic amino acids such that the hydrophobic side chains are oriented towards each other. This gives rise to a "sandwich" structure where 4 strands form one face, and 3 strands form the other face (Fig. 1.1B, right). V domains are essentially the same, but contain two extra strands (labeled C' and C" in Fig. 1.1B) that sit between the two faces. Crystal structures of Ig fragments containing the light chain and the amino-terminal half of the heavy chain (the "arms" of the "Y" in Fig. 1.1A), reveal the V domains to be associated via the "3-strand" faces (containing stands G, F, C, C', and C" in Fig. 1.1B) to give rise to the antigen-binding site, while the C domains associate via the 4-strand faces (containing strands D, E, B, and A in Fig. 1.1B) (*182*, *183*). Crystal structures of Ig fragments comprised of the carboxyl-terminal halves of the two heavy chains (the "stem" of the "Y" in Fig. 1.1A) consist solely of C domains that are associated via the 4-strand faces (*106*). Thus V domains associate with V domains, and C domains with C domains.

The beginnings of the Ig family. β_2-microglobulin (β_2m) was initally characterized as a 100 amino acid serum protein appearing in the urine of patients suffering renal malfunction; when purified and sequenced, β_2m was found to possess homology with the Ig C domain (*43*). β_2m is, in fact, a subunit of the class I major histocompatability antigen (MHC I), a

heterodimeric protein responsible for the presentation of antigen to cytotoxic T cells (*34*). As heavy chains of human MHC I alleles were purified and sequenced, it was found that these too contained a sequence homologous to the Ig C domain (*219*). Fourteen years after the complete sequencing of β_2m, the crystal structure of human MHC I was solved, vindicating the idea of the existence of a family of Ig-like molecules at the structural level (*14*). Both β_2m and the α_3 domain of the MHC I heavy chain adopt tertiary structures similar to those of Ig C domains, and though slightly displaced, they are associated in a manner similar to that of C domain association between Ig heavy chains (*14*). Most recently, the structure of the first two amino-terminal domains of CD4, another member of the IgSF, has been determined from a genetically engineered fragment (*199, 233*). While it was predicted by sequence analysis that the amino-proximal domain (D1) would be V-like, no consensus was reached over the predicted structure of the carboxyl-terminal domain (D2) of the fragment (*107, 236*). Again, the structural data vindicated the prediction that D1 does indeed closely resemble a V domain; D2 clearly possesses Ig-like structure (3 β strand sheet/4 β strand sheet sandwich), but it shares features with both V and C domains, and contains an unusual placement of the requisite disulfide bond.

The general lack of crystallographic data demonstrating the existence of Ig domain structures in non-Ig proteins has not deterred the recognition of Ig domains in non-Ig proteins by primary sequence analysis. Cloning and sequencing of Fc receptors (FcR) (for review see Ref. *157*), as well as the membrane receptor responsible for the transepithelial transport of polymeric Igs (polyIgR) (*169*), suggested an interesting tendency among proteins bearing Ig domains. Both FcR and polyIgR bind Igs, and both contain multiple Ig domains; thus, not only do Ig domains prefer to associate with other Ig domains within single proteins, as in the case of Ig and MHC I, but Ig domain interactions appear to be harnessed in intermolecular associations as well. This observation has become a recurrent theme in the ever growing literature concerning the family of molecules now known as the IgSF (*107, 236*). Further examples of Ig

domain interactions occurring between non-Ig proteins of the IgSF include the association of T cell antigen receptor with MHC antigens (reviewed in Ref. *51*), as well as MHC association with the T cell proteins CD4 (*59*) and CD8 (*81*). It is interesting to note that truly homophilic associations among Ig domains (i.e., direct association of identical subunits) in molecules of the immune system has only been observed in the V domain-containing, T cell antigens, CD8 (*138, 221*) and CD28 (*4*). It is worth remembering, however, that at least one IgSF member, ICAM-1, binds the integrin, LFA-1, rather than another IgSF member (*149*).

IgSF members in the nervous system. In addition to the molecules that mediate immune responses, many molecules mediating intercellular interactions in the nervous system have also been shown to contain Ig domains. Members of the IgSF expressed in the vertebrate nervous system include Thy-1 (*237*), OX-2 antigen (*33*), neural cell adhesion molecule (NCAM) (*42*), myelin-associated glycoprotein (MAG) (*3*), L1 (*163*), P_0 (*132*), contactin (*84, 187*), and the transiently expressed axonal glycoprotein-1 (TAG-1) (*80*). IgSF members have also been identified in the nervous system of *Drosophila melanogaster*, and these include fasciclin II (*99*), and amalgam (*206*). Thy-1, as well as P_0, consists of a single V domain (*132, 237*), while OX-2 antigen resembles an Ig light chain since it contains 1 V domain followed by 1 C domain (*33*). The remaining neural IgSF members, however, display Ig domains that are considered neither V-like nor C-like, but instead are intermediate in nature and thus suggest an independent evolutionary path away from the ancestral single domain gene (*107, 236*).

The most thoroughly characterized of the neural IgSF members is NCAM. Cloning of the NCAM gene (reviewed in Ref. *231*) has demonstrated that the protein can adopt a variety of forms through alternative exon usage. The gene was originally thought to be encoded by 19 exons that give rise to 180 kD and 140 kD transmembrane forms differing only in their cytoplasmic extents, and the 120 kD glycophosphotidylinositol-linked (GPI-linked) form. Further analysis,

however, revealed the existence of 3 small exons that are expressed in the extracellular portion of the protein, together or separately, in a muscle-specific fashion in both the transmembrane and GPI-linked molecules. One additional exon, the usage of which results in a secreted form of NCAM, has also been identified. That such a variety of NCAM isoforms exist was not realized when the purified molecule was first shown to exhibit homophilic binding when incorporated into liposomes (*198*). Nevertheless, recent studies that make use of cells transfected with cDNAs encoding the various surface bound isoforms have shown that all appear to make use of the same mechanism, and only cell surface density (*56*) and degree of glycosylation (*55*) of the protein directly affect the degree of intercellular adhesion. Thus the functions of the muscle-specific segments are unknown. Though the cytoplasmic portions of both the 180 kD and 140 kD forms have been demonstrated to be phosphorylated in vivo by glycogen synthase kinase-3 and casein kinase-1, the function of this portion of NCAM is also unknown (*145*).

In addition to homophilic association, NCAM possesses a heparin/heparan sulfate binding site (*36*). Antibodies directed against this site reduce the aggregation of dissociated, NCAM expressing cells (*37*), and synthetic peptides corresponding to this site inhibit the adhesion of retinal cells to NCAM-coated substrates (*36*). Thus it is clear that sulfated polysaccharides of the extracellular matrix can act as NCAM receptors, and modulate NCAM-mediated interactions. Extensive polysialylation of NCAM also modulates cell-cell interactions; throughout the majority of embryonic development, NCAM is highly polysialylated, but soon after birth, the degree of sialylation drops. The presence of polysialic acid reduces homophilic NCAM interactions, and thus the developmental shift to the less sialylated form promotes adhesion (*46, 55*).

NCAM expression *in vivo* has been best characterized in the developing chick embryo (*46*). It first appears on early blastoderm cells, and as development proceeds, is expressed on both neural and non-neural cell types. Most pronounced among the latter are the notochord, somites, and muscle tissue. Expression of NCAM on striated muscle begins fairly early, and becomes concentrated at the neuromuscular junction after birth.

NCAM is expressed by the neural plate, and subsequently by both the neural tube and pre-migratory neural crest cells. It is down regulated on the crest cell surface as they migrate, but reappears as the cells aggregate and give rise to presumptive PNS ganglia. NCAM is then uniformly distributed on all neural cell bodies and processes of the CNS and PNS, staining intensity decreasing somewhat in the adult. Thus NCAM appears to play the role of a general "glue molecule" in the nervous system.

L1 (also known as NILE, Ng-CAM, and G4) was initially characterized as a neural membrane protein of molecular weight ranging from 180 kD to 200 kD; lower molecular weight forms were also identified and shown to be proteolytic fragments (*200*). Liposomes containing immunopurified L1 have been demonstrated to display a tendency to aggregate, though the same study failed to reproduce such an effect with liposomes containing NCAM (*200*). The "choreography" of L1 expression *in vivo* is more complicated than that of NCAM: in general it is observed on the surfaces of migrating neurons and in regions undergoing neurite extension (*46*). Most nerve fiber tracts in the CNS, while retaining NCAM, lose L1 after the major phases of tract development are complete. Thus it has been suggested that L1 plays an important role in the fasciculation of axons and the formation of fiber bundles (*8*). Support for this idea comes from experiments in which cerebellar microexplant cultures were exposed to Fab fragments derived from antibodies directed against L1. Such Fabs induced a decrease in the extent of fasciculation of neurites extended by the explants and promoted the rate of outgrowth (*72*). Unfortunately, these studies did not include control antibodies that bound the cell surface and did not affect outgrowth, thus the perturbation may have resulted from non-specific effects of binding cell surface antigens. Finally, L1 purified from mouse and chick, when adsorbed to culture substrates, supports excellent neurite outgrowth by a mechanism clearly dependent upon homophilic interactions. This was demonstrated by culturing chick and mouse neurons on L1 of either species, and showing that species-specific mAbs directed against L1 would severely retard outgrowth. These mAbs did not affect outgrowth on a control substrate of

laminin (*133*). Taken with the histological observations, these *in vitro* perturbations do suggest that L1 plays a specific role in fiber tract development, and not in general adhesivity.

That generalizations concerning NCAM and L1 do not do justice to the truly complex nature of their interactions have been demonstrated in a series of *in ovo* perturbations of developing chick limb muscle. As the chick motor nerve trunk invades the iliofibularis muscle, the nerve is observed to stain with both anti-NCAM and anti-L1 mAbs, while the muscle is observed to stain with anti-NCAM mAbs only (*125*). As expected, the *in ovo* injection of activity-blocking mAbs directed against NCAM or L1 induce the defasciculation of axons in the nerve trunk. Interestingly, however, anti-L1 mAbs also bring about an enhancement in the side branching of the defasciculating fibers, while the anti-NCAM antibodies decrease the number and degree of such side branching (*125*). Thus, it is the conjuction of both NCAM and L1 expression, in partially overlapping patterns of expression, that together modulate the overall pattern of nerve branching (as mediated by NCAM and L1 in axon-axon interactions) and local side branching (as mediated by NCAM in axon-myotube interactions). As in the case of the NCAM cDNA transfection studies, polysialic acid also appears to play a modulatory role in the control of nerve side branching *in ovo* since the injection of endoneuraminidase into the developing muscle increases axonal fasiculation; furthermore, neuromuscular activity blockade, which induces local side branching, is associated with increased expression of polysialic acid, and can also be inhibited by neuraminidase treatment (*126*).

Such detailed analyses are not yet available on the remainder of the IgSF members expressed by neurons. The chick protein, contactin (*187*), its murine homolog F3 (*84*), and the closely related chick protein F11 (*21*), each show relatively distinct patterns of expression in the developing nervous system, though all appear to be expressed on specific fiber tracts and fascicles. Thus it is likely that they play roles similar to L1, but mediate interactions concerning very specifc sets of axons. This would be

similar to specific axon labeling observed in the insect nervous system by mAbs generated against neural surface proteins such as fasciclin II (*99*). In fact, the highly restricted pattern of expression of TAG-1 in the rat nervous system supports this hypothesis in vertebrates as well: TAG-1 is expressed by commisural axons as they extend through the neuroepithilium toward the floor plate. As they cross the floor plate and change trajectory, they begin to fasciculate, and this is accompanied by a cessation of TAG-1 expression and onset of L1 (*54*). TAG-1 also supports neurite outgrowth when purified and used as a culture substrate (*80*). There is little data on the expression of OX-2 antigen other than the observation that it is expressed ubiquitously in the rat nervous system as early as embryonic day 13 and down regulates towards birth (*235*).

Two members of the IgSF are components of myelin (reviewed in Ref. *131*). MAG represents approximately 1% of CNS myelin protein, and less than that in PNS myelin. It contains 5 Ig-like domains, one of which contains the RGD sequence, and thus may interact with an integrin receptor. MAG is excluded from compact myelin, but is expressed primarily on the periaxonal sheath of glia, immediately adjacent to the axonal surface. Thus MAG is believed to be an adhesion molecule important for the initiation and maintainence of myelination. Direct evidence for this model comes from the inhibition of neuron-oligodendrocyte adhesion *in vitro* by mAbs directed against MAG (*184*). P_0 contains a single V domain, and is specific to PNS myelin where it constitutes greater than 50% of total protein. The cytoplasmic domain of P_0 is extremely basic, and by interacting with the negatively charged lipid head groups of PNS myelin, is thought to mediate compaction of the Schwann cell sheath major dense line that is observed by electron microscopy (EM). The extracellular V domain has been demonstrated to be involved in homophilic binding (*71*), and is thus thought to mediate the compaction of the intraperiod line observed by EM. Thus both P_0 and MAG are believed to mediate adhesive events necessary for the proper electrical insulation of myelinated nerve fibers.

Among all of these molecules, three have been conclusively demonstrated to participate in truly homophilic binding interactions. To reiterate: NCAM homophilic interactions were shown through the aggregation of liposomes containing purified NCAM (*198*), though more recent studies have revealed that such interactions are clearly modulated by glycosylation and by heparin (*37*). Homophilic L1 interactions have been most convincingly demonstrated through the selective perturbation of neurite outgrowth on substrates of purified L1 with antibodies that specifically bind to either cell surface L1 or substrate-bound L1 (*133*). Microbeads coated with L1 have also been demonstrated to aggregate, with the additional observation that coating beads with both NCAM and L1 enhances their binding to beads coated with L1 alone (*116*). Homophilic interactions involving P_0 were demonstrated by transfecting a nonaggregating, P_0-deficient cell line with the P_0 gene, and showing that resultant cells aggregate in a P_0-dependent fashion (*71*). Unlike the homophilic interactions involving CD8 and CD28 (*4*, *138*, *221*), which appear to take place within the plane of the membrane (*cis* interactions), those involving NCAM, L1, and P_0 all take place between cells and thus make use of *trans* interactions in addition to possible *cis* interactions.

Thy-1: A review of the literature

With our current knowledge of IgSF members in the nervous system, why bring particular focus to Thy-1? As has been made evident by the review on IgSF members that are well characterized, it is not yet clear on a molecular level how any of them carry out their putative functions. The pursuit of such an understanding is hampered by the fact that most of these proteins are relatively large, with multiple domains that may well be involved in very different functions. A simplifying approach to this problem would be to study an IgSF member that contains only one Ig domain.

Biochemical characterization. Thy-1 was the first immune system antigen to be identified serologically and has been used as an allelic marker in mice ever since its initial characterization (*190*). At the time of its identification, it was noted that the brain expresses the antigen abundantly, and thus it is the tissue from which Thy-1 has been purified for physicochemical study. Thy-1 purified from rat brain membranes is 111 amino acids long (*26*), while mouse brain Thy-1 is 112 amino acids long and 81% identical to the rat protein (*237*). The allelic difference in mouse Thy-1 is due to differing amino acids at position 89: Arg in Thy-1.1, and Gln in Thy-1.2 (*237*). A Thy-1 homolog in the chicken has been purified (*194*), and its recent cloning reveals that the molecule is 54% similar to rodent Thy-1 when conservative amino acid substitutions are considered (*58*). By way of similarities in method of purification, approximate molecular weight, and immunological crossreactivity, candidate homologs of Thy-1 have been identified in many species (*211*), including dog (*45*), cow (*243*), and frog (*148*). Amino acid sequencing of a squid brain protein has even revealed a possible invertebrate homolog (*238*).

In rodents, mature Thy-1 is expressed as a GPI-linked glycoprotein (*103*, *141*), of apparent molecular weight 25kD-30kD as determined by denaturing gel electrophoresis (*26*). The heterogeneity in molecular weight is ascribed to differential glycosylation at three N-linked sites and accounts for approximately 30% of Thy-1's apparent molecular weight (*29*, *172*). The glycans are of the simple, complex, and hybrid types with tissue specificity in glycosylation patterns (*172*). Though the cDNA of rat Thy-1 predicts a 142 amino acid protein with a hydrophobic, carboxyl terminal tail (*207*), soon after translation the carboxyl terminal 31 amino acids are cleaved and the GPI-tail attached *en bloc* (for a review on GPI-linkage see Ref.(*140*)). Studies of lymphoma Thy-1 expression show that greater than 90% of cellular Thy-1 is expressed on the cell surface and has a turnover rate greater than two days (*130*). Fluoresence recovery after photobleaching has revealed that approximately 50% of cell surface Thy-1 exhibits mobility comparable to that expected of labeled lipids, while the remainder appears relatively immobile (*113*). This immobile fraction of surface Thy-1 may possibly be explained by the protein's apparent

association, direct or indirect, with the actin cytoskeleton (*76*, *191*), and co-immunoprecipitation with antibodies directed against a band-4.1-like protein (*20*).

Gene organization and regulation of expression. Genomic clones of the rat (*208*), mouse (*31*, *85*), and human (*209*) Thy-1 genes revealed coding regions that agreed with the data derived from protein sequencing and cDNA analysis. All three genes have the same genomic arrangement in that one exon encodes the first half of the signal peptide, the next exon encodes the remainder of the signal peptide and the majority of the mature protein, and the last exon encodes the remainder of the protein, including the cleaved 31 amino acid tail. The mouse Thy-1 gene has been localized to chromosome 9 (*44*), and in humans has been localized to the homologous linkage group on chromosome 11 (*209*).

Initial cloning of the mouse Thy-1 gene (*31*, *85*), the most well characterized gene of the three species, was incomplete. Complete cloning of the gene showed that in addition to the three exons encoding the translated protein (exons II, III, and IV), two exons approximately 2 kb upstream of the start codon (Ia and Ib) contain two distinct promoters that are used alternately (*112*). Steady-state mRNA analysis revealed that exon Ib contributes at most 5% of the total Thy-1 mRNA (*217*). DNase-hypersensitivity analysis showed that the major promoter contains several transcription initiation sites whose frequency of usage varies between brain and other tissues (*217*).

Regulation of Thy-1 gene expression is quite complex. The transfection of the gene into fibroblasts, and neuronal and lymphoid cell lines, revealed that even when all three had stably integrated 2-4 copies into their genomes, the neuronal and lymphoid cells expressed fifty times more antigen than fibroblasts (*67*). This suggests that tissue-specific factors regulate Thy-1 expression. Somatic cell hybridization studies provide evidence for *trans*-acting factors that suppress Thy-1 expression. Thy-1-deficient lymphoma lines can be placed in distinct complementation groups that reexpress the protein when fused to one another (*109*). One Thy-1-deficient lymphoma line, however, does not express Thy-1 even when

fused to "wild type" cells; this line even supresses the wild type Thy-1 expression (*110*). That such a suppressor activity is present in normal cells was demonstrated by the fusion of embryonic rat sensory neurons (cells not yet expressing Thy-1) with a Thy-1-expressing lymphoma line, and showing that the resultant heterokaryons do not express Thy-1 (*201*). The fusion of postnatal Thy-1-expressing neurons with the lymphoma resulted in normal Thy-1 expression. Thus the developmental onset of Thy-1 expression is in part due to the loss of supressor activity. Studies utilizing transgenic mice have made it clear that noncoding regions of the Thy-1 gene control tissue specificity of expression. Homozygous Thy-1.2 mice express a human Thy-1 transgene in a pattern expected for human Thy-1, while control mice express the mouse Thy-1.1 transgene in a manner identical to their endogeneous Thy-1.2 (*88*). When Thy-1.2 mice carried a chimeric transgene containing mouse Thy-1.1 exons Ia, Ib, II, III, part of IV, and the remaining noncoding part of human exon IV and 3' flanking sequence, the transgene was expressed normally in the brain and thymus, but not in mature peripheral T cells as would have been expected for mice (see below) (*120*). The control of Thy-1 expression is likely to require multiple factors that act on several different regions of the Thy-1 gene.

Histological studies. The expression of Thy-1 outside of the nervous system in various species is remarkably inconsistent. In the mouse immune system, Thy-1 is detected at high levels on thymocytes and mature T cells; in the rat it is expressed at high levels on lymphoid stem cells, and on both T and B lineage cells until activated; expression of Thy-1 in the human immune system is very low (*165*). In rodents, in addition to the immune system, Thy-1 is expressed in mammary tissue both on the surface of myoepithelial cells (*195*), and in the extracellular matrix (*160*). Thy-1 immunoreactivity is also present on growing follicles of the rat ovary, corpora lutea, and fallopian tubes (*22*). The kidney shows non-overlapping patterns of Thy-1 expression in the human and mouse (*88*).

The expression of Thy-1 on muscle tissue is particularly interesting. When cultured, primary rat myoblasts stain positively for Thy-1, but as

myotube formation occurs, Thy-1 expression ceases (*134*). Later studies with human muscle tissue found that the cessation of Thy-1 expression correlated not with myotube formation *per se*, but with the exit of these cells from the cell cycle, an event preceding myocyte fusion (*232*). Nevertheless, it has been suggested that Thy-1 may play a role in myogenesis since anti-Thy-1 antibodies have been demonstrated to inhibit myocyte fusion (*104*). Interestingly, it has also been observed that Thy-1 expression by myoblasts in culture is inhibited by a factor secreted by fibroblasts (*205*). Thy-1 may play a role in neuromuscular synaptogenesis since it has been observed *in vivo* that adult skeletal muscle does not express Thy-1, while both neonatal skeletal muscle and denervated adult muscle of rats and mice do express Thy-1 (*17*).

Finally, though histological studies of Thy-1 in the nervous system are largely restricted to the rat (*5*, *6*, *166*, *178*) and the chick (*212*, *213*, *214*), general patterns of expression are much more conserved than in the tissues discussed above: Axons and dendrites tend to stain more brightly for Thy-1 than cell bodies. Long projection axons tend to express greater levels of Thy-1 than do small interneurons; for example, retinal ganglion cells express much higher levels of Thy-1 than horizontal and bipolar cells (*7*, *178*, *214*), and Purkinje cells and climbing fibers of the cerebellum express much more Thy-1 than basket cells (*6*, *212*). Though the modulation of patterns of expression within the nervous system can be quite complicated, Thy-1 is not expressed at significant levels until the major period of neurite outgrowth begins, and is not maximal until the period of outgrowth is complete (*165*, *212*, *213*). In the adult human forebrain, Thy-1 accounts for greater than 0.1% of total protein (*1*), and has been estimated to account for 2.5%-7.5% of total axonal surface protein in the rat (*9*, *165*). It is important to note that in early postnatal life, Thy-1 immunoreactivity within nerves is not restricted to the axonal surface; Thy-1 is also expressed on fibroblasts of the connective tissue and is progressively down-regulated during the first 4-12 weeks of life (*167*). The only notable exception to these observations on neuronal Thy-1 is the olfactory nerve. This nerve consists of axons from the only neurons in the mammalian nervous system known to turn over through adulthood (*89*),

and is completely negative for Thy-1 staining (*166*). Thus histological studies are suggestive of Thy-1 playing a critical role in the nervous system since neuronal patterns of expression are fairly well conserved across species, and nervous tissue expresses Thy-1 so abundantly. Furthermore, the relatively late onset of Thy-1 expression may reflect a role for Thy-1 in the stabilization of neural architecture and connectivity, a function the olfactory nerve may do without.

Functional perturbation. All published attempts to directly perturb Thy-1 *in vitro* as a means of understanding Thy-1 function have made use of antibodies directed against Thy-1. The first studies showed that the binding of Thy-1 by polyclonal antisera induced the activation of primary cultured T cells as measured by the induction of IL-2 production and the upregulation of IL-2 receptor expression (*121*). Subsequent studies utilizing monoclonal antibodies directed against Thy-1 demonstrated similar activation effects on T cell hybridoma lines (*94*, *185*), and cytolytic T cell clones (*144*). In a similar fashion, it has been demonstrated that dissociated mouse cerebellar Purkinje cells (*159*), and rat retinal ganglion cells (*129*) exhibit greatly enhanced neurite outgrowth and viability when cultured on a substrate of anti-Thy-1 mAb. In both of the neuronal experiments, however, the control experiment of culturing these neurons on substrates of mAbs directed against other cell surface antigens was absent, and thus the observed effects need not have been the result of Thy-1 binding, but instead simply a product of enhanced substrate adhesion. Despite this caveat, the neuron and lymphocyte experiments taken together are suggestive of Thy-1 playing a role in signal transduction.

Several experiments have attempted to explore the possible mechanisms by which Thy-1 might act as signal transducing protein. Using a calcium-sensitive dye, it was demonstrated that the binding of multivalent anti-Thy-1 mAbs caused a ten-fold increase in the levels of intracellular free calcium (*127*). Since, this occurred even in the presence of extracellular EGTA, the rise in cytoplasmic calcium may be attributable to its release from intracellular stores. When transfected with the Thy-1.2

gene, a normally Thy-1-deficient murine B lymphoma also displayed an increase in cytoplasmic free calcium in response to anti-Thy-1.2 mAb binding (*122*). The ability to induce a rise in calcium, however, appears to be insufficient for T cell activation. A human T cell line deficient in both the expression of Thy-1 and the CD3/T cell antigen receptor (TCR) complex was transfected with the murine Thy-1.2 gene. When exposed to anti-Thy-1.2 mAb, these cells also displayed an increase in cytoplasmic calcium, but did not produce IL-2. The replacement, by a second transfection, of defective TCR α- or β-chain genes reconstituted both the surface expression of CD3/TCR, and the production of IL-2 in response to anti-Thy-1.2 mAb (*93*). Thus it appears that Thy-1 may mediate different effects by associating with different proteins at the cell surface. Such an idea is reinforced by the observation that though Thy-1 has no structural similarity to known ion channels, anti-Thy-1 mAbs are capable of eliciting a transmembrane calcium flux in rat sensory neurons resembling the T current of voltage-dependent calcium channels (*202*). Recently, it has also been found that Thy-1 is closely associated with the cell surface tyrosine phosphatase, CD45 (*230*).

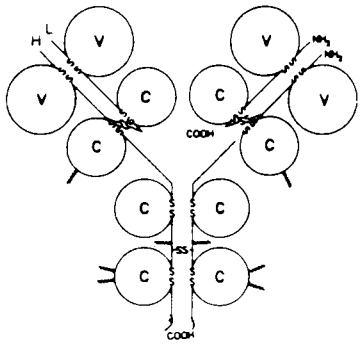
Despite the variety of data available on signal transduction by Thy-1, it must be noted that all of these studies have been carried out with antibodies. The relevance of these studies can only be made clear by efforts to identify physiological ligands of the protein. Though anti-idiotype antibodies have been used to identify candidate Thy-1 receptor molecules (*60, 77*), these proteins have yet to be isolated and studied for their effects on second messenger levels in Thy-1 expressing cells. In addition, Thy-1 has been demonstrated to exhibit high affinity binding to laminin (*137*), fibronectin (*137*), sulfated polysaccharides (*173*), and the protease, plasminogen activator (*137, 234*). None of these ligands has been tested for effects on Thy-1-mediated signal transduction. Whether or not they function through the transduction of second messenger-mediated signals, it is possible that each of these ligands is physiologically relevant. Moreover, Thy-1 may mediate functions that are not involved in intercellular signalling, an issue to be pursued below.

Chapters 2 and 3 comprise submitted manuscripts on *in vitro* pertubations of cell surface Thy-1, and biochemical analyses pertaining to Thy-1 structure and function. Chapter 4 is concerned with the role of the enzyme transglutaminase in neurite outgrowth; these experiments, though not directly involved with Thy-1, were inspired by results of experiments studying Thy-1. Finally, an appendix is also provided, presenting work concerning the effects of NGF and FGF on primary adrenal chromaffin cell cultures, and the potential use of these findings for neural graft therapy.

FIGURE LEGEND

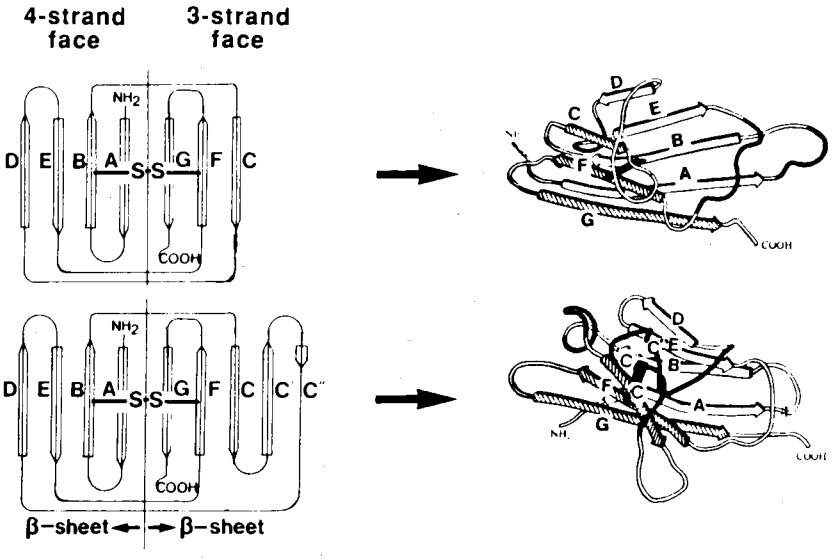
Figure 1.1: Organization of immunoglobulin (Ig) variable and constant domains. A) Schematic organization of a soluble Ig; V, variable domains; C, constant domains; -s-s-, disulfide bonds; and wavy lines, common sites of glycosylation (after Ref.*107*). B) Schematic organization of intradomain β-strand organization and corresponding structures as determined by x-ray crystallography (after Refs.*63* and *236*).

A.



Domain Structure of Immunoglobulin

B.



Schematic Folding Diagram **3° Structure**

Chapter 2:

THY-1 INVOLVEMENT IN NEURITE OUTGROWTH: PERTURBATION BY ANTIBODIES, PHOSPHOLIPASE C, AND MUTATION

Introduction

First characterized in 1964 as a major mouse T lymphocyte surface antigen, Thy-1 is used widely as an allelic marker for these cells. Reif and Allen also noted that Thy-1 is very abundant in the adult mouse brain (*190*). Rat, mouse, and human Thy-1 have been cloned and sequenced (*31*, *164*, *209*), and it is considered to be the simplest member of the immunoglobulin superfamily (*107*, *236*). Though homologues of Thy-1 have been identified in diverse members of the animal kingdom (*45*, *96*, *148*, *194*, *243*), the cellular distribution of the molecule in the immune system is not consistent among the different species. For example, while Thy-1 is expressed at high levels on both the thymocytes and mature peripheral T cells of mice, it is expressed only on immature thymocytes of rats, and shows negligible expression in the human immune system (see Ref.*165* for review). This distribution pattern argues against an essential role for the molecule in the immune system. In striking contrast, however, there are common patterns of expression in the nervous system of several species. In general, the highest levels of Thy-1 are found on long-projection neurons such as retinal ganglion cells, climbing fibers from the inferior olive, and Purkinje cells of the cerebellum, while interneurons with shorter processes, such as retinal horizontal and bipolar cells, express lower levels (*5*, *166*, *168*, *178*). Peak levels of expression are attained in the mature nervous system, where the molecule is estimated to constitute 2.5%-7.5% of total axonal surface protein (*9*). Such patterns suggest a highly conserved role within the nervous system.

In an effort to elucidate the function of Thy-1, polyclonal antisera and monoclonal antibodies directed against Thy-1 have been used to perturb this surface molecule on cells in culture. In the case of T lymphocytes and related cell lines, such antibodies can induce mitogenesis and enhance lymphokine and lymphokine receptor expression (*94*, *121*, *122*). Anti-Thy-1 antibodies have also been used to coat substrates upon which rat retinal ganglion cells and mouse cerebellar Purkinje cells have been cultured. These neurons display enhanced viability and more robust neurite outgrowth when cultured upon anti-Thy-1 antibodies (*129*, *159*). The effect on the neurons may be explained, however, simply by enhanced

substrate adhesion caused by the antibody-antigen interaction. We here demonstrate that soluble anti-Thy-1 antibodies can specifically enhance neurite outgrowth or sprouting, and that this effect requires multivalent antibodies. These antibodies can also induce sprouting of neurites from primary adrenal chromaffin cells and PC12 cells in the absence of NGF, with concommitant shedding of Thy-1 and a Thy-1 dimer. We have further pursued the perturbation of Thy-1 function with phosphotidylinositol-specific phospholipase C (PI-PLC), and mutagenesis. Both of these means of removing cell surface Thy-1 also result in the enhancement of neurite initiation.

Methods and Materials

Cell Culture

All cells were cultured in dishes as described by Mains and Patterson (*147*). Neonatal rat superior cervical ganglion neurons were dissociated and grown by the method of Wolinsky et al. (*240*) in serum-free L15-CO_2 medium containing 100 μg/ml transferrin (Sigma), 5 μg/ml bovine insulin (Sigma), and 1 μg/ml 7S nerve growth factor (NGF) (prepared by Josette Carnahan as described by Bocchini and Angletti (*15*)). Neonatal rat chromaffin cells were cultured as described by Doupe et al. (*57*), in the absence of dexamethasone. PC12 cells were cultured in medium as described by Hawrot and Patterson (*100*) with 10% fetal bovine serum and 5% heat-inactivated horse serum. When cultured in the presence of antibodies (see below), affinity purified monoclonal antibodies (mAbs) or Fabs in PBS were added to the culture medium to a final concentration of 5 μg/ml unless otherwise stated. PI-PLC (purified from the culture supernatants of *Bacillus thuringiensis*) was kindly provided by Dr. Martin Low (Columbia University) in 50 mM Tris-acetate, pH 7.4 mixed with an equal volume of glycerol; this material, with an activity of 20 μmol/min/ml (as measured by the hydrolysis of ^{3}H-phosphatidylinositol), or the glycerol buffer alone, was diluted to the desired concentration directly into culture

medium. Hybridomas were all maintained in RPMI 1640 with 10% fetal bovine serum; clones of pg22 (anti-heparan sulfate proteoglycan, (*152*)), OX-7 (anti-Thy-1, (*150*)), and 19XE5 (anti-Thy-1, (*156*)) were provided by Dr. William Matthew (Duke University School of Medicine), Dr. Alan Williams (Oxford University), and Dr. Irving Weissman (Stanford University School of Medicine), respectively.

Antibody Purification and Fab Production

Antibodies derived from the hybridomas ASCS4 (anti-NILE (*223*)), pg22, OX-7, and 19XE5 were affinity purified from ascites fluid or hybridoma culture supernatants by affinity chromatography on protein A-Sepharose 4B (Pharmacia) by the method of Ey, Prowse, and Jenkin (*68*). The same technique was used to purify from ascites fluid the mAbs, 2G12 (anti-Thy-1, (*7*)), provided by Dr. Colin Barnstable (Yale University School of Medicine), and OX-18 (anti-MHC I, (*79*)), and antibody from polyclonal rabbit anti-Thy-1 serum (the latter two being provided by Dr. Alan Williams). All antibodies used in this study are IgG. Fab fragments were generated by incubation of the purified antibodies with immobilized papain (Pierce Chemical) by the method of Coulter and Harris (*40*) and again purified by protein A-Sepharose 4B chromatography.

Though the mAb pg22 was originally characterized as being directed against an epitope expressed by heparan sulfate proteoglycan (HeSPG) (*152*), it has recently been argued that the pg22 epitope is in fact expressed by Thy-1 (*91*). In order to clarify this issue, an enzyme-linked immunoabsorption assay was performed comparing the binding of OX-7, pg22, and ROCA1 (an IgG directed against a 65 kD glial surface protein (*222*)) to BSA (Sigma), transferrin (Sigma), and murine Thy-1.1 (from the conditioned medium of a thymoma line that secretes the protein (*39, 70*); affinity purified on an immobilized OX-7 column matrix (*26*)). Triplicate wells of a 96-well flexible assay plate were coated with 100 ng of BSA, transferrin, or Thy-1 for 2 hrs. at room temperature, blocked with 5% non-fat dry milk (Carnation) in Tris-buffered saline, and then incubated with the hybridoma culture supernatants of OX-7, pg22, or ROCA1 at 4^{o} overnight. After rinsing with Tris-buffered saline, the wells

were incubated with a 1:500 dilution of alkaline phosphatase-conjugated goat anti-mouse IgG (Boehringer Mannheim) in the 5% milk buffer for 1 hr at room temperature, rinsed, developed using p-nitrophenylphosphate (Sigma) as chromogen, and the absorbance of the reaction product measured at 405 nm. The binding of pg22 to all three proteins (.191±004 absorbance units ± standard error of the mean on BSA, .178±.006 on transferrin, and .251±.015 on Thy-1) was nearly identical to that of ROCA1 (.206±.012 on BSA, .190±.003 on transferrin, and .235±.006 on Thy-1). OX-7, however, did display specificity for Thy-1 (.193±.007 on BSA, .170±.007 on transferrin, and .416±.005 on Thy-1). These data indicate that pg22 is directed primarily against HeSPG, as originally characterized. Additional evidence that pg22 does not bind to Thy-1 is that it does not protect Thy-1 from PI-PLC treatment as do anti-Thy-1 antibodies (see below).

Metabolic Labeling and Immunoprecipitation.

Chromaffin cells were cultured for 24 hrs as described, and then transferred for a further 24 hrs into methionine-deficient L15-CO_2 medium supplemented with 5% dialyzed rat serum, 100 μCi/dish of ^{35}S-methionine (Amersham Corp.), and unlabeled methionine to a final concentration of 10 μM. After three washes with serum-free L15-CO_2, the cells were given serum-free medium with and without affinity-purified OX-7 at a final concentration of 20 μg/ml and incubated for 2 hr. From the two conditions, culture supernatants were collected and 0.5 ml of each incubated with 20 μl (packed volume) of Protein G-Sepharose beads (Pharmacia) coupled to either non-immune rabbit serum, or rabbit anti-Thy-1 antiserum, for 1 hr at room temperature with end-over-end mixing. The bead were pelleted and washed by the method of Wolitzky and Fambrough (*241*), boiled in 30 μl of gel sample buffer, and the resultant solution subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli (*124*). After equilibration in 20% w/v 2,5-diphenyloxazole (Sigma) in acetic acid, the gels were washed in distilled water, dried, and exposed to X-ray film (X-OMAT, AR; Eastman Kodak) for fluorography.

Quantification of Cell Surface Thy-1

Radioiodination of 100 μg of anti-Thy-1 Fab fragments, prepared as described, was performed with 1 mCi of $Na^{125}I$ (Amersham) using Iodogen (Pierce Chemical) as per manufacturer's protocol, and resulted in a specific activity of 3×10^{15} cpm/mmol of Fab. PC12 cells were cultured as described in collagen-coated, 96-well, flat bottom, flexible assay plates (MicroTest III; Falcon) at a concentration of 8×10^5 cells/well. Quantification of cell surface Thy-1 was performed by incubating triplicate wells with 5 μg/ml of ^{125}I-Fab in 50 μl of L15 medium for 1 hr at room temperature, rinsing 3 times with 200 μl of L15, cutting the individual wells apart with scissors, and assayed total bound Fab in each well using a γ-ray emission counter (Beckman). When determining the amount of Thy-1 removed by PI-PLC, cells were first incubated in 1:2000 dilution of PI-PLC in L15-CO_2 for 1 hr at 37°, and then probed with ^{125}I-Fab. To measure the effect of anti-Thy-1 Fab on PI-PLC removal of Thy-1, cells were simultaneously incubated in ^{125}I-Fab and 1:2000 PI-PLC. Non-specific binding was determined by incubating the cells with ^{125}I-Fab in the presence of 250 μg/ml unlabeled Fab. Data are expressed as a percentage of Thy-1 expressed by untreated cells.

Mutagenesis and Selection of Thy-1-Deficient PC12 Cells

Following the procedure of Matthew (*151*), approximately 1×10^7 cells were plated per 100 mm tissue culture dish, exposed to 400 μg/ml ethylmethane sulfonate (EMS) for a total of 20 hr, having exchanged the medium for fresh EMS-containing medium at 10 hr, and then grown for 2 days in normal medium. Since Thy-1 is trypsin-resistant, and complement would be inhibited by cell surface HeSPG, the cells were lightly treated with trypsin (0.25% w/v in PBS, 37° for 5 min). The cells were then incubated in anti-Thy-1 mAb (19XE5) hybridoma supernatant for 45 min at 4°. After rinsing once with cold PBS, the cells were placed in newborn rabbit complement (Pel-Freez Biologicals), diluted 1:10 in serum-free culture medium, for 1 hr at 37°. Cells were rinsed once with regular culture medium and then returned to the incubator in 50% fresh PC12 cell

culture medium (see above)/50% medium conditioned by confluent PC12 cultures for 24 hrs. This complement treatment was repeated again after 7 days. 10 days after the last complement treatment, dishes were fixed with 4% paraformaldehyde in PBS, incubated with anti-Thy-1 mAb (OX-7) ascites fluid diluted 1:1000 for 45 min at 4°, followed by a 45 min incubation with FITC-conjugated goat anti-mouse IgG (Tago) at 4°, and viewed by epifluorescence microscopy. This experiment was performed twice, with 2 dishes being analyzed per experiment.

Counting of Process-Bearing Cells

Cells were counted and scored by phase contrast microscopy at a total magnification of 160x. In the case of antibody perturbation and phospholipase C experiments, all cells in a given dish were counted and total cell number per dish varied from approximately 500 to 800 cells. Individual experiments included 2 or 3 dishes per culture condition and the data presented are the means (± the standard error of the mean) from a minimum of three separate experiments for antibody perturbation, and two separate experiments for phospholipase C treatment. Thus for each data point, the total number of individual cultures scored varied from 6 to 12. Cells were scored as process-bearing if they possessed a neurite greater than two cell diameters in length. In the case of the mutagenized PC12 cells, cells were examined by both phase contrast and epifluorescence microscopy at a magnification of 160x and scored for both neurites and staining by OX-7. Total numbers of mutagenized cells counted are presented in the Results.

Results

Antibodies against Thy-1 specifically induce neurite outgrowth by binding to the cell surface. In an effort to clarify the response of neurons to anti-Thy-1 antibodies, neonatal rat superior cervical ganglion neurons were cultured in serum-free medium either in the

absence or the presence of soluble, affinity-purified, mAbs directed against Thy-1. As can be seen in Fig. 2.1a, three different mAbs to Thy-1 (OX-7, 2G12, and 19XE5) specifically increase the number of sympathetic neurons bearing processes by 30% to 50% after twenty-four hours in culture. These increases are normalized as percentage increases due to variability in the control level of neurite outgrowth from experiment to experiment; the absolute percent of process-bearing neurons under control conditions after twenty-four hours varies from 20% to 60% and was most likely due to variability of the collagen substrate. In addition, neurons were cultured in the presence of mAbs directed against HeSPG (*152*), Nerve growth factor Inducible Large External glycoprotein (NILE) (*223*), and the rat Class I major histocompatibility complex antigen (MHC I) (*79*), all of which are observed by indirect immunofluorescence to stain these cells at comparable intensity. MAbs directed against these proteins, pg22 (anti-HeSPG), ASCS4 (anti-NILE), and OX-18 (anti-MHC I) have negligible effects on outgrowth (Fig. 2.1a). Like Thy-1 (*103*, *141*, *228*), a portion of the cell surface HeSPG is thought to be linked to the membrane by a glycophosphatidylinositide tail (*27*, *114*). The anti-MHC I mAb OX-18 is thought to recognize an invariant epitope on a large number of otherwise highly variable MHC I and MHC I-like molecules (*79*). In the mouse, a subset of the latter, known as the Qa and Tla antigens, is also known to be attached to the cell membrane by a glycophosphotidylinositide tail (*220*). Thus, the anti-HeSPG and anti-MHC I antibodies serve as particularly good controls for the perturbation of Thy-1.

Unfortunately, the requirement for NGF as a trophic agent for primary neurons raises the control baseline values in the assay because it induces neurite outgrowth in the absence of antibodies. Therefore antibody perturbation of Thy-1 was also performed with primary adrenal chromaffin cells, as well as a cell line of adrenal medullary origin, PC12 (*90*). Both cell types are capable of neurite initiation in response to NGF, but do not require it for survival *in vitro* (*57*, *90*, *229*). The effects of the anti-Thy-1 and control mAbs could thus be examined in the absence of NGF. After four days in culture (the time at which the peak effect is observed, and after which chromaffin cells die off rapidly in the absence of

NGF or glucocorticoids (*57*)), the incidence of chromaffin and PC12 cells displaying spontaneous neurite sprouting in control dishes lacking mAbs is approximately 1% of the population. In striking contrast, both primary cells (Figs. 2.1b) and PC12 cells (Fig. 2.1c) display a two- to five-fold increase in the number of process-bearing cells in the presence of anti-Thy-1 mAbs. As in the case of neurons, variability in the control level of sprouting necessitated the normalization of the data in terms of percent increase. The effect of the anti-Thy-1 mAbs was reproducible, and in some cases, the stimulation in the number of sprouting cells is as high as seven- to ten-fold. As with neurons, mAbs directed against control cell surface antigens had little effect on chromaffin and PC12 cells. Since pre-incubating the collagen substrate with the anti-Thy-1 mAbs does not yield enhanced outgrowth (data not shown), it is unlikely that the positive effects observed with soluble mAbs can be attributed to enhanced substrate adhesion by these cells. Indeed, all of these cells are well attached in the absence of antibodies, and can survive for weeks under appropriate conditions (*100, 240*). Thus, in the absence of NGF, the binding of anti-Thy-1 antibodies to the cell surface is sufficient to initiate sprouting of neurites.

The concentration of mAbs used in the above assays, 5 μg/ml, approximates the minimum concentration of mAb required for bright, immunofluorescent staining of these cell types as determined by titration of the corresponding ascites fluids or hybridoma culture supernatants (data not shown). Dose-response studies of the mAb OX-7 on both neurons and primary chromaffin cells demonstrate that this concentration of mAb is sufficient to promote maximal neurite outgrowth and sprouting (Fig. 2.2).

Morphological assessment of the cells served as the primary criterion by which the effects of the antibodies were judged. Cellular extensions two or more cell body diameters in length were scored as neurites in the assays. Figure 2.3 shows representative fields of chromaffin and PC12 cells cultured for 4 days under control conditions (Fig. 2.3a and d), in the presence of OX-7 (Fig. 2.3b and e), and for comparison, in the presence of

1 μg/ml of 7S NGF (Fig. 2.3c and f). Since the processes induced by NGF are significantly longer than those induced by OX-7, the mAb appears to initiate neurite sprouting, but is not capable of inducing the outgrowth of long processes that accompanies NGF-induced differentiation to a fully neuronal phenotype.

Multivalent anti-Thy-1 mAbs are necessary for neurite induction. Previous studies have shown that Fab fragments of anti-Thy-1 mAbs are not capable of inducing T lymphocyte activation (*122*, *127*). We find the same result with neurite outgrowth. The data in Figure 2.4 demonstrate that Fab fragments of the OX-7 mAb do not enhance neurite outgrowth from sympathetic neurons, nor do they induce sprouting from adrenal chromaffin cells. This lack of induction is not due to a loss of the ability of the mAbs to bind to the cell surface since binding can be verified by indirect immunofluorescence microscopy (data not shown). Moreover, the sprouting activity can be reconstituted by incubating the cells in the anti-Thy-1 Fab and a crosslinking, goat anti-mouse immunoglobulin antiserum (Fig. 2.4). In the case of the chromaffin cells (Fig. 2.4b), a nearly complete restoration of the sprouting effect is achieved. Adding secondary antiserum alone has no effect. Crosslinking of intact, divalent, OX-7 mAb by secondary antiserum displayed little further effect on neurite sprouting (data not shown), thus divalence of the mAb is sufficient. As in the case of the T lymphocytes then, the antibody effect displays specificity, and multivalent binding of Thy-1 is necessary.

Treatment of chromaffin cells with anti-Thy-1 mAb results in the shedding of Thy-1. Multivalent mAbs could cause the capping and internalization of Thy-1 and/or the shedding of the protein into the culture medium. To investigate the latter possibility, chromaffin cells were metabolically labeled with ^{35}S-methionine and treated with the anti-Thy-1 mAb OX-7 for 2 hrs. SDS-PAGE analysis of labeled protein in the culture medium which had been immunoprecipitated using polyclonal anti-Thy-1 antiserum, reveals the appearance in the medium of 28kD and 50kD Thy-1-immunoreactive proteins after OX-7 treatment (Fig. 2.5). The low

molecular weight band corresponds to the position we and others observe for Thy-1 by SDS-PAGE (*26*), while the higher band does appear in published immunoprecipitations of T cell Thy-1 (*94*, *144*), and has been demonstrated by us to correspond to a Thy-1 dimer that is stable to boiling in SDS and β-mercaptoethanol (analysis of the dimer is presented in Chapter 3). This mAb-induced shedding of Thy-1 and Thy-1 dimer occurs as quickly as 10 min after OX-7 treatment (data not shown), and we find no evidence of antigen capping.

PI-PLC induces neurite outgrowth from chromaffin and PC12 cells by a Thy-1-specific mechanism. As an alternative means of removing Thy-1 from the cell surface, chromaffin cells and PC12 cells were treated with PI-PLC. As can be seen in Fig. 2.6, a biphasic dose response curve is observed for the induction of neurite sprouting with a maximal effect achieved at an enzyme dilution of 1:2000. Control cultures incubated with corresponding dilutions of the glycerol-containing buffer in which the PI-PLC is stored, show no significant sprouting. The effect appears to depend on a specific interaction of PI-PLC with Thy-1, since Fabs produced from a rabbit anti-rat Thy-1 antiserum block the enzyme-induced outgrowth, but the anti-HeSPG mAb pg22 appears additive (Fig.2.7). As determined by the binding of anti-Thy-1 ^{125}I-labeled Fab fragments to PC12 cells (Fig. 2.8), PI-PLC removes approximately 70% of cell surface Thy-1, while the presence of anti-Thy-1 Fabs results in only 30% removal of surface Thy-1. Scatchard analysis shows that untreated PC12 cells express approximately 1,500 Thy-1 molecules/μm^2 (data not shown).

Thy-1-deficient PC12 cells grow neurites spontaneously. Since PC12 cells respond to anti-Thy-1 mAbs in a fashion similar to that of primary adrenal chromaffin cells, they are a useful cell line in which to manipulate Thy-1 levels and observe the effects on neurite outgrowth. In an effort to generate mutant cell lines deficient in Thy-1 expression, cells were treated with the mutagen, ethylmethane sulfonate, followed by two rounds of complement-mediated lysis with anti-Thy-1 antibodies. The

resultant cells were then allowed to grow for ten days, and assayed for Thy-1 expression by immunohistochemical staining with the OX-7 mAb. In the unmutagenized, wild type population, Thy-1$^-$ cells comprise 0.6%±0.2% (s.e.m.; n=1,922) of the cells. After mutagenesis and selection, the proportion of Thy-1$^-$ cells rises sixteen-fold to 9.8%±0.2% (n=1,218). Our attempts to clone the Thy-1$^-$ cells have not been successful; following identified cells over the course of several days revealed that the Thy-1$^-$ cells were no longer dividing. This finding, coupled with the fact that complement-mediated lysis results in only 90% killing, explains the fact that the majority of the mutagenized and selected population are Thy-1$^+$ several days after treatment. Most importantly, 88.9%±3.4% of mutagenized and selected Thy-1$^-$ cells spontaneously display the morphology characteristic of the initial stage of neurite sprouting (Figs. 2.7 and 2.8). The remainder of the Thy-1$^-$ cells often assumed a round and flattened morphology (Figs. 2.7c and 2.7d) sometimes observed as an alternative response to NGF in unmutagenized PC12 cells. In contrast, less than 1% of Thy-1$^+$ cells sprout neurites (Fig. 2.8). Examination of the very minor population of Thy-1$^-$ cells in the unmutagenized control PC12 cell population revealed that two-thirds (66.2%±13.4%) of these cells also spontaneously sprout neurites.

Discussion

The induction of neurite outgrowth by mAbs, PI-PLC, and mutation/selection may result from the release of an inhibition normally exerted by Thy-1. Initial studies on anti-Thy-1 antibody-induced process outgrowth from neurons (*129*, *159*), as well as studies using such antibodies to induce T lymphocyte activation (*121*, *122*), suggested that Thy-1 may normally serve as a receptor for an unknown ligand that provides a positive stimulus for various cellular responses. Our results, utilizing soluble antibodies to induce outgrowth from several neural crest derivatives (*128*), are consistent with such an idea. The PI-

PLC-induced outgrowth is also consistent with such a hypothesis, since PI-PLC treatment can induce increases in second messengers (*193*). The mutagenesis results, however, suggest an alternative interpretation consistent with all of the data. The correlation between the loss of cell surface Thy-1 and the generation of neurite sprouting in the mutagenesis and PI-PLC experiments, raises the possibility that Thy-1 normally inhibits sprouting. By this hypothesis, multivalent antibody binding also abrogates this function, resulting in enhanced sprouting. Though multivalent antibody binding is often found to promote antigen capping and internalization, we found no evidence for this with the neurons. Rather, anti-Thy-1 mAbs induce the release of some of the protein into the culture medium (Fig. 2.5), providing an alternative mechanism for removal of Thy-1 from the cell surface.

In the case of mAb-induced sprouting of neurites from chromaffin and PC12 cells, a relatively small subset of the total population of cells responds, an average 2%-5% (10% in some experiments). Since this effect is reproducible, it may reflect an underlying heterogeneity among these cells in their ability to express the latent neuronal phenotype. Doupe et al. (*57*) found that when cultured under conditions identical to those used in the study, 10%-40% of chromaffin cells extend neurites in the presence of 1 μg/ml NGF; at a concentration of 0.1 μg/ml, the number of process-bearing cells fell by 50%. In the presence of both 1 μg/ml NGF and serum-free, heart cell conditioned medium, however, the fraction of sprouting cells increased by 60% to 100%. These results support the notion that the chromaffin cell population is heterogeneous, and that multiple factors are required to enable a majority of the cells to sprout neurites. Similar experiments have revealed heterogeneity of responsiveness among PC12 cells (*204*). The reproducibly small subset of chromaffin and PC12 cells that sprout in response to Thy-1 perturbations may be cells with patterns of gene expression or metabolism that are slightly further towards a neuronal phenotype on a continuum of phenotypes between the chromaffin cell and sympathetic neuron (*57*).

Thus, only a slight inhibition must be overcome in order to sprout, and this is mediated by mAbs and PI-PLC.

Our observation that the absence of surface Thy-1 on mutant PC12 cells is strongly correlated with the initiation of process outgrowth, is tempered by the relatively high frequency with which such mutant cells arose, even in the absence of mitoses by the Thy-1$^-$ cells. The observed sixteen-fold increase in the Thy-1$^-$ population after mutagenesis could be due in part to mutations outside the Thy-1 gene itself. In the case of Thy-1$^-$ T lymphoma mutants, lack of Thy-1 expression results primarily from defects in post-translational modifications, changes that do not affect the health of the cells (*109*). If the Thy-1$^-$ PC12 population is, in fact, composed of cells with numerous, diverse genetic lesions all leading to a Thy-1 deficiency, it is quite striking that nearly all of these cells spontaneously sprout neurites.

Possible mechanisms of a Thy-1-mediated inhibition of sprouting. Previous histological studies on the developmental modulation of Thy-1 expression in the vertebrate nervous system provide support for the hypothesis that Thy-1 exerts an inhibition on neurite sprouting and outgrowth. Thy-1 levels gradually increase during axonal outgrowth, reaching peak levels only after the major period of synaptogenesis has been completed (*165*). There is also recent evidence that, while neurons can accumulate Thy-1 mRNA during development, Thy-1 protein appears only after the cessation of axonogenesis (*244*). A striking exception to this generalization is the primary olfactory axon which does not express Thy-1 at high levels at any stage (*166*); this is the only class of neurons known to continually generate long axons in the adult vertebrate (*89*). It is possible that process or membrane stabilization is not desirable or necessary in this case because of the continuous renewal of cells and connections.

In considering possible mechanisms by which Thy-1 could stabilize neurites and inhibit sprouting, the more general question arises as to how lipid-linked membrane proteins mediate ligand-binding effects. There are a number of examples of second messenger changes induced by ligand-

binding to such proteins. The most relevant observations are that lymphocyte activation by anti-Thy-1 mAbs is accompanied by an increase in intracellular free calcium (*122*, *127*), and that anti-Thy-1 mAbs may elicit transient calcium currents across the membranes of rat sensory neurons (*202*). It has been recently reported that Thy-1 in T cell lines is co-immunoprecipitated with the tyrosine phosphatase, CD45, suggesting that this protein could mediate signal transduction (*230*). There is also evidence that Thy-1 is associated with the actin cytoskelton (*76*, *191*), as well as with a band 4.1-like protein in T lymphoma cells (*20*). These data are compatible with the possibility that Thy-1 mediates signals through the cytoskeleton. Our observations suggest that mAb binding and PI-PLC treatment could lead to Thy-1 cleavage and release from the membrane inducing, in turn, second messenger changes related to those just cited. In the case of Thy-1$^-$ PC12 cells, a lack of cell surface Thy-1 could result in alterations of the constituitive levels of these second messengers.

Purified, as well as native membrane-bound Thy-1 can form heat-, detergent-, and disulfide reduction-resistant dimers (*107*, *146*, *243*) as well as higher multimers (this is discussed in great detail in Chapter 3). If Thy-1 is acting as a receptor for a soluble signal, it is possible that signal transduction requires multimerization of Thy-1, and thus multivalent mAb binding promotes such an event while monovalent Fab binding does not. It is also possible that if such signalling is dependent on a PI-PLC pathway, then treatment with exogeneous PI-PLC treatment can mimick antibody binding in a Thy-1-dependent manner. In light of the results from analysis of the Thy-1-deficient PC12 cell mutants, however, it seems more simple to suggest that Thy-1 constituitively inhibits outgrowth, and it is removal that is germane to sprouting.

Homophilic binding is a recurrent theme in the immunoglobulin superfamily (*107*, *236*), and apposed axon membranes could interact by way of intercellular Thy-1:Thy-1 binding. Alternatively, considering the extraordinarily high concentrations of Thy-1 in the adult nervous system (*1*), homophilic interactions among the Thy-1 molecules present on the axon surface could form a kind of "shell" serving to sterically buffer the membrane from surface perturbations. In either case, the removal of Thy-

1 from the cell surface by the various means employed in this study would also serve to disrupt Thy-1:Thy-1 homophilic interactions. The shedding of Thy-1 might also result in local membrane destabilization or mobilization of intramembranous proteins such as that which occurs in PC12 cells within minutes of exposure to NGF (*38*). Interestingly, exposure to NGF causes an up-regulation of Thy-1 expression in PC12 cells, which is maximal after 7-8 days of treatment (*191*). This up-regulation of Thy-1 is an event that occurs well after initial sprouting and is compatible with the molecule's proposed role in stabilizing established neurites.

Heterophilic ligand candidates for Thy-1 have been identified through the use of anti-idiotype reagents (*60, 77*). In addition, Thy-1 binds laminin and fibronectin (*137*), sulfated polysaccharides (*173*), and the growth cone protease, plasminogen activator (*137, 234*). Thus, neuronal Thy-1 could interact with a number of proteins known to regulate cell migration and neurite outgrowth. Here also, the removal of Thy-1 from the neuronal cell surface could modulate sprouting and outgrowth.

In the immune system, the highest expression of Thy-1 is observed within the thymus, both on lymphocytes and on support cells (*165*). Such a localization is consistent with an adhesion function, and may thus facilitate the intercellular interactions necessary for lymphoid maturation events in the thymus (*74*). In testing such an idea, it will be worth considering the hypothesis presented here, that Thy-1 may be normally acting as an inhibitor of particular cellular events.

FIGURE LEGENDS

Figure 2.1: Percentage increase of process-bearing cells after culture in the presence of anti-Thy-1 mAbs. Cells were counted while observed by phase contrast microscopy either 1 day, for neurons, or 4 days, for chromaffin and PC12 cells, after being placed in culture. Values are expressed as the percent increase in the number of process-bearing cells over the average number of process-bearing cells appearing in the absence of mAbs. Each bar represents the average of 4 to 8 dishes, each containing approximately 400 cells; error bars represent the standard error of the mean. (**A**) Neonatal rat superior cervical ganglion neurons, (**B**) neonatal rat chromaffin cells, and (**C**) PC12 cells. Hatched bars represent anti-Thy-1 mAbs (OX-7, 19XE5, and 2G12), and open bars represent control mAbs (pg22, anti-HeSPG; ASCS4, anti-NILE; and OX-18, anti-MHC I).

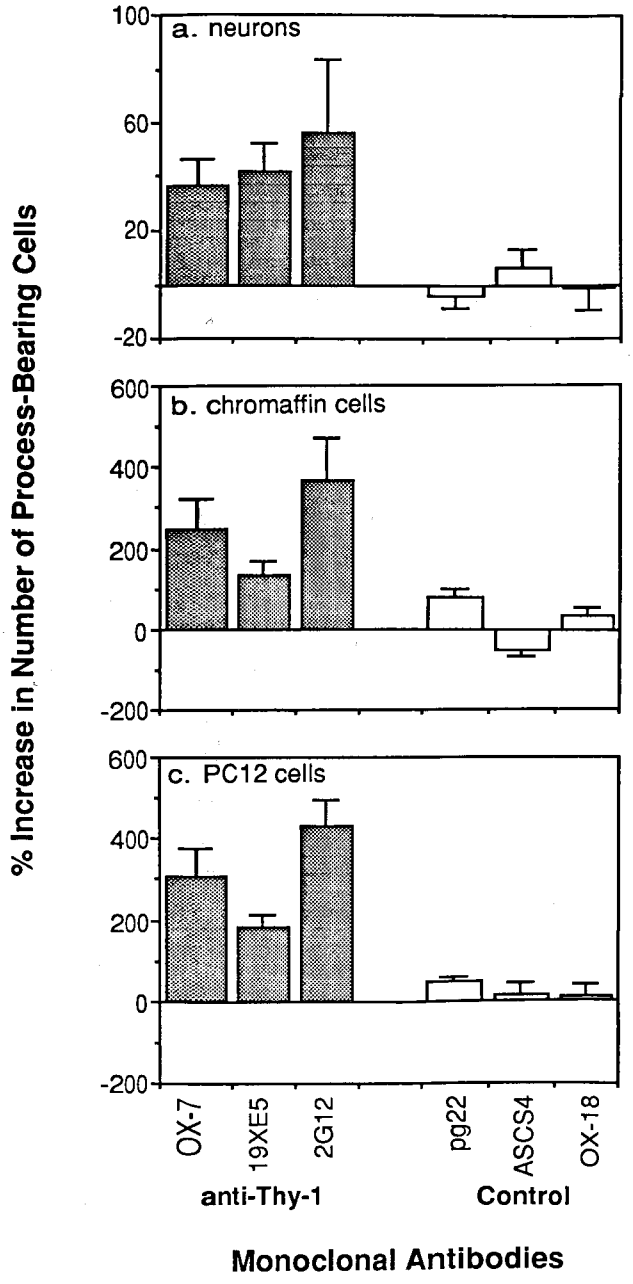

% Increase in Number of Process-Bearing Cells
a. neurons
b. chromaffin cells
c. PC12 cells
100
60
20
-20
600
400
200
0
-200
OX-7
19XE5
2G12
pg22
ASCS4
OX-18
anti-Thy-1
Control
Monoclonal Antibodies

Figure 2.2: The dose response of OX-7's effect on neurite outgrowth is similar for both neurons and chromaffin cells. Open squares represent neuronal responses, dark squares those of chromaffin cells.

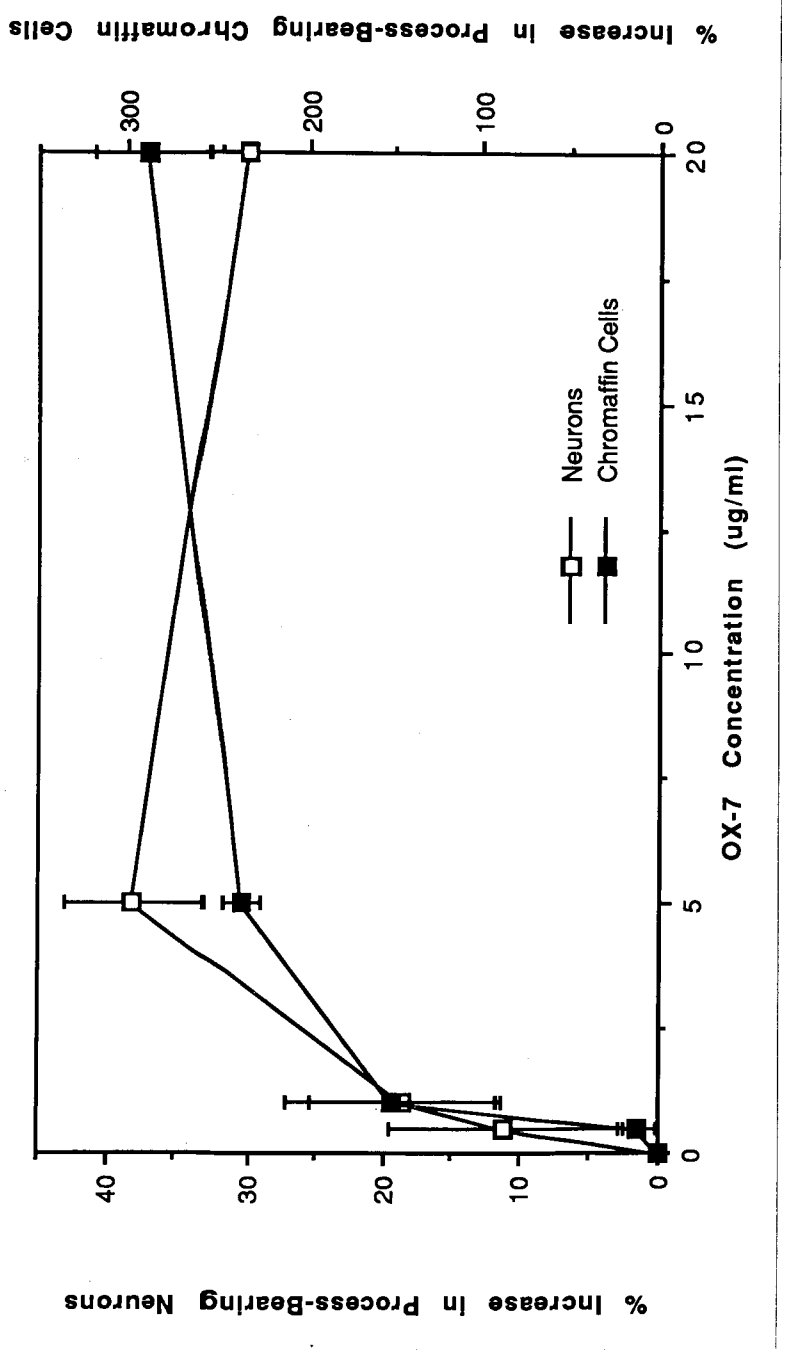

% Increase in Process-Bearing Chromaffin Cells
300
200
100
0
0
5
10
15
20
OX-7 Concentration (ug/ml)
Neurons
Chromaffin Cells
% Increase in Process-Bearing Neurons
40
30
20
10
0

Figure 2.3: Neurite outgrowth induced by anti-Thy-1 mAbs and NGF. Chromaffin cells (**A-C**) and PC12 cells (**D-F**) were cultured as described in Figure 1. In the absence of additional factors, little neurite sprouting is observed (**A** and **D**), but in the presence of 5 ug/ml anti-Thy-1 mAb (OX-7) (**B** and **E**) or 1 ug/ml 7S NGF (**C** and **F**) neurite formation is observed (arrows). Scale bar equals 50 um.

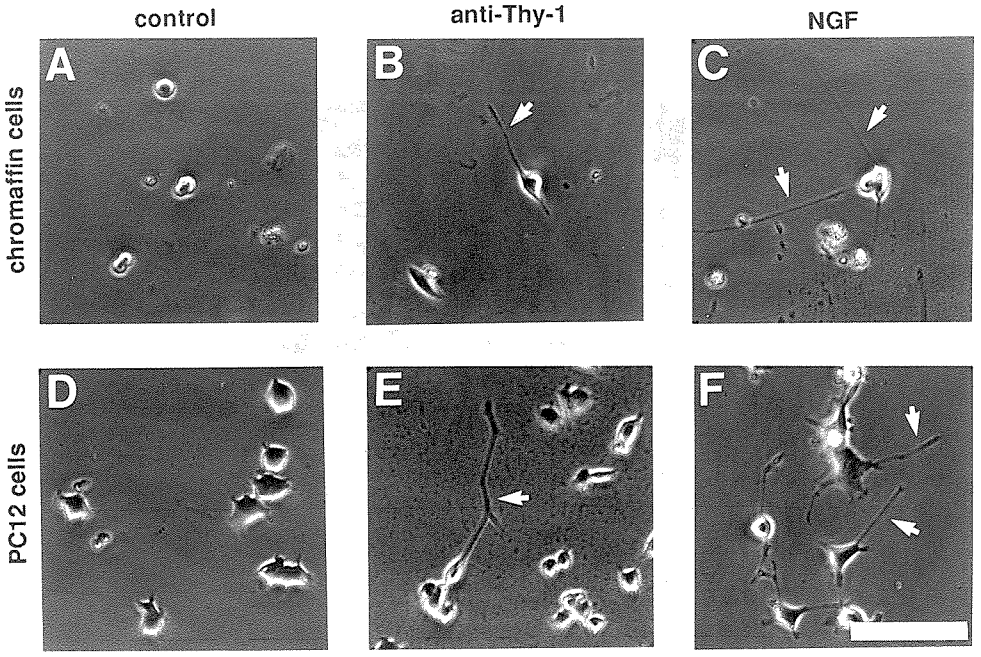

control
anti-Thy-1
NGF
chromaffin cells
PC12 cells
A
B
C
D
E
F

Figure 2.4: Anti-Thy-1 Fabs do not induce neurite outgrowth, but the addition of secondary, crosslinking antiserum reconstitutes the effect. Neurons (**A**) and chromaffin cells (**B**) were cultured as in Figure 1 in the presence of 5 μg/ml anti-Thy-1 mAb (OX-7 IgG), 3.3 ug/ml anti-Thy-1 Fab (a concentration giving a net valence equivalent to the OX-7 IgG; OX-7 Fab), 5 ug/ml goat anti-mouse gamma globulin (Antibodies, Inc.) plus anti-Thy-1 Fab (Fab + 2^o), and the goat anti-mouse gamma globulin alone (2^o alone). Bars represent the percent increase in process-bearing cells over control dishes receiving no antibody and are the average values of 3 to 6 dishes, each containing 100 to 200 cells; error bars represent the standard error of the mean.

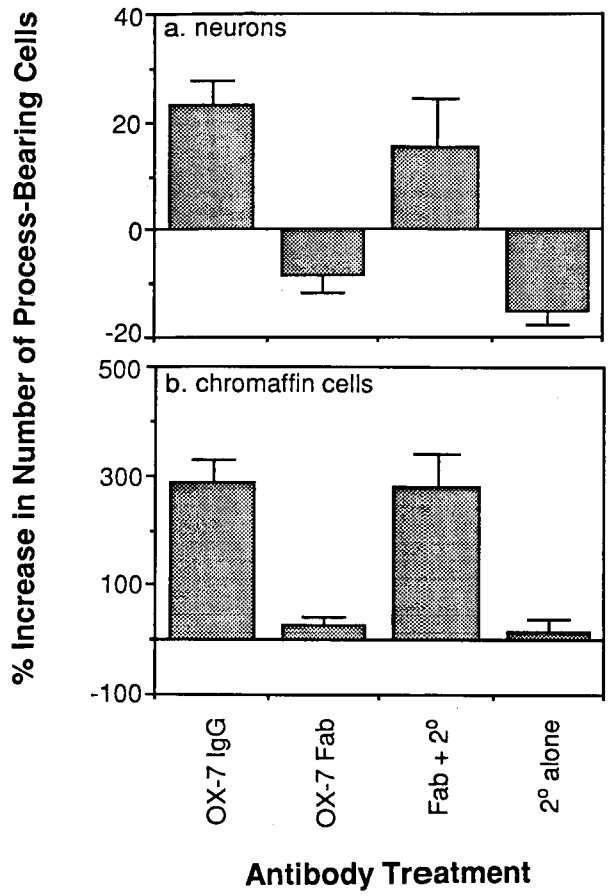

% Increase in Number of Process-Bearing Cells
40
20
0
-20
a. neurons
500
300
100
-100
b. chromaffin cells
OX-7 IgG
OX-7 Fab
Fab + 2°
2° alone
Antibody Treatment

Figure 2.5: Anti-Thy-1 mAb treatment of chromaffin cells results in the shedding of Thy-1 and a Thy-1 dimer. Cells were labeled overnight with ^{35}S-Met, rinsed, and cultured in the presence (lanes 2 and 4) or absence of OX-7 (lanes 1 and 3). Supernatants were then subjected to immunoprecipitation with either polyclonal anti-Thy-1 antiserum (lanes 1 and 2) or with nonimmune rabbit serum (lanes 3 and 4). Arrows, from top to bottom, designate molecular weights of 68 kD, 50 kD, and 28 kD.

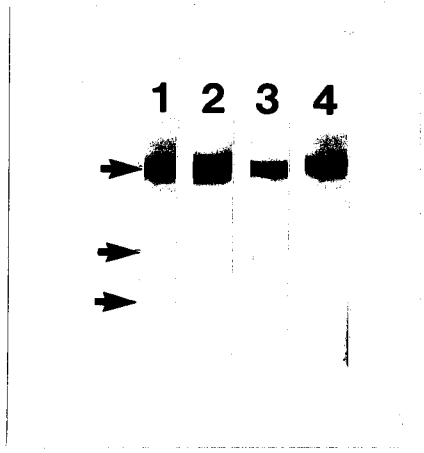

1 2 3 4

Figure 2.6: PI-PLC gives a biphasic dose response for neurite outgrowth from chromaffin (upper panel) and PC12 cells (lower panel). Stippled bars represent dilutions of PI-PLC, open bars represent comparable dilutions of the glycerol-containing buffer alone.

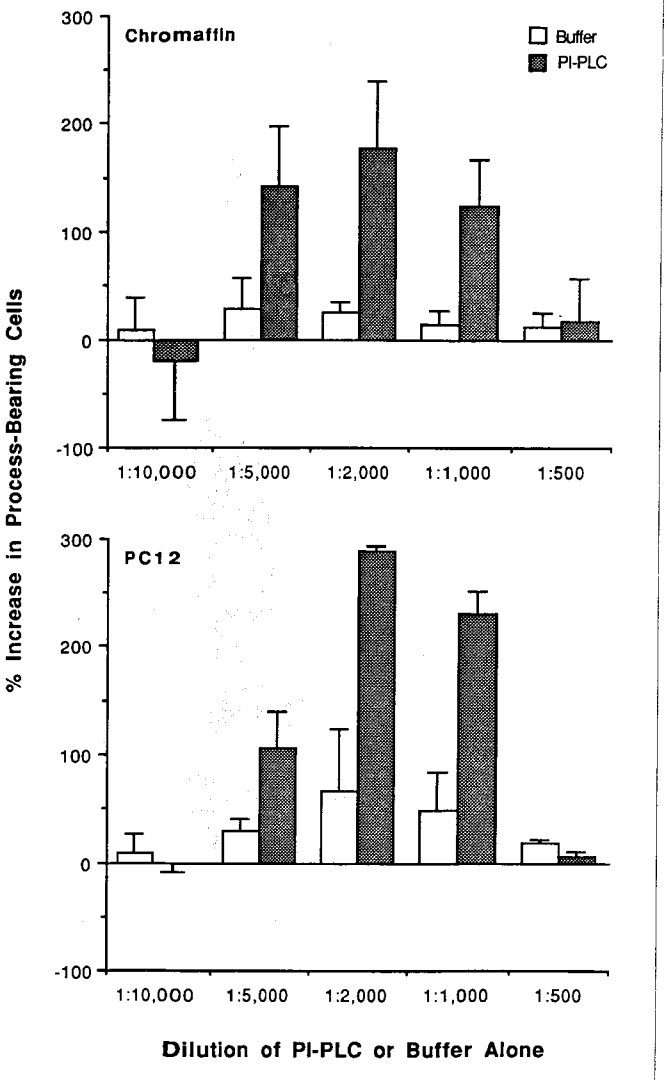

Chromaffin
Buffer
PI-PLC
300
200
100
0
-100
1:10,000
1:5,000
1:2,000
1:1,000
1:500
PC12
300
200
100
0
-100
1:10,000
1:5,000
1:2,000
1:1,000
1:500
% Increase in Process-Bearing Cells
Dilution of PI-PLC or Buffer Alone

Figure 2.7: Fab fragments directed against Thy-1 block PI-PLC-induced outgrowth from chromaffin (open bars) and PC12 cells (stippled bars), while anti-HeSPG mAb pg22 shows an additive effect of outgrowth.

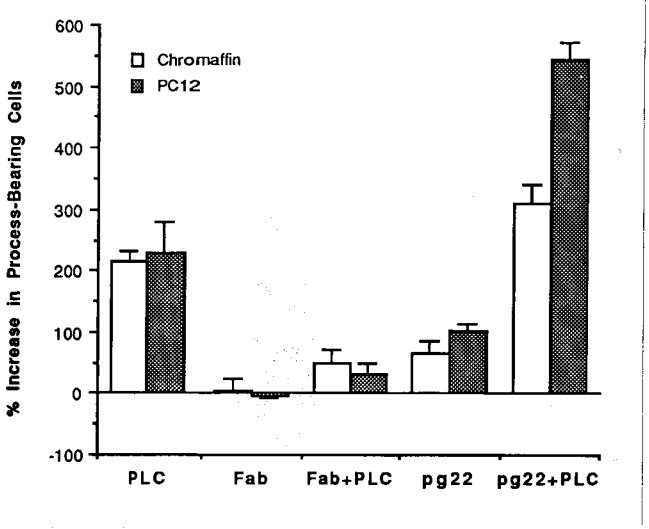

% Increase in Process-Bearing Cells
600
500
400
300
200
100
0
-100
Chromaffin
PC12
PLC
Fab
Fab+PLC
pg22
pg22+PLC

Figure 2.8: Anti-Thy-1 Fab fragments inhibit PI-PLC removal of Thy-1 from the PC12 cell surface. Cell surface Thy-1 was quantified by the binding of polyclonal 125I-Fabs directed against Thy-1 under control conditions, after PI-PLC treatment, or during PI-PLC treatment. Error bars represent the standard error of the mean.

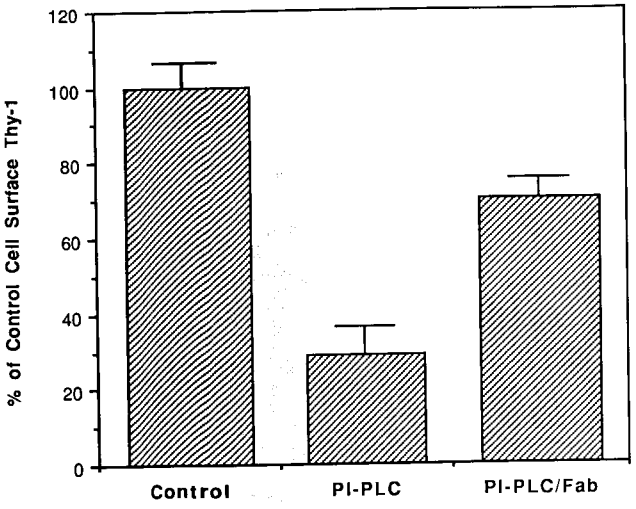

% of Control Cell Surface Thy-1
120
100
80
60
40
20
0
Control
PI-PLC
PI-PLC/Fab

Figure 2.9: Thy-1-deficient PC12 cells show enhanced spontaneous sprouting. Representative fields exhibiting Thy-1-deficient cells are shown in phase contrast optics (**A** and **C**) with their corresponding epifluorescence images after staining with the anti-Thy-1 mAb, OX-7 (**B** and **D**), arrows denote deficient cells. Scale bar equals 50 m.

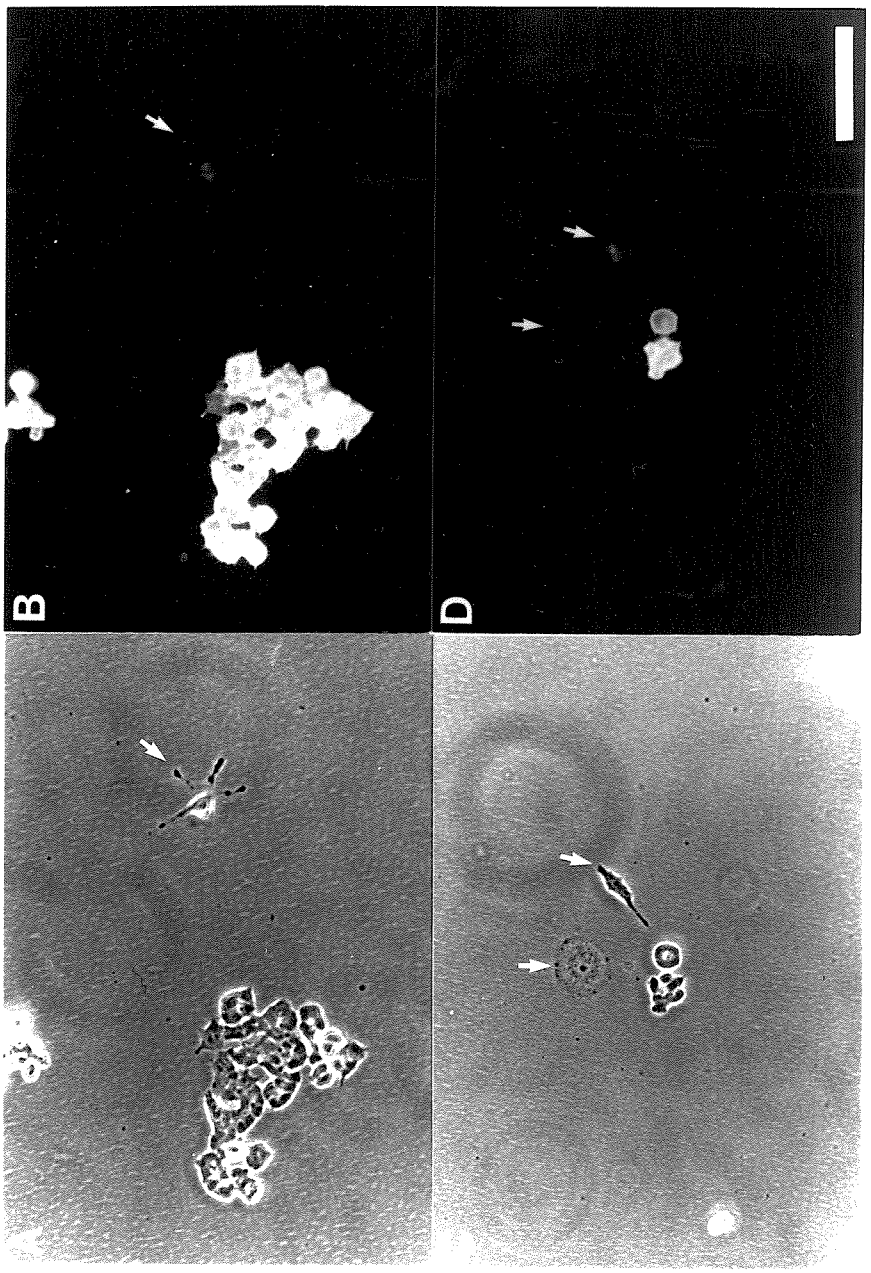
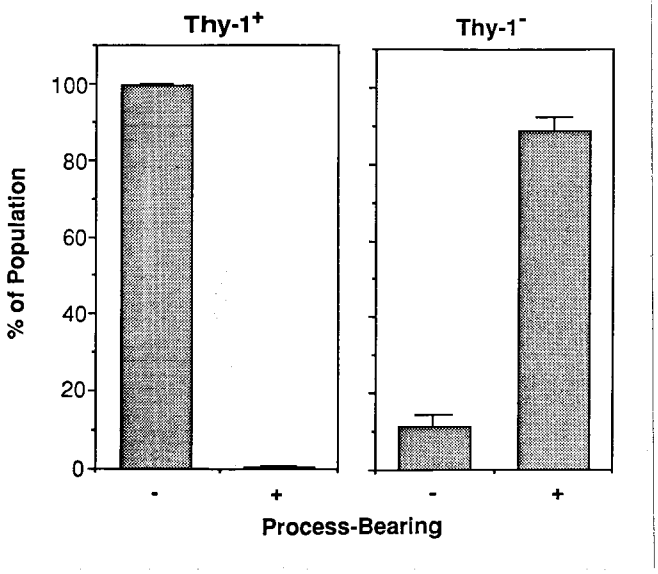

B
D

Figure 2.10: The majority of mutagenized Thy-1-deficient PC12 cells show spontaneous neurite outgrowth. EMS-mutagenized PC12 cells, as described in Figure 6, were counted and scored for Thy-1 staining and the presence of neurites. Two 100 mm dishes each containing approximately 500 cells were counted. The left panel displays the results from all cells scored as Thy-1 positive, and the right panel displays the results from cells scored as Thy-1 negative. "-" denotes cells lacking neurites and "+" denotes cells bearing neurites. Bars represent the percent of the population bearing the given phenotype and the error bars represent the standard error of the mean.


Thy-1+
Thy-1-
100
80
60
40
20
0
% of Population
-
+
-
+
Process-Bearing

Chapter 3:

THY-1 MULTIMERIZATION IS CORRELATED WITH NEURITE OUTGROWTH

Introduction

Thy-1 is a cell surface glycoprotein first characterized as an antigen on the surface of murine T cells (*190*). It is expressed ubiquitously in the nervous system and expressed most abundantly on the axons of long projection neurons. Thy-1 is thought to be the smallest member of the immunoglobulin superfamily (IgSF) (*107, 236*), and homologues have been identified in a variety of species (for review see Ref.*165*). Monoclonal antibodies (mAb) against Thy-1 can induce T cell activation (*94, 121, 144*), and enhance neurite outgrowth from neurons and neuron-like cells (Chapter 2). Furthermore, we have demonstrated that removal of Thy-1 from the cell surface by phosphotidylinositol-specific phospholipase C (PI-PLC), or prevention of Thy-1 expression by mutation, also promotes neurite sprouting (see Chapter 2). These data, and the observation that Thy-1 expression *in vivo* is maximal after the initial period of neurite outgrowth (*165*), suggested the hypothesis that Thy-1 functions to stabilize membranes and inhibit sprouting.

Purified Thy-1 has been reported to dimerize spontaneously *in vitro* (*96*), and immunoprecipitations of Thy-1 often reveal proteins corresponding in size to integral multiples of the molecular weight of Thy-1 (*94, 144, 146*). In addition, lymphocytes shed high molecular weight Thy-1-immunoreactive material (*75*). In demonstrating the necessity for multivalent binding of Thy-1 for the promotion of neurite outgrowth and sprouting by anti-Thy-1 mAbs in culture, we suggested that the mAbs may be perturbing homophilic Thy-1 interactions. We here provide evidence supporting the existence of monomeric, dimeric, and higher multimeric forms of Thy-1 *in vivo*. Moreover, the relative proportions of the different forms shift dramatically in response to known neurite outgrowth promoting factors, and peptides designed to perturb Thy-1 multimerization also affect neurite outgrowth.

Methods and Materials

Cell Culture

Neurons of the neonatal rat superior cervical sympathetic ganglion were dissociated and grown by the method of Wolinsky, et al. (*240*) in serum-free L15-CO_2 medium as described in Chapter 2. For neurite outgrowth assays, neurons were cultured in dishes as described by Mains and Patterson (*147*). For biochemical analysis neurons were cultured on collagen-coated, 35 mm tissue culture dishes (Corning). PC12 cells were grown in L15-CO_2 medium as described by Hawrot and Patterson (*100*); these were cultured on 100 mm tissue culture dishes (Corning) for biochemical analysis, and 24-well Primaria tissue culture plates (Falcon) for neurite outgrowth assays. Where specified, Protein A-purified anti-Thy-1 mAb OX-7 (*150*) was added to 5 μg/ml as described in Chapter 2. Synthetic peptides (see below) were prepared as stock solutions in L15-CO_2 and diluted directly into complete culture medium to the indicated concentrations. The BW5147 Thy-1$^-$e thymoma cell line (kindly provided by Dr. Robert Hyman, Salk Institute) was cultured in Dulbecco's modified Eagle's medium (Sigma) with 10% donor horse serum (Hazleton Biologicals).

Gel Filtration Chromatography and Enzyme-Linked Immunoabsorption Assay (ELISA)

Four 35 mm dishes of sympathetic neurons cultured for 10 d as described above, or one 100 mm dish of PC12 cells treated with the indicated reagent were washed with phosphate buffered saline and lysed at 4° in 10 ml of buffer containing 0.1 M NaCl, 1% Na-deoxycholate, 1% Lubrol-PX, 10 mM Tris-HCl, 1 mM phenylmethylsulfonyl fluoride, 10 mM Na_2EDTA, pH 7.7 (all reagents from Sigma). After ultracentrifugation in a Type 50 rotor (Beckman) at 3 x 10^6 g-min, 0.3 ml of each lysate was loaded on to a Sephadex G-150 (Pharmacia) column (1 x 92 cm) which had been equilibrated in lysis buffer. The column was eluted at 3 ml/hr, and 1.5 ml fractions were collected. Calibration of the collumn was performed with a mixture of β-amylase, alcohol

dehydrogenase, bovine serum albumin, and ovalbumin (all from Sigma) in lysis buffer.

The fractions were dialyzed 4500-fold against 100 mM $NaHCO_3$, pH 9.5 for 24 hrs at 4°. From each fraction 100 µl was applied to wells of flexible assay plates in duplicate (Falcon Microtest III; Becton-Dickinson) and incubated overnight at 4°, blocked with 5% nonfat dry milk (Carnation) in Tris-buffered saline (TBS) for 1 hr, and then incubated serially with a 1:1000 dilution of ascites fluid from the anti-Thy-1 hybridoma cell line OX-7 and a 1:500 dilution of alkaline phophatase-conjugated, goat anti-mouse immunoglobulin G anti-serum (Boehringer Mannheim) in blocking buffer. Wells were washed thrice in between incubations with 200 µl of 0.1% Tween-20 (Sigma) in TBS. In the case of PC12 cells that had been treated with the OX-7 mAb to induce outgrowth (us), cells were lysed and subjected to gel filtration chromatography in pH 11.5 lysis buffer so as to dissociate the mAb from Thy-1, and were then probed by ELISA as above with a 1:500 dilution of rabbit anti-rat Thy-1 antiserum (kindly provided by Dr. Alan Williams, Oxford University) and a 1:500 dilution of alkaline phophatase-conjugated, goat anti-rabbit immunoglobulin G antiserum (Boehringer Mannheim). After a final wash in TBS, the wells were reacted with 100 µl of 2.5 mM p-nitrophenylphosphate (Sigma), 2.5 mM $MgCl_2$, 10 mM diethanolamine, pH 9.5, and the absorbance read at 405 nm in a microplate reader (SLT-Lab Instruments).

Gel Electrophoresis and Immunoblotting

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (*124*); all gels used 5% acrylamide for sample stacking, and as designated, either a 10%-15% linear gradient or straight 10% acrylamide for separation. Electrotransfer of SDS-PAGE-separated proteins to nitrocellulose (BA85, Schleicher and Schuell) was performed in a Mini Protean II electrotransfer appartus (Bio-rad) by the method of Haid and Suissa (*95*). To probe for Thy-1 immunoreactivity, the nitrocellulose was air-dried for 1 hr; blocked with 5% nonfat dry milk in phosphate-buffered saline (PBS/milk) for 1 hr; and

incubated with 20 μg/ml of Protein A-purified, anti-Thy-1 mAb 2G12 (*7*) in PBS/milk overnight at 4°. After washing three times with 0.1% Tween-20 in PBS (TPBS), the blot was incubated with a 1:500 dilution of alkaline phosphatase-conjugated goat anti-mouse immunoglobulin in PBS/milk for 1 hr, rinsed three times with TPBS, and developed with bromochloroindolyl/nitro blue tetrazolium (Sigma) (*98*).

Immunoaffinity Purification of Soluble Thy-1

The Thy-1-negative thymoma cell line, BW5147 Thy-1$^-$e, has been characterized as unable to covalently couple glycophosphotidylinositol to the carboxy-terminus of mature Thy-1 protein. Thus it is a source of soluble Thy-1 which is secreted into the culture medium (*39, 70*). Medium was conditioned by the cells for 2 d and was harvested when the cells were near confluent. Conditioned medium was then 0.22 μm filtered (Corning), NaN_3 added to 0.01%, titrated to pH 8.0 with 10 M NaOH, and then stored at 4° for up to two weeks. The soluble form of Thy-1 was immunoaffinity purified by the method of Campbell, et al. (*26*) from 300 ml of conditioned medium at a time using OX-7 mAb immobilized on CH-Sepharose per manufacturer's protocol (Pharmacia). Protein was eluted with 50 mM diethylamine, pH 11.5, neutralized with solid glycine, and dialyzed 100-fold against 20 mM Na-phosphate buffer, pH 7.0. To remove contaminating OX-7 IgG, the dialyzed material was passed over a Protein G column (Pharmacia), and finally concentrated to 2 ml by ultrafiltration in a stirred cell using a PM-10 membrane (Amicon Corp.).

Two-dimensional Peptide Mapping

Immunoaffinity purified soluble Thy-1 was boiled under reducing conditions (1% β-mercaptoethanol) and subjected to SDS-PAGE (X). Coomassie staining revealed protein bands of apparent molecular weight 25kD and 50kD. These were excised, radioiodinated, chymotryptically digested, and subjected to two-dimensional separation by the methods of Davis and Bennett (*50*), and Elder et al. (*65*).

Synthetic Peptide Preparation

Peptides designated TDP (Thy-1 Derived Peptide)-1,-2, and -3, were synthesized by automated, stepwise, solid phase synthesis employing t-Boc chemistry on an ABI 430A synthesizer at the California Institute of Technology Biopolymer Synthesis Resource Center by the method of Sluka et al. (*215*). The sequences of the peptides, using single-letter amino acid abbreviations, are as follows:

TDP-1: T-R-E-K-K-K-H-V-L-C (36-44)

TDP-2: E-L-R-V-S-G-Q-N-P-C (86-94)

TDP-3: T-T-K-D-E-G-D-Y-M-C (78-87)

The numbers in parentheses indicate the location of these sequences in the Thy-1 protein sequence as deduced from the cDNA of rat Thy-1 (X). TDP-1 and -2 include a carboxy terminal cysteine that does not occur in the Thy-1 sequence; this was intended to facilitate conjugation to carrier proteins by sulfhydryl chemistry. In addition, as controls for the sequence-independent effects of TDP-1 and -3, peptides were synthesized with the identical amino acid compositions, but with their sequence randomized (with the exception of the carboxy terminal cysteine):

TDP-1R: L-R-T-K-H-K-E-K-V-C

TDP-3R: Y-D-T-G-T-E-M-K-D-C

Neurite Outgrowth Assay

Analysis was by phase contrast microscopy at a total magnification of 160x. Cells were scored as process-bearing if they possessed a neurite greater than two cell diameters in length. Individual experiments included 2 to 3 dishes or wells per culture condition. In the case of the neuron cultures, cell number per dish varied between 150 and 200, and for each data point presented (average ± standard error of the mean), the total

number of individual cultures scored varied between 6 and 9. In the case of PC12 cell cultures, approximately 200 cells were scored per dish, and 6 dishes were used for each data point.

Results

Dilute lysates of cultured neurons and PC12 cells contain Thy-1 immunoreactivity at molecular weights greater than 25kD. In order to ascertain the molecular weight of neuronal Thy-1 with minimal manipulation, primary sympathetic neurons and PC12 cells were solubilized in a large volume of lysis buffer so that cellular protein was diluted 100- to 200-fold. After immediate ultracentrifugation to remove insoluble material, an aliquot of the lysate was applied to a Sephadex G-150 column equilibrated in the lysis buffer, and the fractions analyzed by ELISA for Thy-1 immunoreactivity. As is illustrated in Figure 3.1, a peak of immunoreactivity was observed at the molecular weight expected for Thy-1, approximately 25kD (*26*). Thy-1-immunoreactive peaks were also observed at approximately 45kD and 150kD despite the large dilution of cellular protein in a high detergent concentration. In the PC12 extract, the larger forms comprise approximately 80% of the total Thy-1 immunoreactivity. In the case of the neurons, immunoreactivity is evenly distributed among the three peaks. The inclusion of 100 mM iodoacetamide in the lysis buffer does not change the elution pattern of immunoreactivity observed for PC12 cells (data not shown). Thus Thy-1 immunoreactivity in neurons and neuron-like cells exists at molecular weights higher than expected for Thy-1, and the sizes of the observed forms suggest that multimeric forms of the protein exist *in vivo*.

Immunoblotting of PC12 cell lysate gel filtration fractions demonstrates that the 150kD form of Thy-1 immunoreactivity gives rise to 25kD and 50kD immunoreactivities. In order to further analyze the three forms of Thy-1 immunoreactivity observed by gel filtration chromatography, samples from the peak fractions were

subjected to SDS-PAGE and immunoblot analysis with the anti-Thy-1 mAb 2G12. As illustrated in Figure 3.2, each of the samples was run with and without reduction and boiling. Under non-reducing conditions, the fraction corresponding to approximately 150kD gives rise to a Thy-1 immunoreactive band of 150kD (Fig. 3.2, lane 1), but under reduction and boiling gives rise to a 50kD band and a very faint 25kD band (Fig. 3.2, lane 2). Under both reducing and non-reducing conditions, the fractions corresponding to 50kD and 25kD give rise to Thy-1 immunoreactive bands of 50kD and 25kD, respectively (Fig. 3.2, lanes 3-6). Thus the three sizes of Thy-1 immunoreactivity observed by gel filtration are not the product of detergent binding, or Thy-1 micelle formation due to the protein's lipid tail. Furthermore, the 150kD form of Thy-1 immunoreactivity appears to be composed of the 25kD and 50kD forms.

The various multimers of affinity-purified Thy-1 show differential lability to temperature and disulfide-reduction. To explore the ability of an immunoaffinity purified, soluble form of Thy-1 to multimerize, such material was also subjected to SDS-PAGE. Analysis reveals several high molecular weight proteins that are sensitive to disulfide reduction and elevated temperature (Fig. 3.3a). Without disulfide reduction, proteins of 150kD and greater than 200kD are observed, but upon reduction with β-mercaptoethanol, only proteins of 75kD and 25kD are observed. The 75kD and 25kD forms persist at 70°. Upon boiling, however, the 75kD band is greatly diminished, yielding a 50kD band, and a more intense 25kD band. A direct demonstration of the conversion of the 75kD band to the 50kD and 25kD species was performed by excising the 75kD band, eluting its contents by passive diffusion, boiling, and subjecting the sample to SDS-PAGE (Fig. 3.3b). Immunoblot analysis of the boiled and reduced material reveals that all three proteins possess Thy-1-immunoreactivity (Fig. 3.3c). Thus immunopurified Thy-1 appears to exist in extremely stable monomeric and dimeric forms, as well as constituting the higher multimeric forms that are temperature and reduction sensitive.

The 50kD protein generates a peptide map largely identical to that of the 25kD protein. In order to verify that the 50kD molecule observed in Fig. 3.3 is in fact a Thy-1 dimer, both the 25kD and 50kD proteins were isolated from immunoaffinity purified protein by preparative 10% SDS-PAGE. The proteins were radioiodinated, chymotryptically digested, and the resultant peptides subjected to two-dimensional separation and autoradiography. The resulting patterns are quite similar, with the positions of the major spots on both maps corresponding exactly (Fig. 3.4, arrowheads). Most of the unmarked spots are also faintly visible in both maps. Thus the virtual lack of spots unique to the 50kD protein map leads to the conclusion that this protein is composed of two 25kD Thy-1 monomers.

The amount of Thy-1 immunoreactivity in the 25kD and 150kD forms changes with neurite outgrowth. Most Thy-1 immunoreactivity in PC12 cells is found in the 150kD and 45kD fractions, while Thy-1 immunoreactivity is evenly distributed among the 150kD, 45kD, and 25kD peaks in process-bearing sympathetic neurons (Fig. 3.1). To examine whether these differences are indeed correlated with process outgrowth, PC12 cells were treated with NGF for 10 d, or OX-7 for 4 d, treatments known to result in neurite outgrowth (Ref.*90* and Chapter 2). The cells were then lysed, and the distribution of Thy-1 immunoreactivity determined as before. To normalize the results of the different treatments, the immunoreactivity present within each peak (as in Fig. 3.1) is expressed as a fraction of the total immunoreactivity (the results are presented as mean ± standard error of the mean; n=3 for each condition).

As illustrated in Figure 3.5, the treatment of PC12 cells with either NGF or the OX-7 mAb results in an immunoreactivity distribution that is indistinguishable from that of cultured neurons. In untreated PC12 cells, Thy-1 immunoreactivity in the 25kD fraction accounts for less than 20% of the total while both NGF and OX-7 treatment result in values of approximately 30%. Conversely, in the absence of sprouting factors, the 150kD fraction accounts for approximately 50% of PC12 Thy-1 immunoreactivity, while OX-7 and NGF treatment bring this value down

to approximately 35%. Thy-1 immunoreactivity in the 50kD fraction remains constant at 30%-35% irrespective of OX-7 or NGF treatment. Thus, a loss of the 150kD form relative to total Thy-1 and an increase in the 25kD form is correlated with neurite outgrowth. A time course analysis of the response to NGF shows that the change is achieved between 4 and 10 d of treatment (Fig. 3.6).

Synthetic peptides corresponding to candidate sites for potential Thy-1 dimerization interactions affect Thy-1 immunoreactivity size distribution. It is clear that pure, thymoma-derived Thy-1 is capable of forming homophilic multimers, and that neuronal Thy-1 immunoreactivity in cell lysates behaves similarly. Such multimerization is a recurrent phenomenon in the IgSF, and Thy-1 shows significant sequence homology to the Ig variable domain in what has been designated the V-set of such proteins (*236*). In order to generate reagents that could potentially perturb such homophilic interactions, we made use of the extensive crystallographic characterization of variable region:variable region association in the formation of the Ig antigen binding site (as for Fab_{New}, (*182*, *183*)). The Thy-1 sequence can be aligned with the Ig variable region (*236*) to predict potential sites of Thy-1:Thy-1 association. Having identified two such amino acid sequences, corresponding peptides were synthesized to test on cultured cells. Using the nomenclature of Williams (*236*) for Ig structure (see Fig. 1.1), Thy-1-derived peptide-1 (TDP-1) corresponds to a site from the middle of β-strand C into the middle of C', thus aligning with amino acids 337-345 of the Ig heavy chain of Fab_{New}. TDP-3 corresponds to a well-conserved portion of β-strand F and maps approximately to amino acids 386-394 of the Ig heavy chain of Fab_{New}.

The effects of TDP-1 and -3 on the distribution of Thy-1 immunoreactivity in PC12 cells were analyzed first. TDP-1 treatment of PC12 cells gives rise to an approximately 150% increase in the amount of Thy-1 immunoreactivity found in the 25kD fraction (Fig. 3.7a). Concomitant with this increase is a corresponding decrease in the amounts of Thy-1 immunoreactivity in the 50kD and 150kD fractions. In contrast,

TDP-3 has little effect on this distribution. The effect of TDP-1 on Thy-1 size distribution was next compared to that of NGF. Lysates from PC12 cells treated with NGF for 4 d (NGF-primed PC12 cells) were compared with primed PC12 cells treated with synthetic peptide on the fourth day (Fig. 3.7b). NGF treatment for 4 d results in a Thy-1 immunoreactivity distribution similar to that of sympathetic neurons (Fig. 3.5 and 3.6). The addition of TDP-1 gives rise to an even larger size shift, nearly tripling the amount of immunoreactivity found in the 25kD fraction (relative to untreated PC12 cells), and produces a more than 50% decrease in the amount of Thy-1 immunoreactivity in the 150kD fraction. Treatment of sympathetic neurons with TDP-1 (Fig. 3.7c) does not significantly change the distribution of Thy-1 immunoreactivity. Thus TDP-1 causes a dramatic shift in the distribution of Thy-1 immunoreactivity by decreasing the amount of 150kD protein and increasing the amount of 25kD protein relative to the total amount of Thy-1. This is the predicted result of a peptide that interferes with homophilic Thy-1 interactions.

Thy-1-derived synthetic peptides affect neurite outgrowth and sprouting. Given that agents that promote neurite outgrowth (e.g., NGF and OX-7) also increase the proportion of Thy-1 monomer, the ability of TDP-1 to increase the proportion of monomer might also lead to enhanced neurite outgrowth. As can be seen in Figure 3.8, TDP-1 has a stimulatory effect on process outgrowth from sympathetic neurons, while TDP-3 has an inhibitory effect. The dose response curve for TDP-1 is reproducibly biphasic, with maximal stimulation of neurite outgrowth at a concentration of 1 μg/ml. The 50% increase in outgrowth is comparable to the level of stimulation observed with OX-7 in Chapter 2. TDP-3 displayed a dose-dependent inhibition, reaching an approximately 50% decrease at 5 μg/ml. In order to control for non-sequence specific effects, peptides containing the same amino acids in random order (TDP-1R and TDP-3R) were also tested on the neurons. Neither of these peptides affects outgrowth in the doses tested (Fig. 3.9). The sequence of a third peptide, TDP-2, was selected because it could contain the OX-7 binding site. The sequence includes the amino acid arginine at a site shared by both rat Thy-1

and mouse Thy-1.1, but not mouse Thy-1.2 (*26, 237*). OX-7 binds rat Thy-1 and mouse Thy-1.1, but not Thy-1.2 (*150*). As illustrated in Figure 3.8, TDP-2 has no effect on outgrowth.

Figure 3.10 illustrates that TDP-1, -2, and -3 have no effect on sprouting from PC12 cells. In contrast, priming the cells with 1 μg/ml of 7S NGF for 3 d, and then replating with the peptides, reveals that TDP-1, at 1 μg/ml, enhances by 50% the number of process-bearing cells (Fig. 3.10). As with neurons, the TDP-1 dose response curve of PC12 cells is biphasic. TDP-2 appeared to have only a slight inhibitory effect on the primed PC12 cells. TDP-3 displayed a slight inhibition of sprouting from the primed PC12 cells at all concentrations tested. Thus, TDP-1 causes a loss of high molecular weight Thy-1 immunoreactivity and stimulates sprouting. TDP-3, in contrast, decreases sprouting without effecting the Thy-1 immunoreactivity distribution, and TDP-2 has little affect on neurite sprouting.

Discussion

Thy-1 is expressed by cells in both monomeric and multimeric forms. Analysis of both neuronal and PC12 cell lysates reveals the presence of Thy-1 immunoreactivity at the expected molecular weight of 25kD as well as at integral multiples of approximately 50kD and 150kD (Fig. 3.1 and 3.2). The two large forms could result from aggregation of Thy-1 due to its hydrophobic glycophosphoinositide tail (*103, 141*), or alternatively, to the association of Thy-1 with non-Thy-1 proteins with molecular weights of 25kD and 125kD, respectively. These possibilities are unlikely because immunoaffinity purified Thy-1 that lacks the hydrophobic tail also forms a 150kD complex, and this complex is composed of 25kD and 50kD subunits (Fig. 3.3). Two-dimensional peptide mapping of the two smaller proteins shows that the 50kD protein is a dimer of the 25kD protein (Fig. 3.4). It is puzzling that the 25kD protein does not immunoblot as well as the higher molecular weight bands (Fig. 3.2).

This is the case even when equal amounts of the proteins, as determined by protein assay, are loaded on parallel lanes of gels to be blotted (data not shown). Since it has become apparent that protein epitopes recognized by antibodies are usually the product of discontinuous amino acid sequences which are brought into mutual proximity through protein folding (*34*), it is possible that the most common epitopes on Thy-1 are preserved in the dimeric and multimeric forms, but are grossly denatured in the gel-separated monomer; this would result in an inherently weaker signal from monomeric Thy-1 in immunoblots. Thus the 150kD protein is in fact a Thy-1 homomultimer produced by two distinct Thy-1:Thy-1 interactions: (i) a disulfide-mediated association among the 25kD and 50kD subunits, and (ii) an interaction between Thy-1 monomers that is resistant to both reduction and boiling, and thus likely to be covalent.

It is likely that the Thy-1 dimer and hexamer exist in living cells because these forms are observed in very dilute lysates where the probability of artifactual association is low, and because a modulation of the relative quantities of the three observed forms of Thy-1 is altered by physiologically relevant treatment of the cells (e.g., NGF). Moreover, studies of Thy-1 fluorescence recovery after photobleaching have demonstrated that 50% of cell surface Thy-1 does not show the mobility expected of a monomeric, lipid-anchored protein (*113*). This Thy-1 appears to be relatively immobile, possibly due to extensive homomultimeric association. Recent studies have shown that purified Thy-1 incorporated into Thy-1-deficient cells via liposome fusion also displays a comparable lack of mobility (*247*).

The multimeric states of Thy-1 are correlated with neurite outgrowth. In normally dividing PC12 cells, approximately 80% of Thy-1 is in the dimeric and hexameric forms, while in the case of sympathetic neurons, only 65% of Thy-1 is found in the large forms (Fig. 3.5). That this difference is related to the sprouting of neurites by the primary cells was demonstrated by inducing the PC12 cells to sprout with NGF or OX-7, and showing that the resulting size distribution of Thy-1 multimers became identical to that in neurons. Since at least 4 d is required to

generate the neuronal Thy-1 size distribution in PC12 cells (Fig. 3.5 and 3.6), it is possible that control of multimerization takes place on the cell surface or biosynthetically. It is worth noting that the turnover rate of cell surface Thy-1 has been reported to be at least as slow as 2 d (*130*).

Thy-1 multimerization may make use of immunoglobulin variable domain-like interactions. Because Thy-1 is homologous to the V-set of proteins in the IgSF, it is logical to ask whether Thy-1 homomultimer formation is the result of interactions similar to those of Ig variable domain light and heavy chain association. The amino acid sequences of TDP-1 and -3 were selected because they mimic potential association sites and might thereby modify Thy-1:Thy-1 association. These two peptides were synthesized and tested for their effects on multimerization and neurite outgrowth. TDP-1 dramatically reduces levels of hexameric Thy-1 in PC12 cells, and this effect is even more pronounced in NGF-primed PC12 cells. In addition, TDP-1 enhances outgrowth from sympathetic neurons and primed PC12 cells. TDP-3 on the other hand, does not modulate the distribution of Thy-1 multimers in PC12 cells, but does inhibit outgrowth from neurons and primed PC12 cells. The effects of these peptides are very specific since TDP-1R, -3R, and -2 do not affect sprouting. The observed effects of TDP-1 and -3 do require high concentrations of peptide (1-5 μg/ml). Short peptides are generally conformationally unstable in the absence of the intramolecular interactions present in larger proteins (*11*), and only a small subset of the numerous peptide conformations is likely to be biologically active. Supporting this interpretation are our preliminary data which show that ovalbumin conjugates of TDP-1 and -3 affect outgrowth at much lower molar concentrations than the free peptides (N.K.M. and P.H.P., unpublished observations). Although it is possible that TDP-1 and -3 affect multimerization and outgrowth through mechanisms other than direct modulation of Thy-1 homophilic interactions, the data support the notion that Thy-1 forms homomultimers *via* Ig variable domain-like interactions.

A decreased proportion of Thy-1 multimers is permissive for neurite outgrowth, but not causal. Since the amount of Thy-1 dimer remains relatively constant through all the treatments studied, the ratio of Thy-1 monomer to hexamer is a variable that correlates well with the degree of process outgrowth. As illustrated in Figure 3.11, there is indeed a trend towards an increased ratio of monomeric Thy-1 to hexameric Thy-1 as the percentage of cells bearing neurites increases. When all the data points are utilized for the calculation of least-squares fit to a line, the resultant line has a correlation coefficient (R) of 0.36. If, however, the points corresponding to PC12 cells treated with TDP-1 are excluded from the calculation, a line with much greater correlation is generated (R=0.87). Though TDP-1 promotes neurite outgrowth from both neurons and NGF-primed PC12 cells, a very large increase in the amount of monomeric Thy-1 occurs in both primed and unprimed PC12 cells. TDP-1 may cause the "superinduction" of Thy-1 monomer expression by acting as a competitive antagonist of homophilic association. In primary neurons, such a superinduction appears to be constrained by mechanisms not present in PC12 cells. If Thy-1 is a signal transducing protein, it is possible that TDP-1 acts as an agonist of signalling by mimicking the effects of necessary multimerization events (see Chapter 2). Strikingly, the superinduction of Thy-1 monomer does not necessarily bring about a concomitant induction of neurite sprouting, and implies that the state of Thy-1 multimerization does not directly cause outgrowth.

If the Thy-1 monomer-multimer balance is not directly causal, what additional factors are necessary for the induction of neurite outgrowth? The extension of neurites by neonatal rat sympathetic neurons in culture is a regeneration process, and the cellular machinery requisite for neurite extension (e.g., cytoskeletal components and growth cone associated proteins) is clearly present in these cells at the initiation of culture. Given appropriate substrates and trophic support, these neurons put forth neurites. PC12 cells, however, require activation of the neurite producing machinery in order to initiate neurite outgrowth, and this can be achieved by providing the cells with NGF (*62*). Anti-Thy-1 mAbs also induce sprouting from PC12 cells, to a lesser extent (Chapter 2). Both of these

factors trigger changes in the concentrations of various second messengers (*122, 127, 202, 204*). Thus it appears, as mentioned in Chapter 2, that neurite outgrowth can only occur when cells are somehow pushed towards a slightly more neuronal phenotype on a continuum between chromaffin cell and sympathetic neuron. In the case of primary neurons, this is the product of developmental history, and for PC12 cells it can be induced in culture by exposure to appropriate exogenous factors. TDP-1 alone is not able to act as such a factor, though it can affect the state of Thy-1 multimerization and promote neurite sprouting from cells capable of sprouting (i.e., neurons and NGF-primed PC12 cells). This finding demonstrates that modulation of Thy-1 multimerization is not sufficent to induce neurite sprouting. The lack of an example of sprouting cells that express high levels of multimeric Thy-1 suggests that the decreased proportion of hexameric Thy-1 is a permissive condition for sprouting to occur.

Thy-1 as an inhibitor of neurite outgrowth. In Chapter 2, we suggested that the normal role of Thy-1 is to inhibit neurite sprouting and to stabilize processes. This hypothesis accounts for the following findings: (i) multivalent binding by anti-Thy-1 mAbs promotes sprouting from cultured cells and results in the shedding of a molecule of molecular weight appropriate for monomeric Thy-1, (ii) removal of Thy-1 by phosphotidylinositol-specific phospholipase C promotes neurite sprouting, (iii) mutant PC12 cells deficient in the expression of Thy-1 spontaneously sprout neurites with high frequency, and (iv) Thy-1 is expressed late in nervous system development (*165*). In the present study we find that Thy-1 exists in both monomeric and multimeric forms whose relative proportions vary with neurite sprouting. Questions arise as to how Thy-1 multimerization relates to the stabilization of neurites, and how modulation of multimerization releases the inhibition of sprouting.

One manner in which Thy-1 may serve to stabilize the neuronal membrane is to form a "shell" on the extracellular face of the plasma membrane. That Thy-1 may play such a structural role is made plausible by the extremely high levels at which it is expressed: 0.1% of total human forebrain protein (*1*), and up to 7.5% of total axonal surface protein (*9*).

Such a shell could be extensively interconnected through the types of homophilic interactions demonstrated in this study. The association of Thy-1 with both actin (*76*, *191*) and a band 4.1-like protein (*20*) suggests further that a Thy-1 shell could be linked to the submembrane cytoskeleton. This structure would thus mechanically inhibit or buffer extracellular and intracellular perturbations of a neurite. It is also possible that homophilic Thy-1 interactions could occur between Thy-1 molecules on adjacent cells or Thy-1 deposited in the extracellular matrix (*160*). These bonds could further anchor the membrane shell. Such a model predicts that factors that promote neurite outgrowth would loosen or dissolve such a shell. By increasing the proportion of Thy-1 monomers, the level of interconnection within such a structure would decrease and allow neurite sprouting or remodelling. A number of heterophilic interactions have also been proposed for Thy-1 (*137*, *173*, *234*), and whether these interactions involve monomeric or multimeric forms of Thy-1 remains to be studied. Such heterophilic interactions need not, of course, be related to the inhibitory function of Thy-1.

FIGURE LEGENDS

Figure 3.1: Size exclusion chromatography and fractionation, followed by immunoassay reveals three peaks of Thy-1 immunoreactivity in the lysates of PC12 cells and sympathetic neurons (SCG). Peaks occur at molecular weights of (from left to right) approximately 150kD, 45kD, and 25kD. Immunoreactivity was measured by the optical density of p-nitrophenylphosphate, a chromogenic alkaline phosphatase substrate, at 405 nm. Arrows at top indicate molecular weight standards (from left to right): 200, 150, 67.5, and 44.5kD.

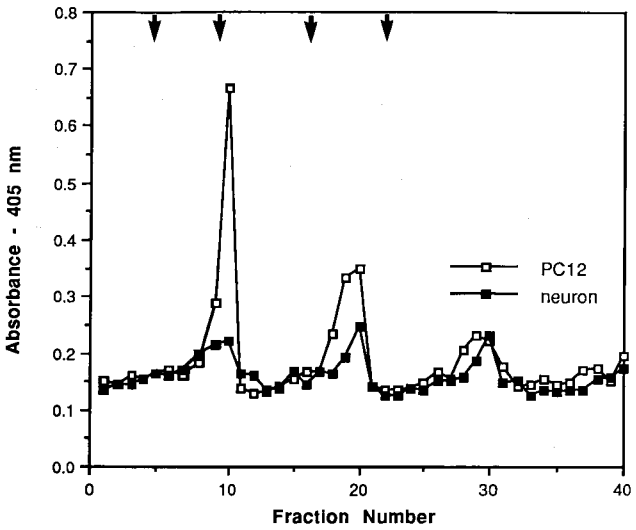

Absorbance - 405 nm
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0
10
20
30
40
Fraction Number
PC12
neuron

Figure 3.2: Immunoblot analysis of the three peak fractions of Thy-1 immunoreactivity from size exclusion chromatography of PC12 cell lysate. From left to right are the fractions corresponding to the 150kD (lane 1-2), 50kD (lane 3-4), and 25kD (lane 5-6) peaks. Samples in lanes 2, 4, and 6 were subjected to disulfide reduction and boiling. The arrows, from top to bottom, correspond to molecular weights of 150kD, 50kD, and 25kD.

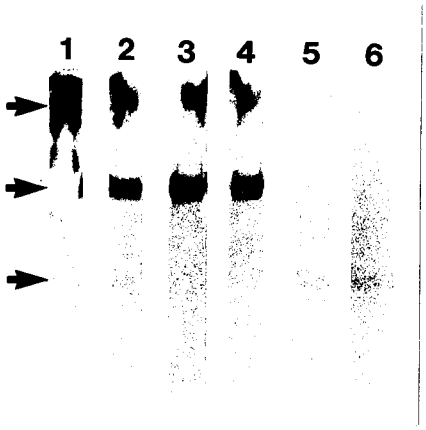

1
2
3
4
5
6

Figure 3.3: Characterization of Thy-1 purified from the culture supernatant of the BW5147 Thy-1$^-$e mutant thymoma. (**a**) Affinity-purified Thy-1 was subjected to the following conditions and analyzed by 5-15% polyacrylamide gradient SDS-PAGE: 20° under non-reducing conditions (lane 1); and the remainder in the presence of β-mercaptoethanol at 20° (lane 2), 37° (lane 3), 45° (lane 4), 70° (lane 5), and 100° (lane 6). Arrows indicate (from top to bottom) the top of the gel, 150kD, 75kD, 50kD, and 25kD. (**b**) Preparative 5-15% gradient SDS-PAGE was performed to separate anti-Thy-1 affinity column eluate after heating to 70° under reducing conditions (Coomassie stain, lane 1). The 75kD band was excised, eluted, and the eluted protein boiled and subjected to 5-15% gradient SDS-PAGE again (silver stain, lane 2). Arrows designate molecular weights of 75kD, 50kD, and 25kD. (**c**) Immunoblot with anti-Thy-1 antiserum of electrotransferred protein eluted from an anti-Thy-1 affinity column and separated by 10% SDS-PAGE. Arrows designate, from top to bottom, molecular weights of 75kD, 50kD, and 25kD.

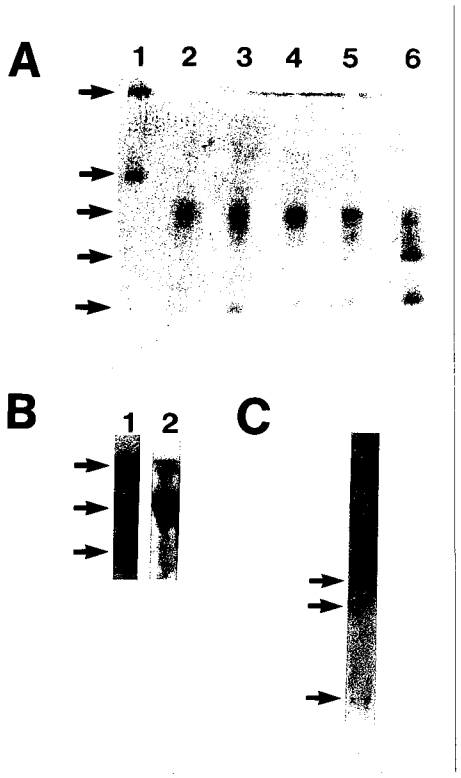

A
1
2
3
4
5
6
B
1
2
C

Figure 3.4: Two-dimensional peptide maps of the 25kD and 50kD proteins purified by anti-Thy-1 affinity chromatography. Electrophoresis was performed in the horizontal dimension, and thin layer chromatography was performed in the vertical dimension. The open arrow designates the origin, and the small arrows designate prominent spots in common between the two maps.

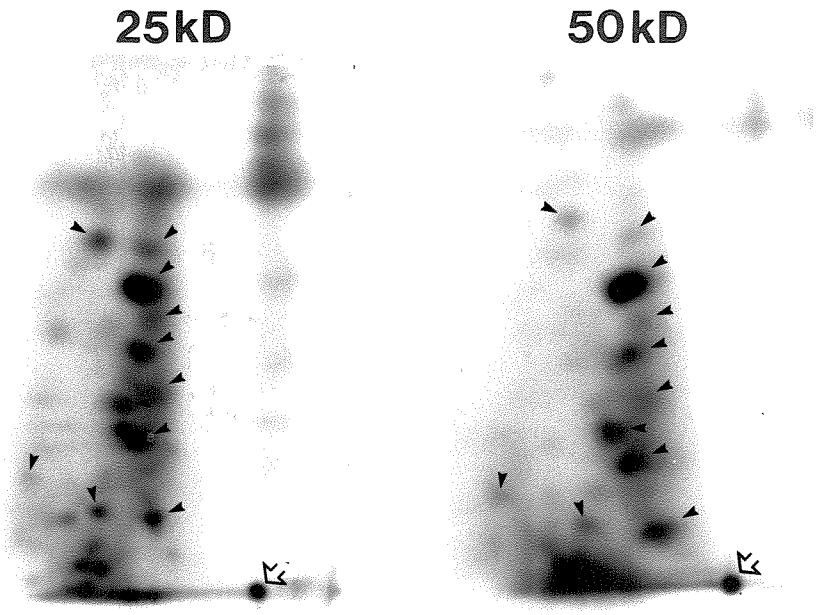

25kD
50kD

Figure 3.5: Comparison of the relative amounts of Thy-1 immunoreactivity found among the 25kD, 50kD, and 150kD fractions of cell lysates analyzed as in Fig. 3.1. Shown are cultured sympathetic neurons, PC12 cells, PC12 cells grown in NGF for 10 d (PC12 + NGF), and PC12 cells grown in anti-Thy-1 mAb OX-7 for 4 d (PC12 + OX7).

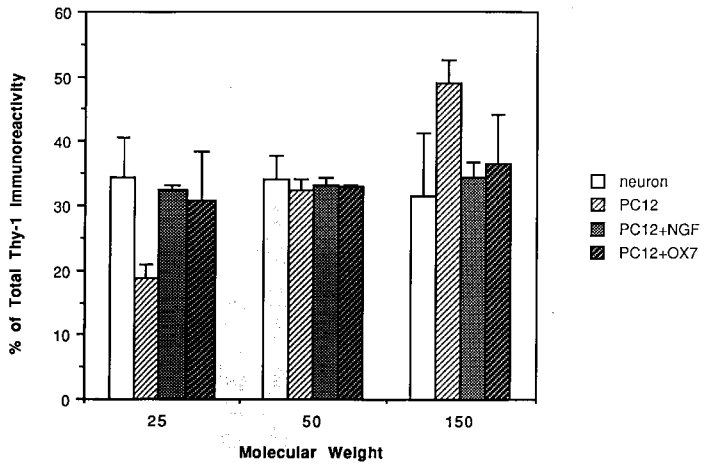

60
50
40
30
20
10
0
% of Total Thy-1 Immunoreactivity
25
50
150
Molecular Weight
neuron
PC12
PC12+NGF
PC12+OX7

Figure 3.6: Time course of NGF effects on Thy-1 multimer composition in PC12 cells. PC12 cells cultured for 0, 2, 4, and 10 d in NGF were lysed and subjected to immunoreactivity analysis as in Fig.1. Data for 10 d points are the same as in Fig.3.5.

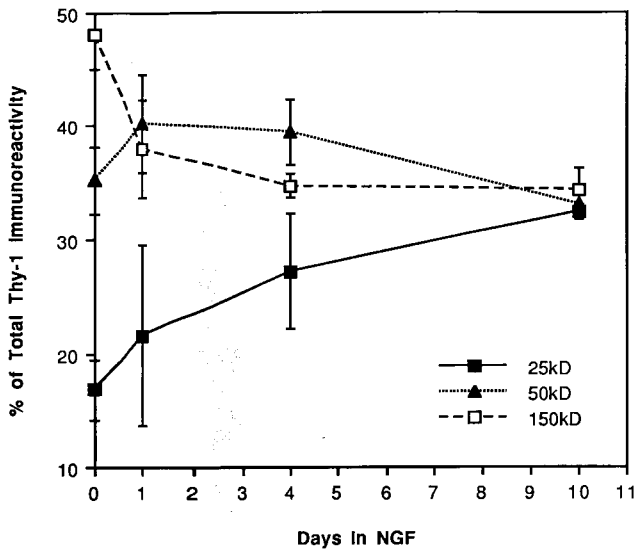

% of Total Thy-1 Immunoreactivity
50
40
30
20
10
0
1
2
3
4
5
6
7
8
9
10
11
Days In NGF
25kD
50kD
150kD

Figure 3.7: Comparison of the relative amounts of Thy-1 immunoreactivity found among the three molecular weight forms in PC12 cells and sympathetic neurons after peptide treatments. PC12 data is the same in all three panels. (a.) Comparison of untreated PC12 cells (PC12) with PC12 cells treated with either 1 μg/ml TDP-1 (PC12, TDP1) or 5 μg/ml TDP-3 (PC12,TDP3). (b.) Untreated PC12 cells compared to PC12 cells raised in NGF for 4 d (pPC12), and PC12 cells primed in NGF for 3 d and TDP-1 for an additional day (pPC12,TDP1). (c.) Untreated PC12 cells compared to sympathetic neurons cultured for 24 hr in the absence (neuron) or presence (neuron,TDP1) of TDP-1.

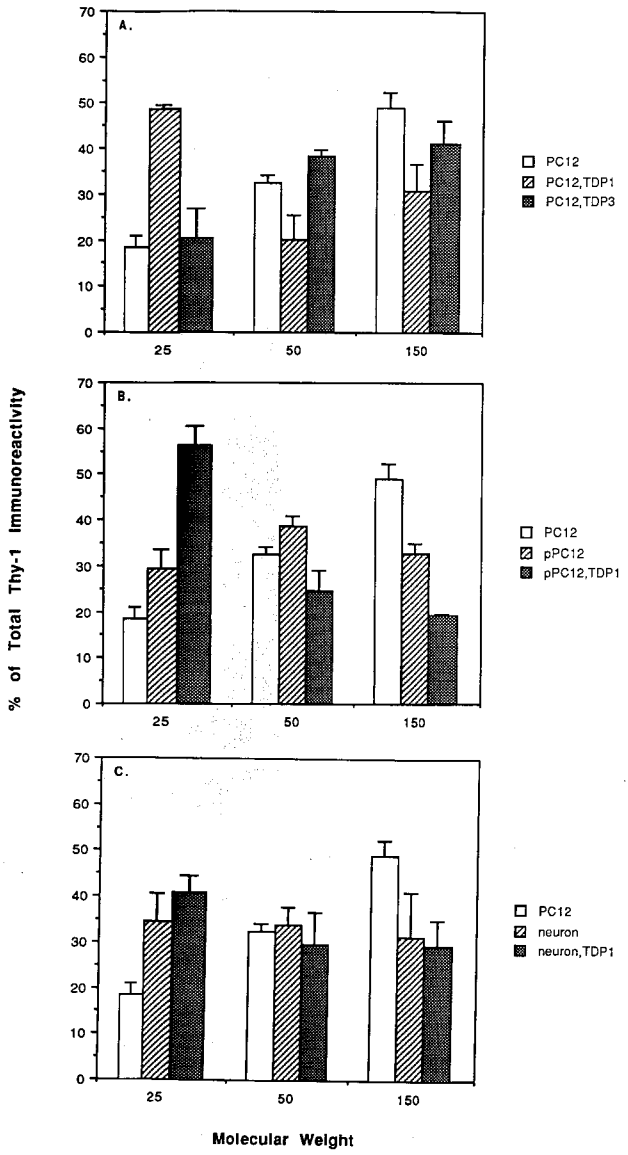

A.
B.
C.
% of Total Thy-1 Immunoreactivity
Molecular Weight
25
50
150
0
10
20
30
40
50
60
70
PC12
PC12,TDP1
PC12,TDP3
pPC12
pPC12,TDP1
neuron
neuron,TDP1

Figure 3.8: The effects of Thy-1 derived synthetic peptides on neurite outgrowth from sympathetic neurons. Neurons were cultured in the presence of peptides TDP-1, -2, and -3 for 24 hrs. at the concentrations shown, and the number of process-bearing cells were scored. The horizontal dashed line corresponds to the baseline level of sprouting observed in the absence of peptides.

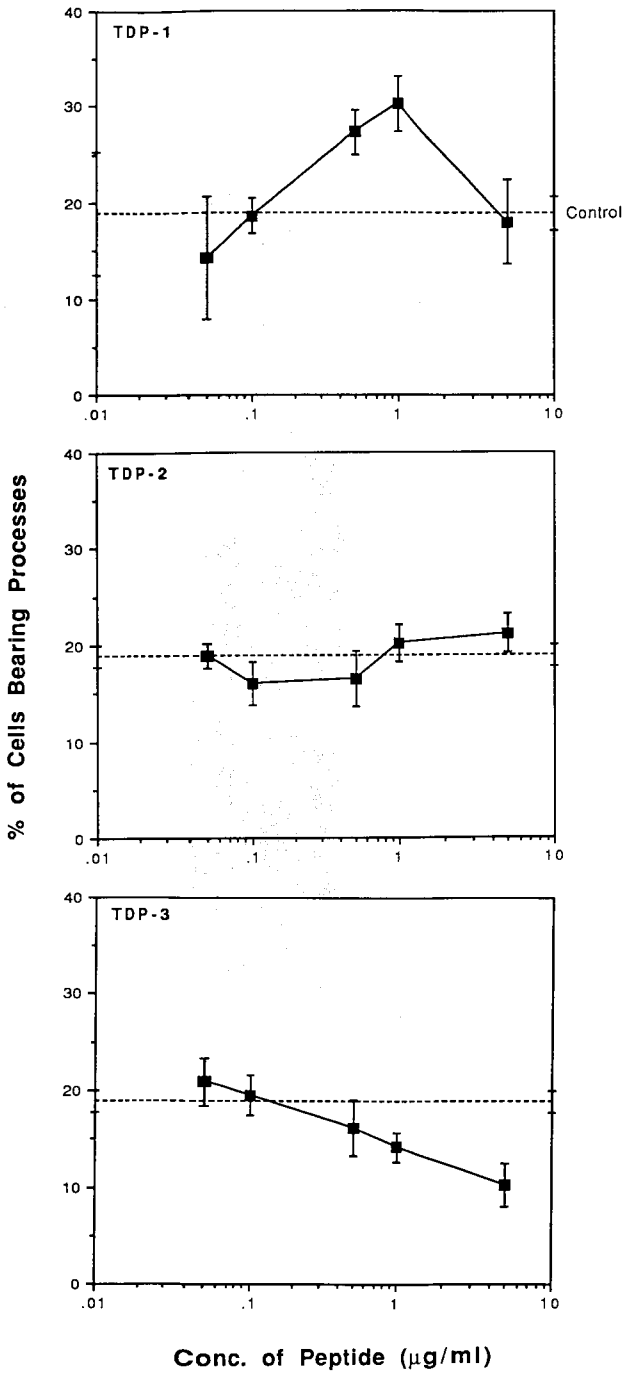

TDP-1
TDP-2
TDP-3
Control
40
30
20
10
0
.01
.1
1
10
% of Cells Bearing Processes
Conc. of Peptide (μg/ml)

Figure 3.9: Randomized counterparts of TDP-1 and -3, TDP-1R and -3R, respectively, have no effect on neurite outgrowth. Peptides were tested over the same dose range as in Fig. 7, and the horizontal line in each box corresponds to the baseline level of sprouting.

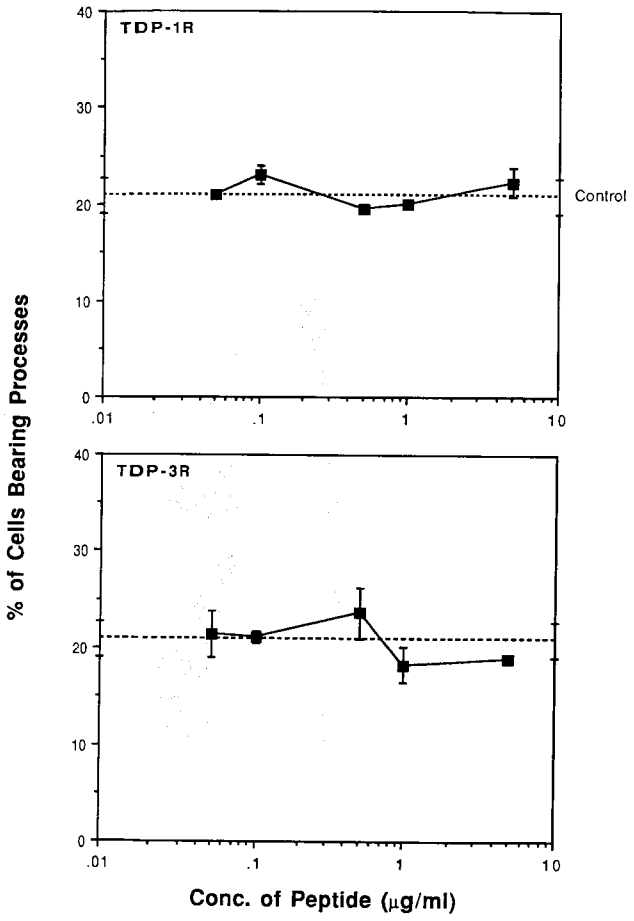

TDP-1R
TDP-3R
Control
% of Cells Bearing Processes
Conc. of Peptide (µg/ml)
40
30
20
10
0
.01
.1
1
10

Figure 3.10: Thy-1 derived synthetic peptides affect outgrowth from NGF-primed PC12 cells, but do not affect outgrowth from unprimed PC12 cells. PC12 cells were cultured in TDP-1, -2, and -3 for 4 d and the number of process-bearing cells were scored. Primed cells were cultured in NGF for 3 d prior to exposure to peptides. The upper and lower horizontal dashed lines correspond to the baseline levels of sprouting from primed and unprimed cells, respectively.

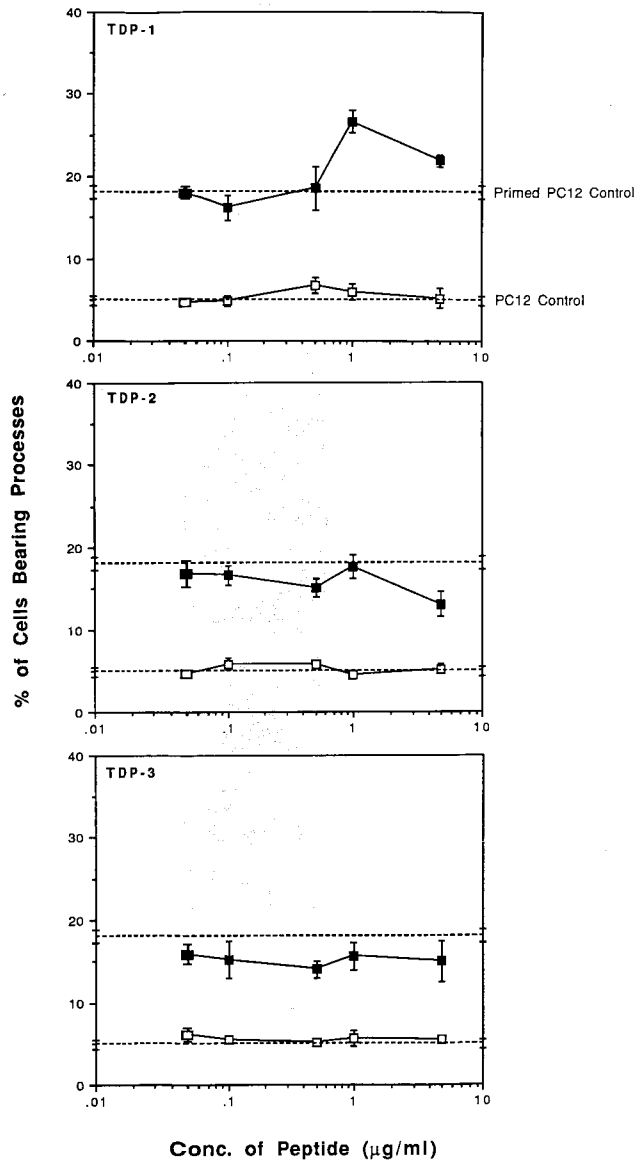
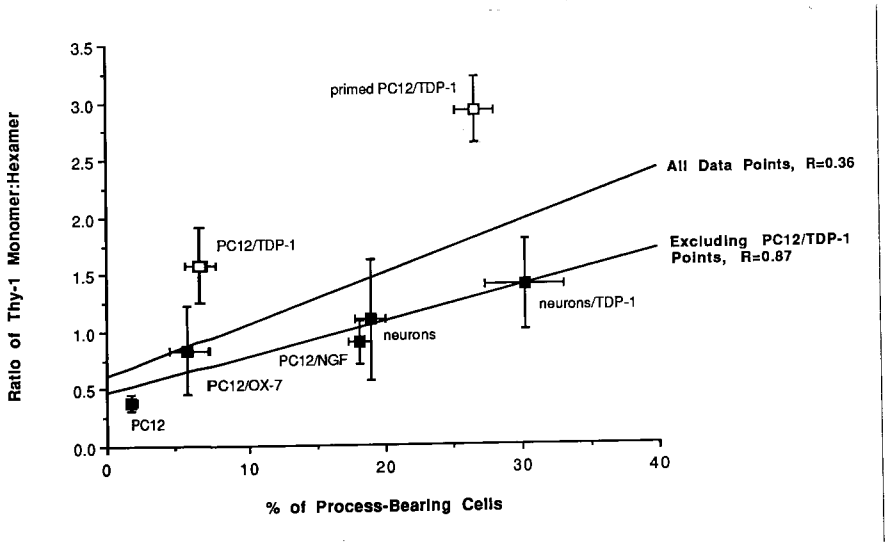

TDP-1
TDP-2
TDP-3
Primed PC12 Control
PC12 Control
% of Cells Bearing Processes
Conc. of Peptide (μg/ml)
0
10
20
30
40
.01
.1
1
10

Figure 3.11: Relative quantities of Thy-1 monomer and hexamer vary in correlation with degree of neurite outgrowth. Graphed are the ratios of the relative amounts of the 25kD form of Thy-1 to the 150kD form of Thy-1 as determined in this study, as a function of the percentage of process-bearing cells under similar culture conditions as determined in this study and in Chapter 2. Empty squares represent PC12 cells treated with TDP-1, and black squares represent all other data points.


Ratio of Thy-1 Monomer:Hexamer
% of Process-Bearing Cells
primed PC12/TDP-1
All Data Points, R=0.36
Excluding PC12/TDP-1 Points, R=0.87
PC12/TDP-1
neurons/TDP-1
neurons
PC12/NGF
PC12/OX-7
PC12
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
10
20
30
40

Chapter 4:

EVIDENCE FOR A ROLE OF TRANSGLUTAMINASE IN NEURITE OUTGROWTH

Introduction

Transglutaminases are Ca^{++}-dependent enzymes that catalyze the crosslinking of endo-γ-glutaminyl to endo-ε-lysyl residues of proteins, and result in the production of γ-glutaminyl-ε-lysine isopeptide side-chain bridges (Fig. 4.1). Since free glutamine is not a normal substrate for this reaction, the Enzyme Commssion recommends that these enzymes be referred to as R-glutaminyl-peptide:-amine-γ-glutamyl-transferases (EC 2.3.2.13) (*139*), but for the purposes of this report, the enzyme will be referred to by the simpler name, transglutaminase (TGase). TGase activities are also capable of incorporating low molecular weight amines, such as putrescine, into proteins, and these molecules are often used as inhibitors of TGase activity by acting as competitive acyl acceptor substrates.

The physiological relevance of TGases has been demonstrated in a wide variety of systems. Probably best known is the example of blood Factor XIII, a TGase responsible for the crosslinking of fibrin in plasma clot formation (*16*). Rodent prostate TGase is responsible for the crosslinking of seminal proteins in the copulatory, vaginal plug (*239*). TGase activities have been characterized in hair follicles and epidermis, and are thought to catalyze crosslinking of hair (*239*) and epidermal keratins (*87*). A TGase expressed by a wide variety of tissues was first characterized as liver TGase, but is now known as tissue TGase. Tissue TGase is thought to be responsible for the permanent changes in shape and rigidity suffered by erythrocytes in response to aging-related or ionophore-mediated increases in intracellular free Ca^{++} (*139, 216*). Ionophore treatment of erythrocytes leads to the production of γ-glutaminyl-ε-lysine crosslinked, high molecular weight polymers which contain the proteins band 3, ankyrin, band 4.1, and hemoglobin (*13*). Hence it has been suggested that tissue TGase may play a general role in cellular aging (*139*).

TGase activities have also been characterized in the nervous system. In the case of *Aplysia californica*, TGase activity has been demonstrated through the injection of ^{3}H-putrescine into the identified neuron, R2, and

showing that a small number of proteins, including tubulin, become ^{3}H-labeled (*2*). TGase activity in mouse brain homogenates, as measured by the incorporation of ^{3}H-putrescine into dimethylcasein or endogenous proteins, is observed to increase 2.5-fold from postnatal day 3 to adulthood (*143*). A correlation with neurite outgrowth was demonstrated with mouse N18 neuroblastoma cells. Serum wtihdrawal results in neurite sprouting and a ten-fold increase in TGase activity, the maximum increase occurring once extensive neurites have formed (*143*). In N18 cells, ^{3}H-label was primarily observed in high molecular weight, polymeric material, but label was also found in a band corresponding to tubulin. Purified polymeric tubulin has also been demonstrated to be a good TGase substrate (*143*). The rat pheochromacytoma line, PC12 constituitively expresses low levels of epidermal-like TGase, and shows a dramatic increase in the expression of tissue TGase in response to sodium butyrate (*25*). This increase coincides with growth arrest and increased intercellular adhesion. Induction of growth arrest and neurite outgrowth by NGF, however, was not accompanied by a modulation of either of the TGase activities (*25*). Thus the involvement of TGase in neurite outgrowth remains to be proven. Nevertheless, there is the clear suggestion that TGase may play an important role in neurite outgrowth.

Evidence was presented in Chapter 3 that the neuronal surface protein, Thy-1, acts to inhibit neurite outgrowth and stabilize the plasma membrane through homomultimerization. The multimerization is caused by a number of different interactions, one of which may be a covalent association of Thy-1 monomers into a Thy-1 dimer. Since Thy-1 contains several endo-γ-glutaminyl and endo-ε-lysyl residues, it is possible that a TGase-mediated isopeptide crosslink could yield the Thy-1 dimer. In an effort to address this hypothesis, sympathetic neurons and PC12 cells were cultured in the presence of known inhibitors of TGase or exogenous TGases, and the effects on neurite outgrowth and Thy-1 multimerization assessed. TGase inhibitors cause a very significant enhancment of neurite outgrowth, but there is no obvious change in Thy-1 multimerization. Exogenously added Tgases do not affect outgrowth. The results support a

role for TGase in neurite outgrowth, but the mechanism does not appear to involve Thy-1.

Methods and Materials

Cell Culture and Counting of Process-Bearing Cells

Neonatal rat superior cervical ganglion neurons and the PC12 cell line were cultured and scored for neurites as described in Chapter 3. The TGase inhibitor dansylcadaverine (Sigma) was prepared as a 10 mM stock solution in 100% ethanol, while bacitracin and ethylamine (Sigma) were prepared as 1 mM stock solutions in L15-CO_2 culture medium. The inhibitors were diluted the desired concentration in complete medium. Guinea pig liver TGase (Sigma), and human placental Factor XIII (Calbiochem) were also prepared as stock solutions in L15-CO_2 and diluted in complete medium to the indicated concentration. In the neuron experiments, 6-12 dishes per condition were scored for neurites, with approximately 100 cells scored per dish. In the PC12 cell experiments, 10 dishes were scored per condition, with approximately 100 cells scored per dish.

Size Exclusion Chromatography and Enzyme-Linked Immunoabsorption Assay (ELISA)

Size exclusion chromatography and ELISA for Thy-1 immunoreactivity were performed on cell lysates as described in Chapter 3. All experiments were performed in triplicate.

Results

Inhibitors of TGase promote outgrowth from primary neurons in culture. As a first approach towards asking whether TGase activities are involved in the growth of neuronal processes, sympathetic neurons were cultured in the presence of three chemically distinct

inhibitors of TGase. In order of their known potency of inhibition (*49*), these were dansylcadaverine (DC), bacitracin, and ethylamine. Each of the drugs was tested at doses of 10 μM, 100 μM, and 1 mM. As illustrated in Figure 4.2, all three drugs are able to induce a ten-fold increase in the number of process-bearing neurons. In the case of both DC and bacitracin, a maximal effect is observed at the lowest dose (10 μM), and the highest dose tested (1 mM) is cytotoxic. Ethylamine, being the least potent inhibitor of TGase, was approximately half as effective as the other inhibitors in the enhancement of process outgrowth at 10 μM, but showed a similar degree of enhancement at higher doses. Thus the dramatic increase in process outgrowth in response to the TGase inhibitors suggests that TGase does play a role in neurite sprouting.

TGase inhibition enhances NGF-induced neurite outgrowth from PC12 cells. In order to examine the relative roles of TGase and NGF in neurite sprouting, PC12 cells were cultured for 24 hrs. in the presence or absence of 100 μM DC, with and without NGF. PC12 cells cultured in DC alone show no neurite sprouting over control levels (Fig. 4.3). Cells treated with NGF alone for 24 hrs., show a slight increase in sprouting, but when treated with NGF and DC simultaneously, a ten-fold increase in the number of process-bearing cells is observed. To examine whether the action DC on PC12 cells requires prior activation by NGF, cells were primed with NGF for 3 d and then cultured under the same conditions as above. Primed PC12 cells show a higher level of baseline sprouting than unprimed PC12 cells, but even in the absence of NGF, DC induces a 50% increase over the new baseline (Fig. 4.3). NGF alone is a more potent inducer of outgrowth from primed PC12 cells than dansylcadaverine alone, but as in the case of unprimed PC12 cells, NGF and DC together promote the greatest degree of neurite sprouting (Fig. 4.3). Thus the promotion of neurite outgrowth from PC12 cells by DC is dependent upon the pretreatment of the cells with, or the simultaneous presence of, NGF.

Exogenous TGases do not inhibit neurite outgrowth. The ability of TGase inhibitors to promote neurite outgrowth suggests the converse experiment, in which exogenous TGase added to the cultured cells might inhibit sprouting. As illustrated in Figs. 4.4 and 4.5, however, the addition of two TGases, guinea pig liver TGase and human placental Factor XIII, do not significantly affect outgrowth from sympathetic neurons over a wide range of concentrations. It is possible that these TGases do not possess the substrate specificity necessary to affect neurite outgrowth, or they may not have access to the relevant substrates when added to living neurons.

DC does not affect the proportions of Thy-1 monomers and multimers. As demonstrated in Chapter 3, a correlation exists between a decreased proportion of Thy-1 in the multimeric state and an increased degree of process outgrowth. By acting as a competitive acyl acceptor (*139*), DC might be expected to inhibit the covalent association proposed to occur in Thy-1 dimers, and thus facilitate the sprouting of neurites by preventing Thy-1 multimerization. Figure 4.6 illustrates that DC has no effect on Thy-1 size distribution in PC12 cells, NGF-primed PC12 cells, and neurons. Thus DC promotes neurite outgrowth by a mechanism other than direct modulation of Thy-1 multimerization.

Discussion

TGase inhibitors promote neurite outgrowth from activated cells. The data presented in Figure 4.2 make clear that diverse inhibitors of TGase cause a large increase in the number of process-bearing neurons. Each of the three TGase inhibitors is likely to affect the cells in numerous ways, but the observation that all three agents lead to the stimulation of outgrowth strongly supports the argument that the inhibition of TGase activity is what is germane to the enhancement of sprouting. Thus the subsequent experiments with PC12 cells were performed with DC

only. Strikingly, DC can indeed enhance neurite outgrowth from PC12 cells, but only in the presence of NGF, or after PC12 cells had been primed with NGF (Fig. 4.3). In Chapter 3, the finding that the synthetic peptide TDP-1 could promote sprouting from NGF-primed PC12 cells but not unprimed cells was discussed. It was argued that TDP-1 can promote outgrowth only from cells in which the machinery necessary to form processes is already present; in PC12 cells this must be induced by factors such as NGF. The same discussion applies to the results with DC and PC12 cells. DC alone does not induce the state of activation necessary for process outgrowth, but if the cells are activated by NGF, DC can promote outgrowth. In sum, TGase may indeed be involved in the regulation of neurite sprouting and process outgrowth from cells capable of sprouting.

Exogenous TGases do not affect neurite outgrowth. The observation that the three TGase inhibitors promote outgrowth suggested the possibility that exogenously applied TGases may do the opposite, namely inhibit neurite outgrowth. However, culturing neurons in a wide concentration range of both guinea pig liver TGase, and human placental Factor XIII, has no effect on outgrowth (Figs. 4.4 and 4.5). There are two likely explanations for this finding: (i) The exogenously applied TGases simply may not have access to the substrates involved in neurite outgrowth. In the case of the TGase inhibitors, all three are likely to be membrane permeable since both DC and ethylamine are reasonably hydrophobic, and bacitracin was originally characterized as an antibiotic that interferes with bacterial metabolism (*115*). Therefore, the inhibitors are likely to have access to cytoplasmic TGase and thus mediate their effects. Both liver TGase and Factor XIII, however, are fairly large proteins (150kD and 75-80kD, respectively (*73*)) and are unlikely to directly enter the cytoplasm. If taken up by endocytosis, it is probable that they would still lack access to the cytoplasm and would be targeted to lysozomes and degraded. Thus, if substrates relevant to neurite outgrowth are primarily cytoplasmic, it is unlikely that exogenous TGases would have access to them. Alternatively, (ii) if TGase substrates involved in outgrowth are in fact present on the cell surface, it is possible that the exogenously applied TGases do not have the

substrate specificities required to act on the relevant proteins. It will be important to determine if these classes of TGases are present in neurons.

The promotion of neurite outgrowth by DC is not due to direct modulation of Thy-1 multimerization. Since the application of DC on cultured PC12 cells, NGF-primed PC12 cells, and neurons has no effect on the proportions of Thy-1 in monomeric and multimeric forms, it is unlikely that DC promotes outgrowth by affecting Thy-1. This observation does not negate the possibility that the Thy-1 dimer may be a product of a γ-glutaminyl-ε-lysine crosslink between two Thy-1 monomers; if such a crosslink exists, it may occur biosynthetically in a compartment inaccessible to DC, or DC may be incapable of inhibiting the TGase activity responsible for catalyzing Thy-1:Thy-1 bond formation. Further structural analysis of the Thy-1 dimer may resolve this issue.

The possible role of TGase in neurite outgrowth. Since a direct action of TGase on Thy-1 is unlikely to regulate neurite outgrowth, the question arises as to how TGases do affect sprouting. As noted earlier, in the case of differentiating mouse neuroblastoma cells, ^{3}H-putrescine was incorporated primarily into very high molecular weight polymeric protein and tubulin, as well as actin and low molecular weight derivatives (*143*). The same study demonstrated that purified microtubules could incorporate ^{3}H-putrescine when added to neuroblastoma extracts containing TGase activity, while purified free subunits of tubulin and microtubule-associated proteins showed much lower levels of incorporation. Moreover, free tubulin subunits preincubated with putrescine and neuroblastoma TGase, then dialyzed with microtubule assembly buffer, polymerized at a greater rate than control tubulin subunits (*143*). The preincubation of tubulin subunits presumably blocks sites of normal crosslinking which would inhibit or slow polymerization. Thus these results suggest that neuroblastoma TGase serves to crosslink assembled microtubules and slow polymerization. The addition of TGase inhibitors such as DC to cultured neurons, may thus override the control of polymerization and lead to the large increase in process outgrowth. Similarly, it has been demonstrated

that NGF treatment of PC12 cells leads to increased expression of both tubulin mRNA and protein (*61*, *62*); thus the requirement for NGF treatment or pretreatment in order for DC to affect PC12 outgrowth may again be attributable to interference with normal microtubule assembly. It is important to realize that neural TGases probably act on other substrates in addition to acting on microtubules. The relationship between these as yet unstudied post-translational modifications and neural function may provide fruitful ground for future research.

FIGURE LEGENDS

Figure 4.1: The reaction catalyzed by transglutaminase. An isopeptide linkage between two proteins is formed through the crosslinking of endo-γ-glutaminyl and endo-ε-lysyl residues and gives rise to the γ-glutaminyl-ε-lysine bridge.

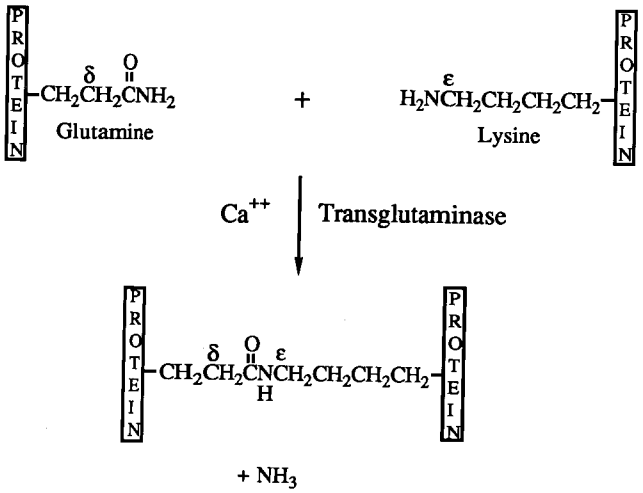

PROTEIN
δ
CH2CH2CNH2
O
Glutamine
+
ε
H2NCH2CH2CH2CH2
Lysine
PROTEIN
Ca++
Transglutaminase
PROTEIN
δ
CH2CH2CNCH2CH2CH2CH2
O
H
ε
PROTEIN
+ NH3

Figure 4.2: Process outgrowth from sympathetic neurons in culture is stimulated by TGase inhibitors. Neurons were cultured in serum-free medium with the indicated concentrations of dansylcadaverine, bacitracin, and ethylamine for 24 hr and the number of process-bearing cells counted. Data is expressed as percent of total cells with processes ± standard error of the mean.

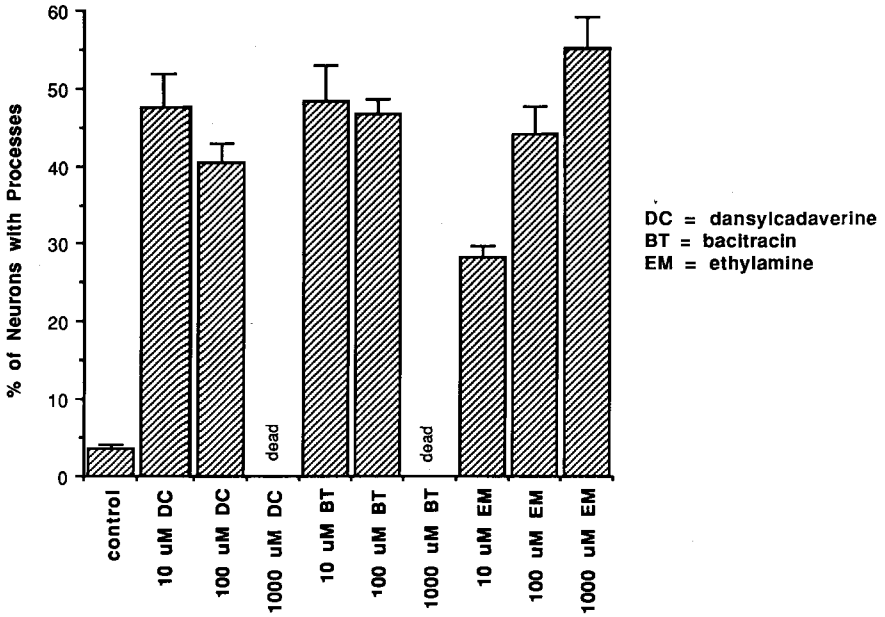

% of Neurons with Processes
60
50
40
30
20
10
0
dead
dead
control
10 uM DC
100 uM DC
1000 uM DC
10 uM BT
100 uM BT
1000 uM BT
10 uM EM
100 uM EM
1000 uM EM
DC = dansylcadaverine
BT = bacitracin
EM = ethylamine

Figure 4.3: The effect of DC on NGF-primed and unprimed PC12 cell neurite outgrowth in the presence and absence of NGF. Primed cells were cultured in NGF for 3 d prior to the experiment and then cultured as the unprimed cells for 24 hrs. as indicated. The number of process-bearing cells was scored and presented as the percent of total cells with processes ± standard error of the mean.

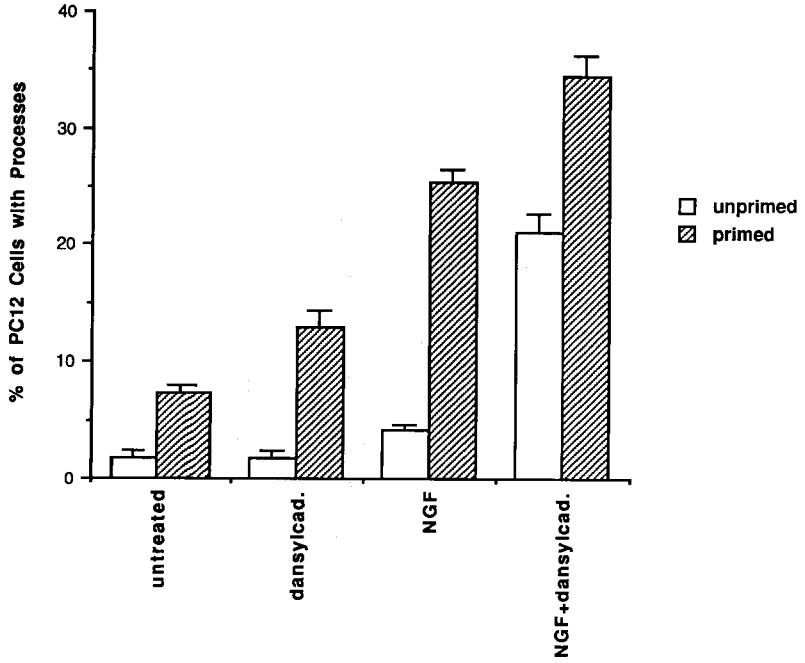

% of PC12 Cells with Processes
40
30
20
10
0
untreated
dansylcad.
NGF
NGF+dansylcad.
unprimed
primed

Figure 4.4: The effect of guinea pig liver TGase on outgrowth from cultured neurons. Neurons were cultured for 24 hrs. in serum-free medium with the indicated concentration of TGase and the number of process-bearing cells scored. Error bar represents the standard error of the mean.

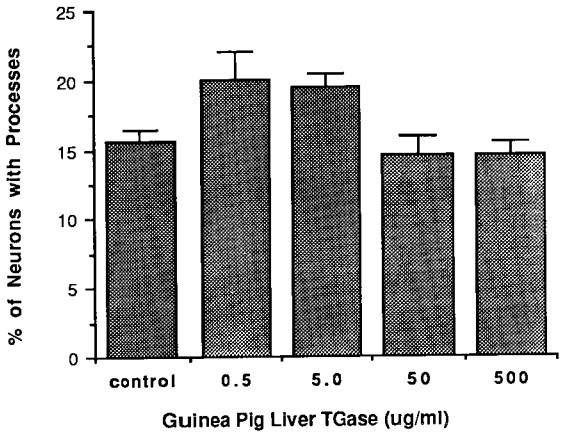

% of Neurons with Processes
25
20
15
10
5
0
control
0.5
5.0
50
500
Guinea Pig Liver TGase (ug/ml)

Figure 4.5: The effect of human placental Factor XIII on outgrowth from cultured neurons. Neurons were cultured for 24 hrs. in serum-free medium with the indicated concentration of TGase (1 U = 568 μg) and the number of process-bearing cells scored. Error bar represents the standard error of the mean.

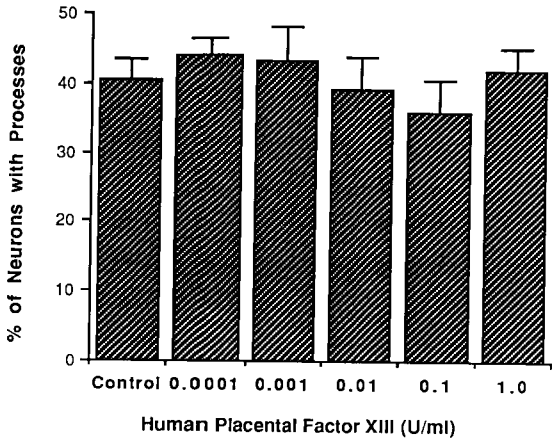
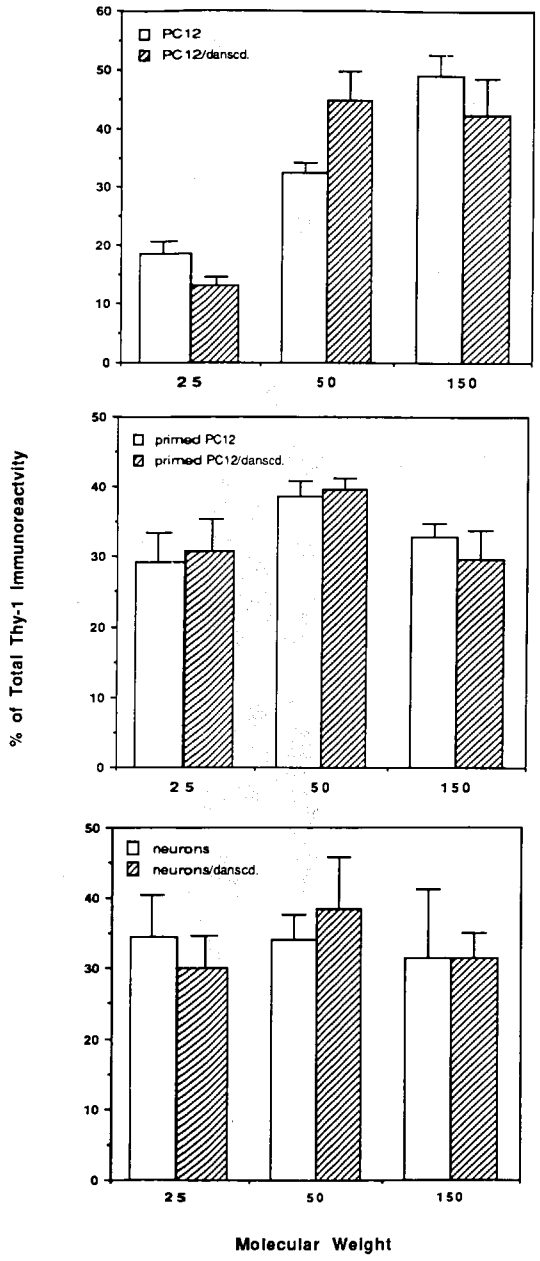

% of Neurons with Processes
50
40
30
20
10
0
Control
0.0001
0.001
0.01
0.1
1.0
Human Placental Factor XIII (U/ml)

Figure 4.6: DC does not affect Thy-1 multimerization. PC12 cells, NGF-primed PC12 cells, and neurons were cultured in the presence or absence of DC, lysed, and then subjected to size exclusion chromatography followed by immunoassay of the fractions for Thy-1 immunoreactivity. The amount of Thy-1 found in the monomer, dimer, and hexamer forms is expressed as a percentage of the total Thy-1 immunoreactivity. Error bar represents the standard error of the mean.


PC12
PC12/danscd.
primed PC12
primed PC12/danscd.
neurons
neurons/danscd.
% of Total Thy-1 Immunoreactivity
Molecular Weight
25
50
150
0
10
20
30
40
50
60

APPENDIX

The following two papers contain research I have performed as a graduate student, but do not directly pertain to studies on the function of Thy-1. These papers are, however, interrelated and concern the effects of growth factors on the survival and differentiation of primary adrenal chromaffin cells in culture. The first paper, "Basic FGF induces neuronal differentiation, cell division, and NGF dependence in chromaffin cells: A sequence of events in sympathetic development" (*Neuron* 1:517-525 (1988)) presents the results of a series of experiments performed by Derek Stemple and myself, and discusses the relevance of these findings for understanding phenotypic differentiation of cells in the sympathoadrenal lineage; it is reproduced here by the kind permission of Cell Press. The second paper, "Adrenal chromaffin cells as multipotential neurons for autografts" (*Prog. Brain Res.* 82:33-39 (1990)), presents data that is a continuation of the first paper, as well as data concerning the transplantation of sympathoadrenal tissue into the CNS. It is a product of preliminary, collaborative experiments with Dr. Fred H. Gage (University of California Medical School, San Diego) that seek therapeutic applications of the *in vitro* findings; it is reproduced here by the kind permission of Elsevier Science Publishers.

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Basic FGF Induces Neuronal Differentiation, Cell Division, and NGF Dependence in Chromaffin Cells: A Sequence of Events in Sympathetic Development

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Summary

To define further the molecules that control sympathoadrenal differentiation, we have investigated the effects of FGF, NGF, and glucocorticoid on cultured neonatal rat adrenal chromaffin cells. Basic FGF (bFGF), like NGF, induces cell division and neurite outgrowth from these cells. Dexamethasone inhibits neuronal differentiation but not proliferation induced by bFGF. Unlike NGF, bFGF will not support the survival of chromaffin cell–derived sympathetic neurons. However, bFGF induces a dependence on NGF. The overlapping but distinct responses to NGF and bFGF may underlie a sequence of events in sympathetic differentiation. bFGF (or another factor) may act locally in developing ganglia to stimulate mitotic expansion and initial axon outgrowth. Subsequent survival and maturation are then controlled by NGF, which is provided by peripheral targets of innervation. In the adrenal gland, glucocorticoids may permit bFGF to amplify the chromaffin population, while preventing neuronal differentiation.

Introduction

The neural crest is a transient neurectodermal structure of the vertebrate embryo that gives rise to a variety of cell types including those of the sympathoadrenal lineage: the neurons of the sympathetic ganglia and the chromaffin cells of the adrenal medulla (Le Douarin, 1982). In vitro studies have shown that chromaffin cells and sympathetic neurons can be derived from a common precursor (Doupe et al., 1985b; Anderson and Axel, 1986). Interestingly, mature chromaffin cells can be induced to transdifferentiate into sympathetic neurons by addition of nerve growth factor (NGF) and a factor in heart cell–conditioned medium (Unsicker et al., 1978; Doupe et al., 1985a). This effect is blocked by glucocorticoid, suggesting that the high local concentration of steroids in the adrenal medulla may be important for the maintenance of an endocrine phenotype. In support of this, glucocorticoids are required for the survival of rat chromaffin cells in vitro (Doupe et al., 1985a). In addition to inducing neuronal differentiation, NGF can simultaneously serve as a mitogen for these cells (Lillien and Claude, 1985). A cell line of adrenal medullary origin, PC12, is also capable of adopting a neuron-like phenotype in the presence of NGF (Greene and Tischler, 1976). These findings, in conjunction with the original characterization of the role of NGF in the embryo (Levi-Montalcini, 1976), have established NGF as a mitogen and differentiation agent, as well as a survival factor.

Acidic and basic fibroblast growth factors (aFGF and bFGF), though originally characterized as potent mitogens for a variety of cell types of mesodermal origin (for review, see Gospodarowicz et al., 1986b), have recently been demonstrated to support the survival of a variety of vertebrate neurons. Embryonic rat hippocampal neurons, cerebellar granule cells, and Purkinje cells, as well as cortical neuronal precursor cells, can all be sustained by bFGF in vitro (Walicke et al., 1986; Morrison et al., 1986; Gensburger et al., 1987; Hatten et al., 1988). In addition, FGF supports the survival of chick parasympathetic ciliary ganglion neurons (Unsicker et al., 1987). The response of PC12 cells to aFGF and bFGF is very similar to their response to NGF (Togari et al., 1985; Wagner and D'Amore, 1986; Neufeld et al., 1987; Rydel and Greene, 1987; Schubert et al., 1987). Thus far, FGF and NGF are the only proteins known to be capable of inducing neuron-specific mRNAs in PC12 cells (Leonard et al., 1987; Stein et al., 1988a).

The response of PC12 cells to FGF raises the possibility that this factor may play a previously unanticipated role in the normal development of the sympathoadrenal lineage. To address this question, the actions of bFGF on primary cultures of neonatal rat adrenal chromaffin cells were examined and compared with those of NGF.

Results

bFGF Induces the Neuronal Differentiation of Chromaffin Cells

The effects of bFGF (purified some 140,000-fold from bovine pituitary) on chromaffin cells were initially examined by measuring neurite outgrowth, a morphologic index of neuronal differentiation (Figure 1; Figure 2). bFGF at a concentration of 10 ng/ml rapidly induced process outgrowth from cultured chromaffin cells (Figure 1E). The kinetics of this induction were roughly equivalent to those observed with NGF (Figure 2A, compare NGF and FGF, filled symbols), except that in bFGF the percentage of process-bearing cells reached a plateau of 83% at day 6, whereas in NGF it continued to increase to 99% by day 10. In agreement with observations reported for PC12 cells (Neufeld et al., 1987; Rydel and Greene, 1987), it was found that the dose-response for neurite outgrowth from chromaffin cells was biphasic with respect to bFGF (Figure 2B), although this was less apparent after longer periods of exposure to the factor.

Dexamethasone (dex) inhibits NGF-induced neurite outgrowth from chromaffin cells (Unsicker et al., 1978; Doupe et al., 1985a). It was therefore of interest to determine whether this synthetic glucocorticoid would have a similar effect on process outgrowth induced by bFGF. When cultures were simultaneously exposed to bFGF plus 1 μM dex, the extent of process outgrowth was in-

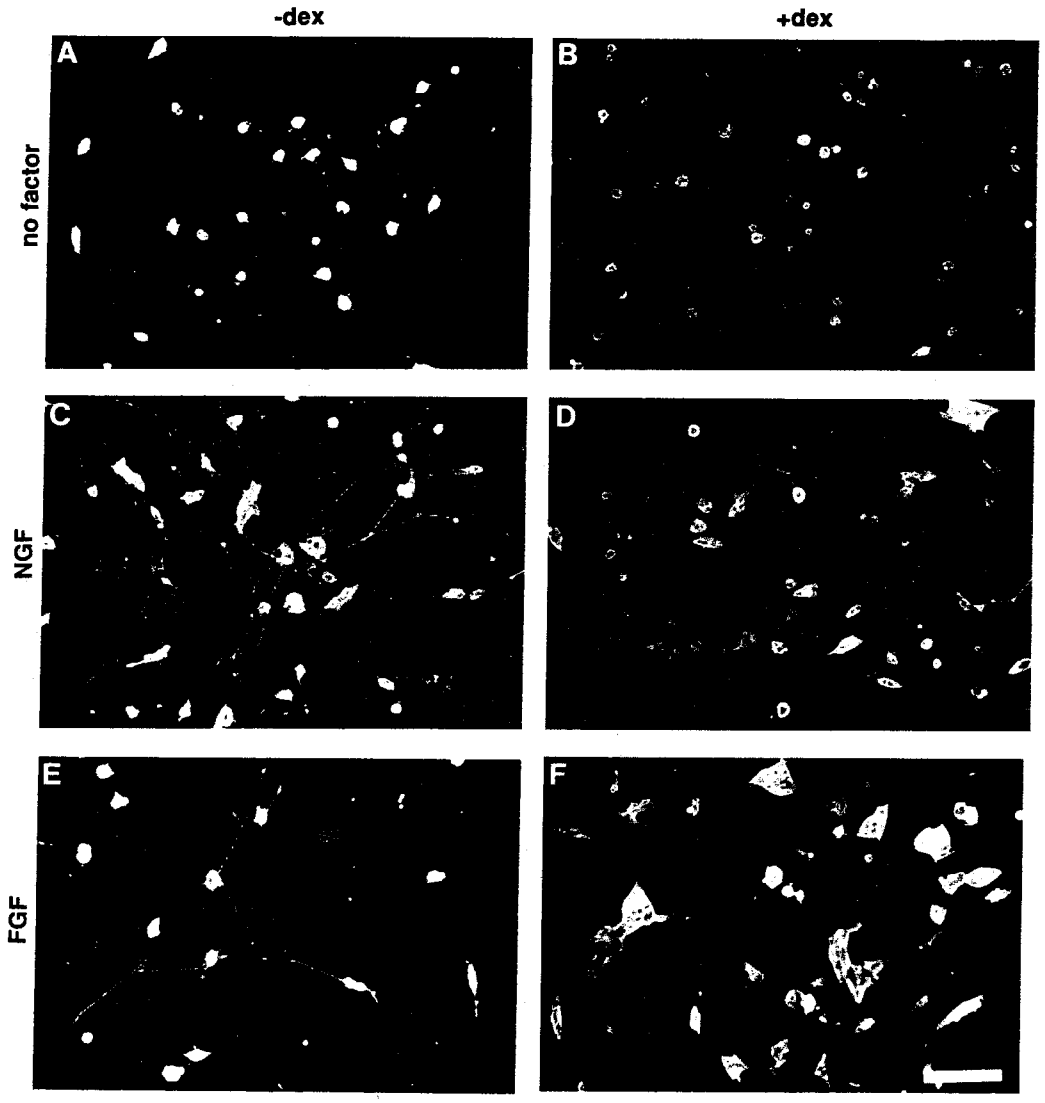

Figure 1. Morphology of Chromaffin Cells Treated with bFGF, NGF, and/or dex

Cells were grown for 6 days in growth medium alone (A) or in growth medium supplemented with the following factors: 1.0 μM dex (B), 1.0 μg/ml 7S NGF (C), NGF plus dex (D), 10.0 ng/ml bFGF (E), and bFGF plus dex (F). Fluorescence micrographs were taken of cultures stained with OX-7 monoclonal anti-Thy-1 antibody. Bar, 50 μm.

hibited by about 50% (Figure 2A, FGF, open symbols). However, the rate of accumulation of process-bearing cells was only slightly diminished by the steroid. In this culture condition, chromaffin cells were often found in large, flattened clusters bearing processes that were thicker, shorter, and less arborized than those observed in bFGF alone (compare Figures 1E and 1F). Furthermore, most process-bearing cells in bFGF plus dex did not express immunohistochemically detectable amounts of the neural-specific marker protein SCG10 (Stein et al., 1988b) (data not shown). The quantitative data may therefore underestimate the inhibition of neuronal differentiation caused by dex. The inhibitory effect of dex on NGF-induced neuronal differentiation was more pronounced than in the case of bFGF, affecting both the rate and the extent of neurite outgrowth (Figure 1D; Figure 2A, NGF, open symbols).

To distinguish whether the neuronal differentiation caused by bFGF reflects the phenotypic conversion of chromaffin cells (as observed for NGF [Doupe et al., 1985a]) or the selection for preexisting process-bearing cells (the adrenal medulla contains a small proportion of ganglionic neurons [Anderson and Axel, 1985; Trojanowski et al., 1986]), the number of cells in each dish was measured as a function of time in bFGF. This experiment revealed that between days 2 and 6, the number of cells

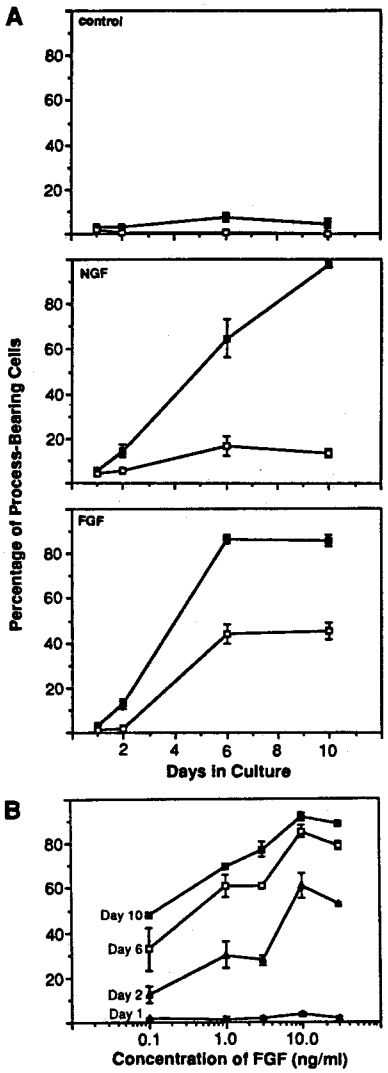

Figure 2. Effects of bFGF and NGF on the Time Course of Neurite Outgrowth

(A) Cells were counted and scored for the presence of neurites. For each of the experimental conditions, the percentage of cells bearing a process is plotted against the number of days in culture. In all panels, filled squares correspond to the presence of the factor indicated in the upper left corner of the panel and open squares correspond to the factor in combination with 1.0 μM dex. Control indicates that cells were grown in growth medium alone; NGF indicates the presence of 1.0 μg/ml 7S NGF; FGF indicates the presence of 10.0 ng/ml bFGF. The values plotted are averages of several dishes for each condition on each day, obtained from two independent experiments (days 1 and 2, n = 10; day 6, n = 8; day 10, n = 4). Error bars indicate the standard error of the mean.

(B) Dose-response characteristics of bFGF-induced neurite outgrowth. Cells were counted and scored as in (A). Each point represents the average value of counts from at least two dishes with the exception of day 10, 0.1 ng/ml and 1.0 ng/ml conditions (n = 1). Error bars indicate the standard error of the mean.

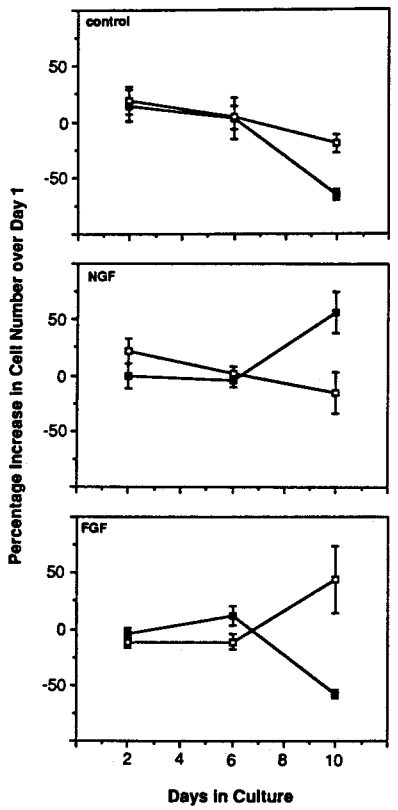

Figure 3. Effects of bFGF and NGF on Chromaffin Cell Number

For each of the experimental conditions, the percent increase in the number of cells over day 1 (to normalize for initial differences in plating efficiencies) is plotted against the number of days in culture. In all panels, filled squares correspond to the presence of the factor indicated in the upper left corner of the panel and open squares correspond to the factor in combination with 1.0 μM dex. Control indicates that cells were grown in growth medium alone; NGF indicates the presence of 1.0 μg/ml 7S NGF; FGF indicates the presence of 10.0 ng/ml bFGF. The values plotted are averages of several dishes for each condition on each day, obtained from two independent experiments (days 1 and 2, n = 10; day 6, n = 8; day 10, n = 4). Error bars indicate the standard error of the mean.

in bFGF increased only slightly (Figure 3, FGF, filled symbols), whereas the percentage of process-bearing cells rose to over 80% during this same interval (Figure 2, FGF, filled symbols). It is therefore likely that bFGF, like NGF (Figure 2; Figure 3; Doupe et al., 1985a), acts by inducing the phenotypic conversion of chromaffin cells to neurons, rather than by selecting for preexisting neurons in the culture. This interpretation is supported by serial observations of identified chromaffin cells (described below).

bFGF Is a Chromaffin Cell Mitogen

In cultures exposed to bFGF, a striking increase in the frequency of cell clusters was observed. This raised the possibility that, in addition to stimulating neuronal differentiation, this factor might also be a mitogen for chro-

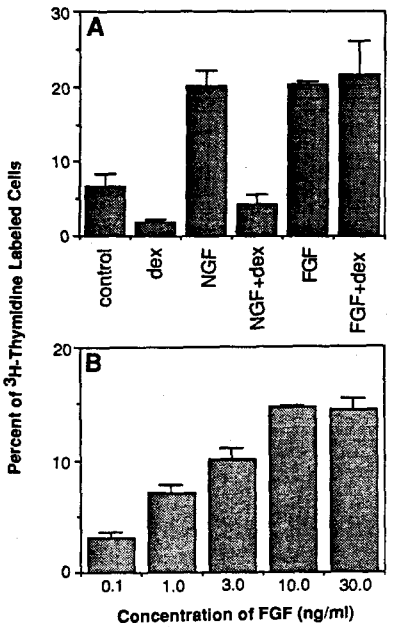

Figure 4. Effects of bFGF and NGF on the Incorporation of [^{3}H]Thymidine in Chromaffin Cells

Cells were grown for 6 days in the various culture conditions and then exposed to [^{3}H]thymidine for 24 hr. Cultures were processed, and OX-7-positive cells were scored for the presence of silver grains over the nuclei. The percentage of chromaffin cells that incorporated thymidine was calculated. (A) Control indicates that cells were grown in growth medium alone; dex indicates the presence of 1.0 μM dex; NGF indicates the presence of 1.0 μg/ml 7S NGF; NGF + dex indicates the both NGF and dex were present; FGF indicates the presence of 10.0 ng/ml bFGF; FGF + dex indicates that both bFGF and dex were present in culture. The average of data from five dishes in each condition is plotted. Error bars indicate the standard error of the mean. (B) Dose-response for thymidine incorporation. Data represent the average from three dishes for each condition. Error bars indicate the standard error of the mean.

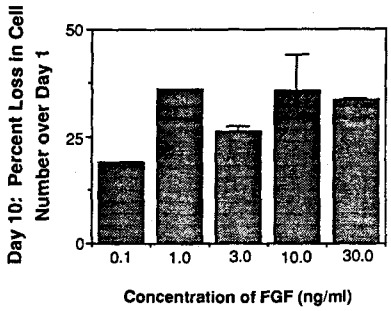

Figure 5. Effect of Varying bFGF Concentration on Chromaffin Cell Survival

The percent decrease in cell number at day 10 relative to day 1 is shown. Data for 3, 10, and 30 ng/ml represent the average from two sister cultures, while data for 0.1 and 1.0 ng/ml are from a single culture; all are based on initial counts of 350–400 cells per culture on day 1. The apparent slight decrease in cell loss at 0.1 ng/ml bFGF may not be statistically significant. However, at this concentration a significantly smaller proportion of the cells were process-bearing (48% compared with 95% for 10 ng/ml bFGF); therefore it is possible that at low concentrations bFGF may improve the viability of those chromaffin cells which have not yet converted to neurons.

maffin cells. This possibility was also suggested by the previous observation that NGF is a chromaffin cell mitogen (Lillien and Claude, 1985). [^{3}H]thymidine labeling of cultures on day 6 confirmed that bFGF, like NGF, stimulated DNA synthesis about 3-fold in chromaffin cells (Figure 4A). The dose-response for mitogenic stimulation confirmed that a concentration of 10 ng/ml was saturating for this effect (Figure 4B). Although dex strongly inhibited the mitogenic action of NGF (as previously reported [Lillien and Claude, 1985]), it did not do so for bFGF (Figure 4A). This difference in the inhibitory effect of dex was further reflected in cell number measurements: in bFGF plus dex, cell number increased significantly by day 10 in culture (Figure 3, FGF, open symbols), whereas dex inhibited the increase in cell number caused by NGF (Figure 3, NGF, open symbols).

bFGF Induces a Dependence on NGF but Does Not Itself Promote Survival

Although bFGF and NGF had similar effects on chromaffin cells between days 2 and 6 in vitro, between days 6 and 10 a significant difference was observed. In bFGF, the number of cells decreased precipitously during this later interval, while the cell number increased in NGF (Figure 3, filled symbols). One possible explanation for this difference is that bFGF can induce the neuronal differentiation of chromaffin cells, but it cannot support their subsequent survival as sympathetic neurons. In support of this interpretation, cultures of neonatal sympathetic neurons (derived from superior cervical ganglia) cannot be maintained by bFGF (data not shown), consistent with the findings of Rydel and Greene (1987). Thus, although bFGF can support the survival of some CNS neuronal populations (Walicke et al., 1986; Morrison et al., 1986; Hatten et al., 1988), and even parasympathetic periperal neurons (Unsicker et al., 1987), it does not do so for neonatal rat sympathetic neurons.

In a separate experiment (Figure 5), it was determined that varying the concentration of bFGF over a wide range (0.1–30 ng/ml) did not significantly improve the viability of cells when measured at day 10, by which time most have converted to a neuronal phenotype. In all concentrations of bFGF tested, a net cell loss occurred, whereas in the experiment of Figure 3, a 60% increase in cell number at day 10 was observed in the presence of NGF. Although under control conditions (no added factors), the extent of cell loss at day 10 was similar to that seen in 10 ng/ml bFGF (Figure 3), the attrition in control cultures reflects the elimination of chromaffin cells, which are glucocorticoid-dependent (Figure 3, control versus dex); in contrast, the loss of cells in bFGF-containing cultures occurs in a population expressing a neuronal phenotype.

The simplest interpretation of the decline in cell number in bFGF after day 6 is that at about this time, the chromaffin-derived neurons have acquired an absolute

Table 1. NGF Rescues Chromaffin Cells Previously Exposed to bFGF

	Mass Culture	Identified Cell
Condition[a]	% of Cells at Day 6 Present at Day 10[b]	% of Cells at Day 6 Present at Day 12[c]
NGF → NGF	147 ± 25	100
bFGF → bFGF	37 ± 7	16
bFGF → NGF	108 ± 1	92

[a] Cells were switched to the indicated factor on day 6 of culture.
[b] Cell counts were made as for Figure 3. Values reflect the average cell numbers obtained from at least two dishes for each condition. Cells (200–400 in each dish) were scored on day 6. The number of cells at day 10 after switching from bFGF to NGF on day 6 was 404 ± 59; in cultures maintained in NGF it was 455 ± 115; in those maintained in bFGF it was 172 ± 28.
[c] Numbers based on following individual identified cells as described in the text. The mass culture data and the identified cell data are from two separate experiments.

dependence upon NGF, which cannot be substituted by bFGF. A prediction of this hypothesis is that NGF should rescue cells previously triggered to convert to sympathetic neurons by exposure to bFGF. To test this prediction, cells were exposed to FGF for 6 days and then switched to medium containing NGF. This treatment prevented the steep decline in cell number that ocurred after day 6 in bFGF, maintaining about 73% of the total number of cells maintained in control cultures exposed only to NGF (Table 1, Mass Culture column).

To exclude the possibility that bFGF and NGF may act on different subpopulations of chromaffin cells, in a separate experiment individual cells were identified during exposure to bFGF and then serially observed after switching to medium containing NGF. These experiments confirmed that most cells which had initially extended processes in response to bFGF died after day 6 when maintained in bFGF, as suggested by the cell counting data (Figure 6, FGF → FGF, days 8 and 12). In dramatic contrast, however, process-bearing cells in sister cultures survived when the medium was switched to NGF after day 6 (Figure 6, FGF → NGF, day 12). Of the identified cells, 92% survived when switched to NGF (n = 13), while only 16% survived when maintained in bFGF (n = 19) (Table 1, Identified Cell column). The rescued cells acquired the large cell bodies characteristic of cells maintained continuously in NGF (Figure 6, NGF → NGF). These single-cell tracking data thus strongly support the idea that when chromaffin cells are triggered to undergo neuronal differentiation by bFGF, they acquire a dependence upon NGF for survival that is not satisfied by bFGF.

The effects of the purified bovine pituitary bFGF preparation reported here could be mimicked by a preparation of recombinant human bFGF at similar concentrations (data not shown). As discussed previously elsewhere (Kimelman and Kirschner, 1987), this control makes it highly unlikely that the biological activities observed with the purified bovine preparation are due to any molecule other than bFGF. Also, we observed a similar response to bFGF when chromaffin cells were plated on a simple collagen substrate rather than Matrigel (data not shown), making it unlikely that the effects we observed in the latter case are due to a synergistic interaction (Kimelman and Kirschner, 1987) between bFGF and other growth factors which may be present in Matrigel (Hynda Kleinman, personal communication).

Discussion

aFGF and bFGF have mitogenic and/or differentiation-inducing effects on a variety of precursor cell types, including myoblasts and chondroblasts (Linkhart et al., 1980; Kato and Gospodarowicz, 1985; for review, see Gospodarowicz et al., 1986b). It was found that bFGF exhibits both activities on cultured adrenal chromaffin cells, which function as stably differentiated endocrine cells in the adult organism. (A preliminary report indicates that aFGF has similar effects [Claude et al., 1987, Soc. Neurosci., abstract].) In this case, bFGF induces the transdifferentiation of chromaffin cells into sympathetic neurons, rather than the further differentiation of an endocrine phenotype. This effect, as well as the mitogenic effects, is similar to the action of NGF on chromaffin cells (Doupe et al., 1985a; Lillien and Claude, 1985). Similarly, both NGF and bFGF cause PC12 cells to extend neurites (Togari et al., 1985; Wagner and D'Amore, 1986; Neufeld et al., 1987; Rydel and Greene, 1987; Schubert et al., 1987) and to increase the expression on neuron-specific mRNAs (Leonard et al., 1987; Stein et al., 1988a). The present work also reveals a difference between the actions of bFGF and NGF: bFGF cannot support the long-term survival of chromaffin cell–derived neurons, while NGF can (Table 2). However, bFGF does induce a dependence on NGF for survival, perhaps as part of a program of neuronal differentiation that it triggers in chromaffin cells.

The changes in the size of the chromaffin cell population in response to NGF and bFGF (Figures 1–3) can be analyzed in terms of the contributions of cell division, survival, and death. The increase in total cell number in NGF (Figure 3) reflects the mitotic expansion of the differentiating population, as well as its subsequent survival as neurons. However, the proliferation effect is delayed, as indicated by both [^{3}H]thymidine incorporation on day 2 (data not shown) and single-cell tracking analysis (Figure 5). This delay in the mitogenic effect of NGF yields the flattened early portion of the growth curve (Figure 3). Since many cells extend neurites during this interval, this lag indicates that proliferation is not necessary for neuronal differentiation, consistent with the fact that neurite outgrowth from chromaffin cells can be observed in the presence of anti-mitotic agents (Doupe et al., 1985a). For bFGF, the decline in cell number after day 6 (Figure 3) reflects the fact that as these cells become neurons, they acquire a dependence on NGF for survival that is not satisfied by bFGF. In support of this interpretation, serial observations of identified cells revealed that NGF can rescue cells that have been triggered to undergo neuronal differentiation by bFGF (Table 1; Figure 6). In contrast to the case of bFGF alone,

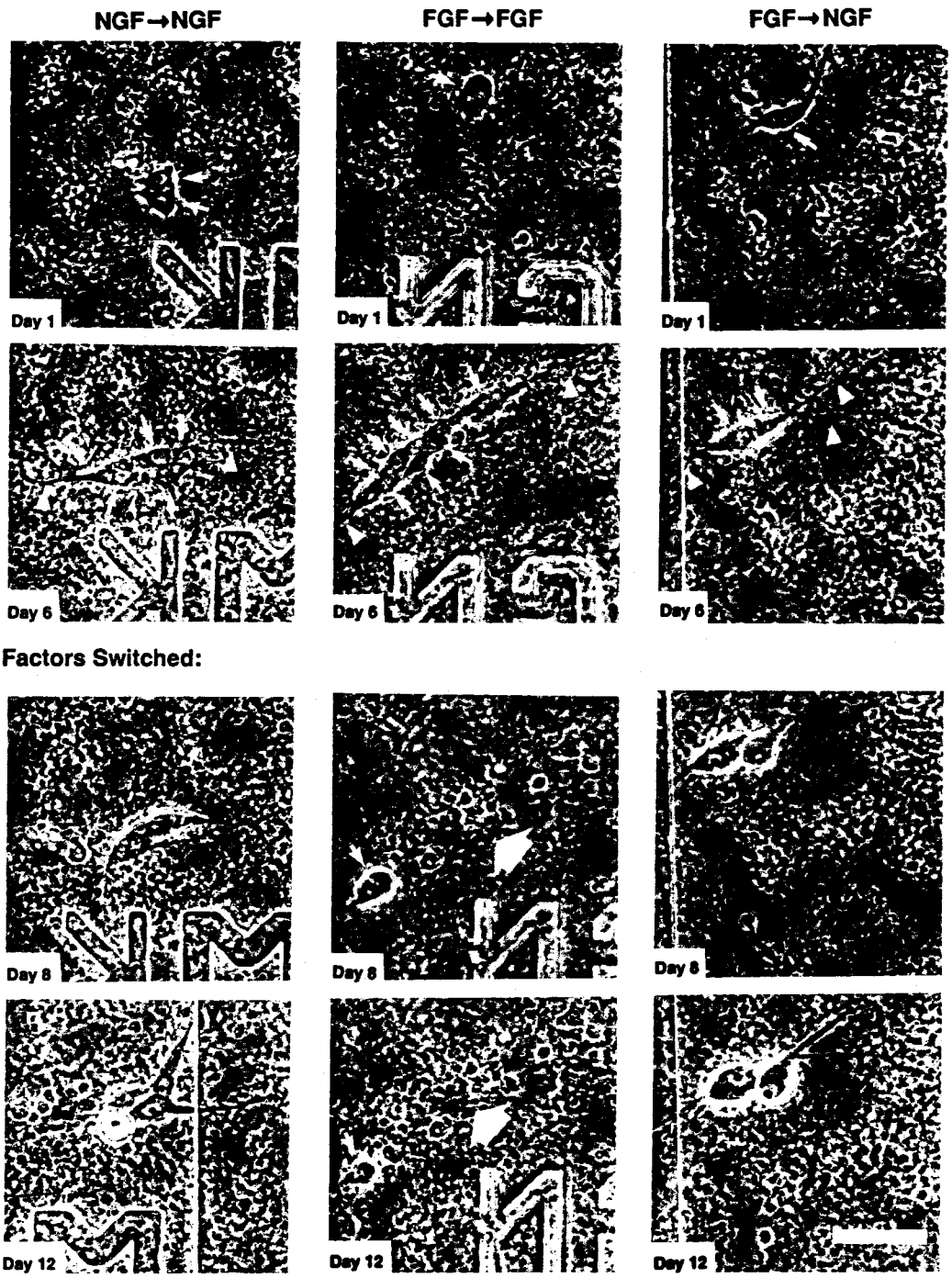

Figure 6. Chromaffin Cells Induced to Become Neurons by bFGF Are Rescued by Treatment with NGF

For each of these conditions, cells were photographed on the first day and on several subsequent days in culture. For the NGF → NGF condition, cells were maintained in 1.0 μg/ml 7S NGF throughout the series. For the FGF → FGF condition, cells were maintained in 10.0 ng/ml bFGF throughout the series. For the FGF → NGF condition, cells were initially treated with FGF and then switched on day 6 to medium containing NGF instead of bFGF. The small triangles indicate neurites emerging from the chromaffin cell clusters. The small arrows indicate the nuclei of some of the cells in the clusters. The large arrows indicate cellular debris left where clusters once existed. Bar, 25 μm.

Table 2. Summary of NGF and bFGF Effects on Neonatal Chromaffin Cells

Factor	Neuronal Differentiation	Mitogenic	Neuronal Survival
NGF	+	+	+
bFGF	+	+	−
NGF + dex	−	−	−
bFGF + dex	−	+	−

the combination of bFGF and dex causes cell number to increase dramatically by day 10 (Figure 3). This difference can be explained by two different effects of the glucocorticoid. In the presence of dex, the neuronal differentiation effect of bFGF is suppressed (Figure 1; Figure 2), while the mitogenic effect is not (Figure 4). Thus, cells remain in the chromaffin state and continue to proliferate. (As in the case for NGF, the mitogenic effect of bFGF is delayed.)

NGF was originally characterized as a survival factor for sympathetic neurons (Levi-Montalcini, 1976). Glucocorticoids, on the other hand, have been shown to function in part as a survival factor for adrenal chromaffin cells (Doupe et al., 1985a; Figure 3, compare control versus dex, day 10). In the absence of glucocorticoids, but in the presence of NGF, chromaffin cells survive and extend neurites. This initially suggested that NGF might substitute for dex as a chromaffin cell survival factor, but that in the absence of the inhibitory influence of glucocorticoid (Unsicker et al., 1978), the endocrine cells would undergo neuronal differentiation by default. The data presented here suggest a more complex mechanism. In the absence of any added factors, chromaffin cells survive for several days but do not undergo spontaneous neuronal differentiation. Therefore, the neuronal differentiation of chromaffin cells is not a default pathway, but requires both the presence of an inducer (bFGF or NGF) and the absence of an inhibitor (dex). The fact that bFGF can induce neuronal differentiation but is not sufficient for subsequent survival suggests that the ability of NGF to induce neuronal differentiation may be distinct from its effects on survival (Doupe et al., 1985b; Patterson, 1987).

Developmental Implications of the Chromaffin Cell Response to FGF

The similar but distinct responses of chromaffin cells to NGF and FGF (Table 2) imply that these two factors may activate different intracellular signaling pathways. The existence in chromaffin cells of such alternative signal transduction cascades, producing overlapping but nonidentical sets of biological responses, could be simply coincidental. Alternatively, if the response properties of chromaffin cells are similar to those of embryonic sympathetic precursors, these properties would fit well with some of the spatial and temporal constraints that recent data have placed on the embryonic development of sympathetic neurons. For example, NGF, which is synthesized by targets of sympathetic innervation (Heumann et al., 1984; Shelton and Reichardt, 1984), is not produced until the time at which embryonic neurons have grown axons to these targets (Davies et al., 1987; Korsching and Thoenen, 1988). Therefore, some other factor(s) may act at or near the forming ganglia to trigger the initial outgrowth of axons from neuroblasts to the periphery. Our results suggest that FGF, or some other molecule exerting identical effects, could serve such a function in vivo. The intracellular signaling pathway activated by this factor would not only initiate axon outgrowth, but would expand the population of ganglionic neuroblasts as well. The adrenal gland is also known to be a source of bFGF (Gospodarowicz et al., 1986a), and thus the established dex insensitivity of the mitotic response would allow the expansion of the developing medullary cell population within the glucocorticoid-rich adrenal gland, while simultaneously preventing neuronal differentiation.

In chromaffin cells, the FGF response pathway also leads to the acquisition of NGF dependence. By extension, in embryonic sympathetic precursors, the timing of this NGF-dependence might coincide with the arrival of sympathetic axons at their NGF-enriched peripheral targets. Simultaneously, the inability of the FGF signaling pathway ultimately to support neuronal survival would ensure the elimination of those neurons that failed to reach an appropriate peripheral source of NGF. Local NGF secretion by the target would then activate an alternative intracellular signaling pathway in sympathetic neurons, which would allow their subsequent survival and further maturation. In this way, a combination of factors, acting at different stages of development and triggering overlapping but distinct response cascades, could control a crucial sequence of steps in the early development of sympathetic neurons.

Preliminary observations suggest that the response properties of chromaffin cells to bFGF and NGF may indeed reflect the response properties of embryonic sympathetic precursor cells. In particular, it has been observed that bFGF stimulates neuronal differentiation in cultures of E14.5 paravertebral and superior cervical sympathetic ganglia (D. L. S. and J. Carnahan, unpublished observations). Moreover, sympathetic neurons at this stage do not require NGF for survival in vitro (Coughlin and Collins, 1985). In addition, bFGF appears to be mitogenic for E14.5 precursors in the presence of dex (A. Michelsohn, unpublished data). Thus, bFGF seems to induce a set of responses in embryonic sympathetic precursors similar to those induced in neonatal chromaffin cells. Confirmation of such a role for bFGF in vivo, however, awaits the development of methods for the sensitive detection of FGF in the mammalian embryo as well as the perturbation of its function.

Experimental Procedures

Cell Preparation

The adrenal medullae of neonatal (1–3 days) Simonsen albino rats (Simonsen Laboratories Inc., Gilroy, CA) were dissected free of cortex in L-15 air medium (Hawrot and Patterson, 1979). The medullae were dissociated by a modification of the method of Unsicker et al. (1978) in Hank's buffered saline solution without Ca^{2+} and Mg^{2+} (GIBCO, Grand Island, NY) containing 1.0 mg/ml collagenase

(Worthington Diagnostics, Freehold, NJ). Cell suspensions were centrifuged twice at 1000 rpm for 10 min and resuspended into growth medium. To remove the more substrate-adherent cortical cell population, the cell suspension was preplated on 100 mm Corning tissue culture plates (Corning Glass Works, Corning, NY) for 6 hr in growth medium in a humidified incubator at 37°C and 5% CO_2. The chromaffin cells were removed from the plates by gentle trituration, centrifuged at 1000 rpm for 10 min, resuspended into growth medium, and plated on previously prepared plates at a density of 400–3000 cells per well.

Culture Media

The growth medium used in this study was prepared as described by Mains and Patterson (1973; Hawrot and Patterson, 1979) and was supplemented with 5% adult rat serum and 5% fetal calf serum (HyClone, Logan, UT). To generate the various experimental media, the growth medium was supplemented with one or more of the following additives: dexamethasone phosphate (Merck, Sharpe & Dohme, Teterboro, NJ) at a final concentration of 1.0 μM, NGF (7S) (Mains and Patterson, 1973) at a final concentration of 1.0 μg/ml, or bovine bFGF, prepared according to Gospodarowicz (1975), or recombinant human bFGF at a final concentration of 10 ng/ml. The bovine bFGF was a gift of Gera Neufeld, University of California, San Francisco, and the recombinant human bFGF was a gift of Forrest Fuller, California Biotechnology, Inc., Mountain View, CA.

Culture Dishes and Substrate

For the experiments in which the time course of process outgrowth was assayed, cells were growing on Aclar (Allied Chemical Corp., Morristown, NJ) coverslips in 35 mm Falcon petri dishes (Becton Dickinson and Company, Oxnard, CA) in which an 8 mm well had been lathed (Hawrot and Patterson, 1979). The dishes were sterilized by UV irradiation. To prepare the substrate, dishes were cooled to 4°C and coated with 50 μl of Matrigel (Collaborative Research, Bedford, MA), an Engelbreth-Holm-Swarm sarcoma extracellular matrix (Hadley et al., 1985), mixed 1:1 with growth medium. After 10 min, the coated dishes were flooded with 2 ml of experimental media and left for 1 hr at 4°C to allow the substrate meniscus to flatten. They were then transferred to a 37°C incubator for at least 1 hr to induce substrate polymerization and equilibration with the 5% CO_2. After 1 day in culture, the cells were overlaid with Matrigel mixture as follows. The medium and dishes were chilled to 4°C. Most of the medium in the dishes was drawn off, and an additional 50 μl of the Matrigel mixture was spread over the cells. The dishes were then flooded with cold medium and returned to the incubator, where they were maintained throughout the experiment. Cultures were fed every 4 days.

For [^{3}H]thymidine incorporation experiments, 15 × 3 mm glass rings (Thomas Scientific, Swedesboro, NJ) were attached to the bottoms of 35 mm Corning tissue culture dishes with high-vacuum silicone grease (Dow Corning, Midland, MI). The bottoms of the wells formed by the glass rings were coated with 100 μl of Matrigel mixture as described above.

For identified cell studies, a BB-Press petri dish press (World Precision Instruments, Inc., New Haven, CT) was used to imprint a grid pattern on the bottoms of 35 mm Corning tissue culture dishes. Glass rings were attached to the dishes outside the grid pattern as described above.

Cell Counting

For the time course of process outgrowth studies, chromaffin cells were distinguished on the basis of their phase-dense granular cytoplasm under phase-contrast optics at a total magnification of 200×. A cell was counted as bearing a process if the process extended >3 cell body diameters from the soma. Where cells were clustered, the number of nuclei was recorded as the total number of cells, and the number of emerging processes was recorded as the number of process-bearing cells. Cell numbers were measured by counting all of the cells in two parallel strips constituting ~30% of the total growth surface area. In this way, from 100 to 600 cells were scored for each dish, depending upon time and condition.

[^{3}H]Thymidine Labeling

At day 6 after plating cultures were exposed to [^{3}H]thymidine (ICN Radiochemicals, Irvine, CA) at a final concentration of 2 μCi/ml for 24 hr. Cultures were rinsed with PBS and fixed with 3.7% formaldehyde in PBS. The cultures were then incubated with OX-7, an anti-Thy-1 monoclonal antibody, and strained with an FITC-conjugated goat anti-mouse IgG secondary antibody (TAGO, Burlingame, CA) to label specifically the chromaffin cells in the cultures. The OX-7 hybridoma cell line was a gift of Alan Williams, MRC Cellular Immunology Unit, Oxford University. The labeled cultures were coated with photographic emulsion, Kodak NTB2 (Eastman Kodak Company, Rochester, NY), diluted 1:1 in 0.5 M sodium acetate, and exposed for 7 days at 4°C. The dishes were developed with Kodak D-19 developer and scored. OX-7-positive cells were counted with epifluorescence illumination, and those cells that had incorporated [^{3}H]thymidine were scored under bright-field illumination. All positive cells in all cultures were counted at a final magnification of 200 ×.

Identified Cells

Cells were identified by means of a gridded dish and photographed with 35 mm Plus-X film at ASA-125 under phase-contrast optics at a final magnification of 400×. For several days after initial plating, the same fields were photographed.

Acknowledgments

The authors wish to express great thanks to Paul H. Patterson for his advice, support, and enthusiasm throughout the course of this work and for his critical comments on this manuscript. We thank Doreen McDowell for the preparation of tissue culture media and collagen and Josette Carnahan for the preparation of 7S NGF. We would also like to thank Forrest Fuller (California Biotech, Inc.), Gera Neufeld (UCSF), and Alan Williams (Oxford University) for the provision of the recombinant human bFGF, purified bovine bFGF, and the OX-7 hybridoma line, respectively. D. L. S. is supported by an NIH Training Grant, and N. K. M. is a recipient of an NSF Graduate Fellowship. This work is supported by grants from the NINCDS and the McKnight Foundation to Paul H. Patterson and by NIH grant No. RO1 NS23476-01 and Searle Scholar and NSF Presidential Young Investigator awards to D. J. A.

Received March 21, 1988; revised May 23, 1988.

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S.B. Dunnett and S.-J. Richards (Eds.)
Progress in Brain Research, Vol. 82

CHAPTER 4

Adrenal chromaffin cells as multipotential neurons for autografts

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There are a number of advantages in using a patient's own tissue for grafts to repair damage to the nervous system. Autografts alleviate the need for immunosuppression and the ethical, legal and availability problems associated with the use of fetal tissue are avoided. Peripheral nerve autografts have been used for many years in the treatment of peripheral nerve damage (Sunderland, 1978). In animal models of Parkinson's disease, grafts of adrenal chromaffin cells can successfully ameliorate behavioral deficits (Freed, et al., 1981; Freed, 1983; Kamo, et al., 1987; Becker and Freed, 1988; Nishino, et al., 1988), although there are questions as to the mechanism of this effect (Bohn, et al., 1987; Fiacanda, et al., 1988; Hansen, et al., 1988; Pezzoli, et al., 1988). Clinical trials of adrenal autografts with parkinsonism have yielded very positive or mixed results (Backlund, et al., 1985; Madrazo, et al., 1987; Allen, et al., 1989; Goetz, et al., 1989; Kelly, et al., 1989; Peterson, et al., 1989).

The chromaffin cell grafts in the parkinsonism models were used primarily as living depots of catecholamines. Because of their phenotypic plasticity, however, chromaffin cells have considerably more potential as donor tissue than has been utilized heretofore. These cells can be maintained in their normal chromaffin phenotype by addition of corticosteroids (Doupe, et al., 1985) and they can grow neurites when the steroid is removed and nerve growth factor (NGF) or fibroblast growth factor (FGF) is added (Olson, 1970; Unsicker, et al., 1978; Tischler, et al., 1980; Lillien and Claude, 1985; Notter, et al., 1986; Shaw and Letourneau, 1986; Claude, et al., 1988; Stemple, et al., 1988). Moreover, when the appropriate conditioned medium (CM) is supplied along with NGF, the efficiency of conversion of neonatal chromaffin cells into sympathetic neurons is enhanced and chromaffin cells taken from adult rats can also be converted (Doupe, et al., 1985). In addition, the chromaffin cell-derived neurons can be converted to the cholinergic phenotype when an instructive differentiation factor purified from cultured heart cells is added (Doupe, et al., 1985; Fukada, 1985; see also Boska, 1985). These converted, cholinergic sympathetic neurons can form functional cholinergic synapses (Ogawa, et al., 1984).

The switch from noradrenergic to cholinergic transmitter phenotype was first studied using primary neurons obtained from sympathetic ganglia of neonatal or adult rats (Patterson, 1978; Matsumoto, et al., 1987). These neurons have subsequently been found to be capable of expressing other transmitters (serotonin, adenosine), as well as a variety of neuropeptides (vasoactive intestinal polypeptide, somatostatin, Substance P, neuropeptide Y, enkephalins, cholecystokinin) when grown in CM (Kessler, 1985; Wolinsky and Patterson, 1985; Marek and Mains, 1987; Sah and Matsumoto, 1987; Nawa and Patterson, 1990). Several of the instructive differentiation factors in CM have now been separated and shown to be

distinct (Nawa and Patterson, 1990), allowing the production of sympathetic neurons with defined neuropeptide as well as neurotransmitter phenotypes. Since the adrenal-derived neurons respond to the cholinergic signal and are thus far indistinguishable from sympathetic neurons, it is probable that they will also respond to the newly defined neuropeptide-inducing signals. Thus, chromaffin cells are a potential source of neurons whose phenotype is under experimental control.

To test the efficacy of using these pluripotential cells as donor neurons for grafts into the adult rat brain, we are first using cholinergic sympathetic neurons. There is reason to predict that such neurons grafted into the denervated hippocampus will survive and innervate that structure. These neurons respond to NGF as a trophic factor (Patterson and Chun 1977) and the hippocampus has the highest level of NGF protein and mRNA in the brain, with significant increases upon cholinergic denervation (Korsching, 1986). In addition, septal lesions in adult rats cause endogenous noradrenergic sympathetic axons to sprout and grow into the hippocampal neuropil (Crutcher, 1987; Crutcher and Marfurt, 1988). Most importantly, noradrenergic axons from grafted sympathetic ganglia can innervate a denervated adult hippocampus (Björklund and Stenevi, 1977, Gage et al., 1984). In fact, the distribution of sympathetic axons was found to resemble both that of the normal noradrenergic innervation originating in the locus coeruleus, as well as that of the septal cholinergic afferents. It remained to be determined if cholinergic sympathetic neurons would also innervate the hippocampus and whether their pattern of growth would be different from that observed for noradrenergic sympathetic neurons. In addition, we were interested in whether large numbers of chromaffin cells could be produced from few donor adrenal glands, by causing the cultured cells to multiply before they differentiated into neurons.

Neurons, chromaffin and heart cells (for CM) are prepared by standard enzymatic dissociation methods (Doupe et al., 1985) and grown in a modified L-15 medium (Hawrot and Patterson, 1979). The chromaffin cells are grown in rat serum (from the same rat strain used for the chromaffin cells and for the graft recipients). The synthetic corticosteroid dexamethasone is added if the cells are to be maintained in the chromaffin phenotype. For conversion into neurons, NGF and heart cell CM is added (Doupe et al., 1985). The CM is serum-free, containing epidermal growth factor and insulin and it is concentrated and dialysed before addition to the chromaffin cells (Doupe et al., 1985). In this initial study, cholinergic sympathetic neurons are produced from neonatal ganglia and grown in the presence of ganglionic non-neuronal cells and CM (Hawrot and Patterson, 1979).

Mitosis and conversion of adult chromaffin cells

In order to produce more adrenal-derived neurons from fewer donors, we tested various conditions to determine if chromaffin cells from adult rats could be stimulated to multiply in culture and then be converted into neurons. It had been previously shown that FGF can induce mitosis in chromaffin cells from neonatal rats (Claude et al., 1988; Stemple et al., 1988) and fetal humans (Crickard et al., 1981) and that subsequent ad-

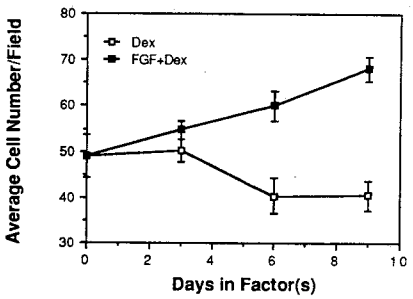

Fig. 1. The growth of adult rat adrenal chromaffin cells in the presence of dexamethasone (dex), or FGF plus dexamethasone. Adrenal medullae are dissected from the adrenal glands of adult rats, subjected to enzymatic dissociation with serial exposure to 0.1% collagenase, followed by 0.1% trypsin, preplated on tissue culture plastic for 6 hr and finally plated on a collagen substrate. Random fields in each culture were counted; error bars represent the standard error of the mean ($n = 9$ dishes for day 0; on subsequent days, $n = 6$ dishes for cells in dex and $n = 12$ dishes for cells in FGF plus dex).

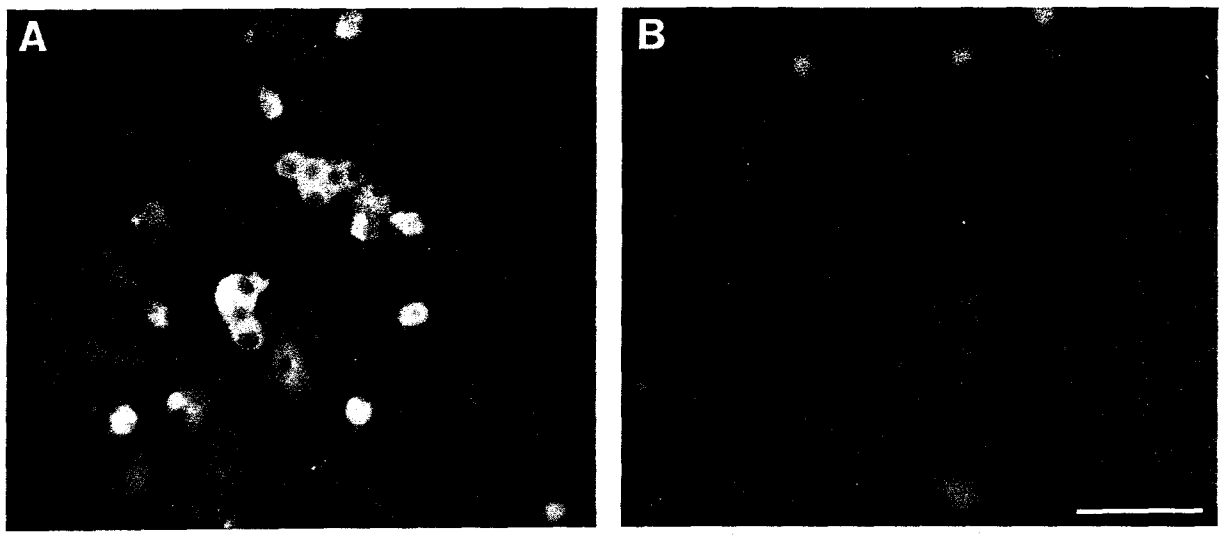

Fig. 2. The conversion of adult rat adrenal chromaffin cells to cholinergic neurons. Cells were generated as described for Fig. 1 and grown in FGF plus dex for 9 days. At that time, the medium was changed to one containing 10 ng/ml 2.5S NGF and 50% heart cell CM (Doupe, et al., 1985). On day 30 the cells were stained with a rabbit anti-choline acetyltransferase antiserum and a peroxidase-conjugated goat anti-rabbit secondary antibody (A), or nonimmune rabbit serum (B). Magnification bar is 50 μm.

dition of NGF can rescue the cells for long-term survival as neurons. It was also known that corticosteroid addition can keep chromaffin cells alive (Doupe et al., 1985) and inhibit neuronal differentiation (Unsicker et al., 1978; Doupe et al., 1985). Therefore, chromaffin cells from adult rats were plated into medium containing FGF and dexamethasone. Many single cells and small clusters of cells are seen to expand in numbers, yielding large clusters or islands of cells. The increased cell number is quantified by counting chromaffin cells, as illustrated in Fig. 1. Therefore, this paradigm is an effective way to increase the number of cells for grafting. After 9 days the medium is changed to one containing NGF and CM. During the week that follows, the chromaffin cells grow

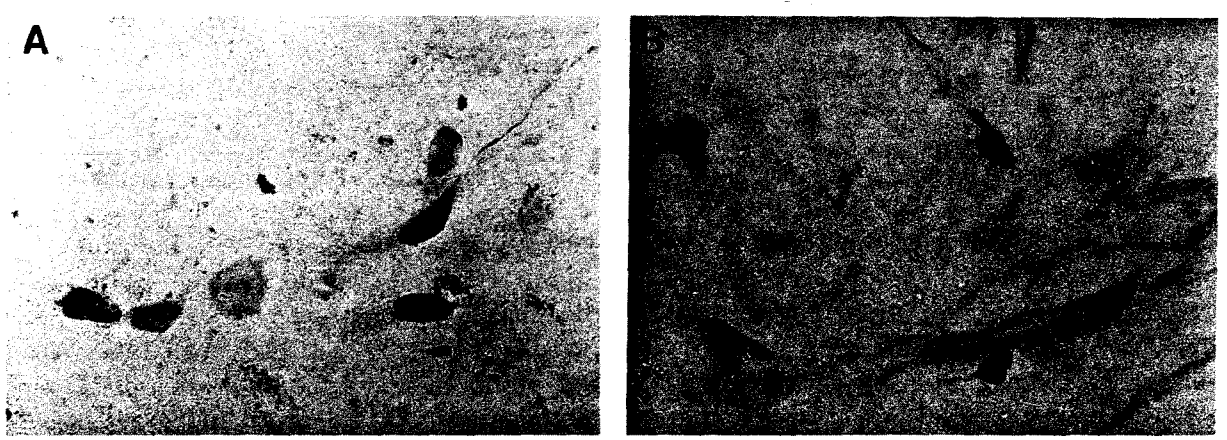

Fig. 3. Graft-derived cells in fimbria-fornix lesion cavity. Sections were taken from an animal 5 weeks following the lesion and grafting and stained for (A) acetylcholinesterase histochemistry and (B) for tyrosine hydroxylase immunoreactivity.

processes, enlarge and become morphologically indistinguishable from sympathetic neurons (Fig. 2).

Grafting of cholinergic sympathetic neurons

For the intial grafting experiments, we have prepared cholinergic sympathetic neurons from dissociated, neonatal ganglia. Such neurons are indistinguishable from adrenal-derived, sympathetic cholinergic neurons (Doupe et al., 1985). To enhance their cholinergic differentiation, the neurons are grown in mixed cultures with ganglionic non-neuronal cells and CM. Although sympathetic neurons and chromaffin cells can begin to express cholinergic properties within a few days of CM addition, the maximum conversion requires several weeks (Patterson and Chun, 1977; Johnson et al., 1980; Landis, 1980; Doupe et al., 1985). Therefore, the neurons are cultured for 2–4 weeks before harvesting and grafting. Several methods of harvesting were tested; enzymatic dissociation from the dish produced low yields of healthy neurons, while mechanically rolling the cell layer up in a ball with a microscalpel appeared to damage the neurons less. This ball of cells, with intact processes, is placed directly on the dorsal surface of the hippocampus that had been lesioned in the fimbria-fornix 2 weeks prior to grafting (Gage and Björklund, 1986). To enhance neuronal survival further, the cultured cells are mixed with 2×10^5 fibroblasts that have been genetically modified to secrete NGF (Rosenberg et al., 1988). The total volume of the grafted material is approximately 1 mm^3.

The survival and growth of the grafted neurons is assessed by several histological procedures. After 4–5 weeks, the general health and disposition of the graft is visualized by Nissl and acetylcholinesterase staining (Gage et al., 1989). Noradrenergic fibers are visualized by staining for tyrosine hydroxylase (Armstrong et al., 1987). These assays reveal that grafted sympathetic neurons do survive in the hippocampus. The extent of survival and the stimulation caused by denervation will require future quantification. Immunohistochemistry reveals positive staining for both noradrenergic fibers and acetylcholinesterase in the graft (Fig. 3). It is likely that most of these neurons are dual-function, expressing both enzymes simultaneously (Landis, 1976; Higgins et al., 1981). While the precise nature of the innervation of the hippocampus will require further study, it is encouraging that the grafted neurons grow axons in the appropriate areas of the hippocampus (Fig. 4).

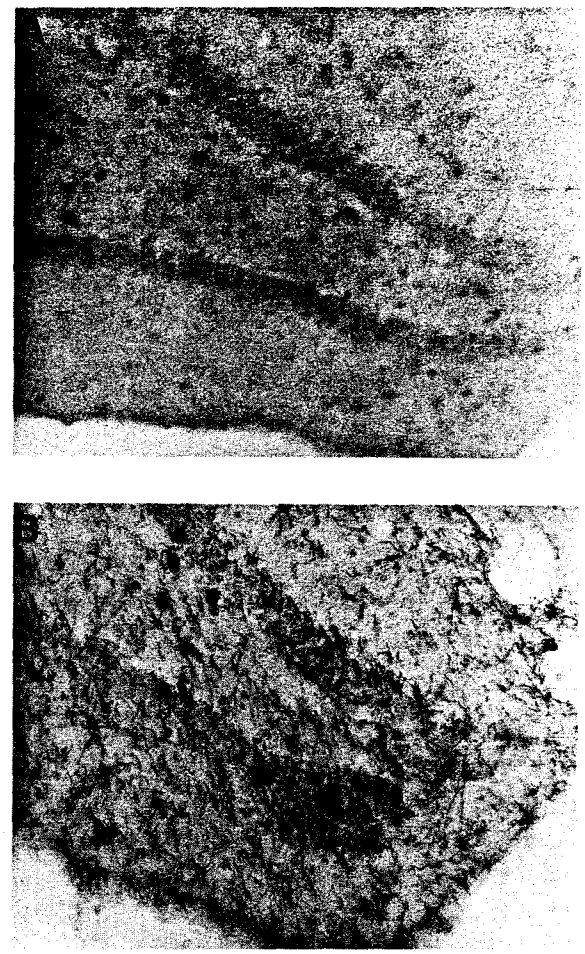

Fig. 4. Acetylcholine esterase histochemistry of dorsal hippocampus 5 weeks following fimbria-fornix lesion and grafting. Comparison is made between (A) graft of neurons without added fibroblasts and (B) graft of neurons plus transfected fibroblasts.

Conclusions

The results of this preliminary study suggest that future grafting work with populations of adrenal-derived neurons, expanded in culture and directed into desired phenotypic pathways by instructive factors, is merited. It will also be of considerable interest to determine if and how various host environments may influence the differentiation pathways taken by these multipotential neurons. That is, the grafted neurons can also be viewed as sensitive probes for instructional differentiation cues residing in the adult brain.

Acknowledgements

We thank Doreen McDowell for preparation of tissue culture materials, S. Forbes for help with the animals and the histology, Josette Carnahan for NGF and M. Rosenberg and T. Freidmann for collaborative efforts on generating the transfected fibroblasts. The project was supported by grants to PHP from a NIA PPG, a McKnight Foundation Neuroscience Research Project Award, the Joseph Drown Foundation and the Gustavus and Louise Pfeiffer Research Foundation and grants to FHG from the Pew Foundation, the McKnight Foundation, the Margaret and Herbert Hoover Foundation and the NIA. NKM was a NSF predoctoral fellowship awardee.

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