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Role of Laminin and Integrin Interactions in Growth Cone Guidance

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Abstract

Laminin is well known to promote neuronal adhesion and axonal growth, but recent experiments suggest laminin has a wider role in guiding axons, both in development and regeneration. In vitro experiments demonstrate that laminin can alter the rate and direction of axonal growth, even when growth cone contact with laminin is transient. Investigations focused on a single neuronal type, such as retinal ganglion cells (RGCs), strongly implicate laminin as an important guidance molecule in development and suggest the involvement of integrins. Integrins are receptors for laminin, and neurons express multiple types of integrins that bind laminin. Morphologically, integrins cluster in point contacts, specialized regions of the growth cone that may coordinately regulate adhesion and motility. Recent evidence suggests that the structure and regulation of point contacts may differ from that of their nonneuronal counterpart, focal contacts. In part, this may be because the interaction of the cytoplasmic domain of integrin with the cytoskeleton is different in point contacts and focal contacts. Mutational studies where the cytoplasmic domain is truncated or altered are leading to a better understanding of the role of the α and β subunit in regulating integrin clustering and binding to the cytoskeleton. In addition, whereas integrins may regulate motility through direct physical linkages to the growth cone cytoskeleton, an equally important role is their ability to elicit signaling, both through protein tyrosine phosphorylation and modulating calcium levels. Through such mechanisms integrins likely regulate the dynamic attachment and detachment of the growth cone as it moves on laminin substrates.

Index Entries: Neuronal integrins; point contacts; adhesion; laminin.

Introduction

The pioneering observations of Ramón y Cajal *(1)* identified the neuronal growth cone as the sensory-motor apparatus that directs axonal growth, both in development and in regeneration. Since that time it has become clear that a growth cone is a rather autonomous structure that possesses all of the essential proteins that enable it to move and seek

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cues in the environment. The guidance molecules may either be attractive or repulsive signals, and the molecular identity of many of the signals is still being elucidated. For example, two recently discovered families of molecules, the netrins and semaphorins, are under intense investigation *(2–6)*. In this article we do not seek to catalog and discuss the molecular identity of different types of guidance molecules. Many excellent recent reviews on these topics and other new molecules have recently appeared *(2)*. Instead, we focus here on the neuronal response to a very well characterized molecule, laminin, and how recent experiments on the laminin receptors are shedding light on the events within the growth cone that alter growth cone motility and direct axonal guidance.

Laminin is a widely expressed component of the extracellular matrix, and in the mammalian nervous system it is present both in developing peripheral and central tracts at times when axons grow. In addition, although laminin is retained in the adult peripheral nervous system (PNS), which has a good capability for repair after injury, it is not widely expressed in the mature central nervous system (CNS). Therefore, the general expression of laminin *in vivo* correlates well with the capacity of these different environments to support neurite growth. In this article we will focus on the evidence that laminin promotes axon growth and that it can act as a guidance molecule. Then we turn to the integrins, which are a major class of receptors for laminin. The integrins are present on various types of cells and have been the focus of research in many different cellular contexts. Their multiple roles in cell adhesion and cell motility have been extensively investigated, particularly with respect to cancer biology and immunology. Significant progress has been made in understanding their role in adhesion and signaling and their relationship with the underlying membrane cytoskeleton. Several excellent reviews of integrins exist and cover these topics in depth *(7–9)*. We will restrict the scope of this article to the aspects of integrin and laminin function that are most relevant to neurons, and highlight some unique aspects with respect to axonal guidance. Although in this article we focus exclusively on integrin–laminin interactions, clearly other neuronal integrins bind to other substrate molecules; some of these have been reviewed elsewhere *(3,10,11)*.

Growth of Neurons on Laminin

It has long been realized that axons respond to adhesive and guidance molecules that are present as components of the extracellular matrix. Among the many molecules of the extracellular matrix, laminin has received particular attention because virtually all types of neurons respond to it. Different genetic variants for each laminin subunit now have been identified, an exciting advance in laminin research because of the large number of potential laminins, and likely many new laminins have yet to be discovered. This explosion in our knowledge about laminin created problems with nomenclature, but a recently adopted nomenclature designates the laminin forms in the order discovered *(12)*, such that laminin isolated from the mouse Engelbreth-Holm-Swarm (EHS) tumor *(13)* is called laminin-1, merosin is laminin-2, s-laminin is laminin-3, and so forth.

Laminin-1 is the best understood variant and has been extensively investigated because of its widespread availability *(14,15)*. Designated $\alpha1\beta1\gamma1$ (Fig. 1), it has three distinct polypeptide chains (classically named A, B1, and B2) that associate by noncovalent bonds, and in the electron microscope it appears as a cross-shaped structure. It has three short arms formed by the N-terminal regions of $\alpha1$, $\beta1$, and $\gamma1$ chains, each of which consists of EGF-like repeats interrupted by several globular regions. The fourth and longest arm is where all three chains associate, with the $\alpha1$ chain being the longest polypeptide, and the α chain extends further to form five globular loops with shared homology (*see* refs. *14,16–18*, for reviews). Overall, there are many different

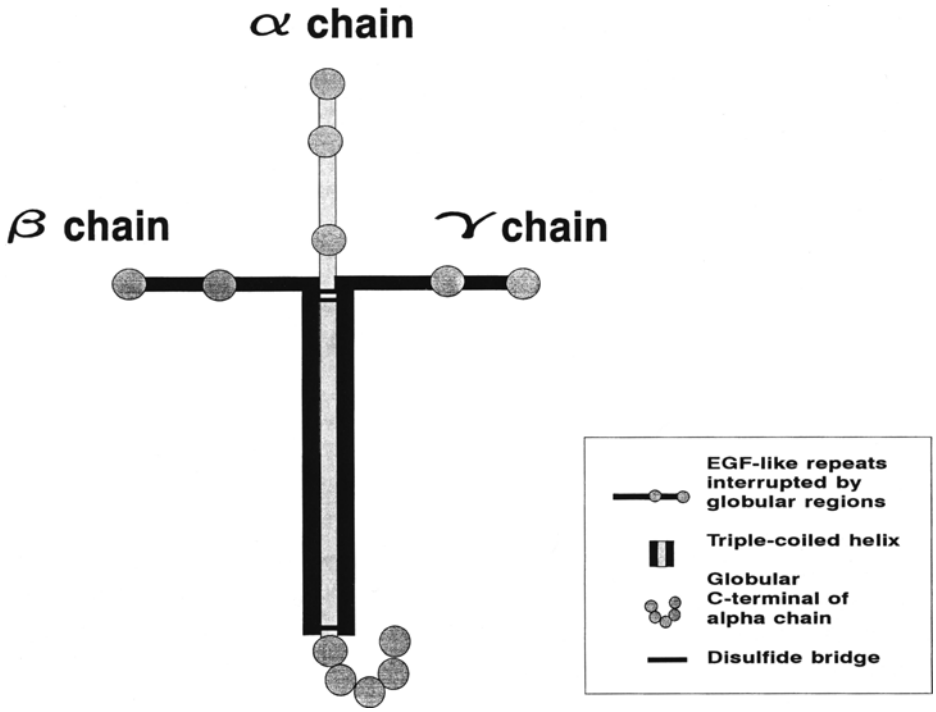

Fig. 1. Schematic representation of laminin structure showing that laminin has three chains, referred to as α, β, and γ. Different forms of laminin, including laminin-1, laminin-2, and laminin-3, have a similar overall structure, but each form has at least one genetically distinct chain. Data for this this figure was taken from several more detailed reviews of laminin structure *(16,18,151)*.

active regions of laminin-1, and separate domains of the laminin-1 molecule have distinct biological activities. A strong neuronal outgrowth-promoting domain is located at the distal long arm of laminin and is recognized by the α6β1 integrin *(19–21)*. The α3β1 integrin also binds near this region *(22)*. Another important domain for neurons is the site recognized by α1β1 integrin, which is near the center of the cruciform laminin molecule *(23)*. A less characterized region resides on the γ1 chain and has been shown to be important for the migration of cerebellar granule cells *(24)*. Other regions important for cell neurite growth, such as the YIGSR sequence *(25,26)*, have been identified, but it is not known how widely they are utilized by neurons. Given the complexity of laminin receptors of the integrin type, and additional receptors of the nonintegrin type that are being identified, it will not be surprising if additional regions of laminin-1 are found to promote axon growth and to guide growth cones.

Laminin-1 not only promotes the adhesion of neurons, but it stimulates outgrowth of neurites from neurons plated in culture *(27–29)*. In vivo, laminin-1 is a component of basement membranes that are known to support axon growth. For example, proteolytic treatment of the basement membrane can cause certain growth cones to retract in grasshopper embryos *(30)*. The ability of laminin-1 to promote neurite outgrowth, when compared to that of other extracellular matrix components, can be quite impressive. Such is the case when retinal explants are grown on laminin-1, fibronectin, or collagen, where striking differences in the number and length of extending neurites are observed *(31)*. More generally, laminin-1 has been found to be the best sub-

strate for most types of neurons when compared with various collagens, proteoglycans, and fibronectin in similar neurite outgrowth assays *(32–36)*. However, when dorsal root ganglion (DRG) neurons, which are a heterogeneous population of neurons, are plated on patterned substrates where they must make a choice at borders between laminin-1 and fibronectin, some axons do cross from laminin-1 to fibronectin *(37)*. Such is also the case when retinal neurons meet borders between laminin-1 and L1 or laminin-1 and N-cadherin *(38)*. Therefore, although laminin-1 is generally an excellent substrate, it is not always the substrate of choice for a particular neuron or under certain growth conditions.

Laminin-2 ($\alpha2\beta1\gamma1$) is a component of the basal lamina of the peripheral nerve. It is made by Schwann cells (*see* ref. *39*) and also, in the developing CNS, likely by astrocytes *(40)*. The presence of laminin-2 in the basal lamina surrounding Schwann cells is likely important for the successful regeneration of injured peripheral nerve *(41)*. Laminin-2 is also present in the retina and optic nerve at the appropriate developmental time to be a guidance molecule *(40)*. The growth of retinal ganglion cells (RGCs) is promoted by laminin-2 in addition to laminin-1 *(43)*, and therefore, it seems likely that laminin-2 may be the relevant biological form of laminin for RGCs.

Laminin-3 ($\alpha1\beta2\gamma1$), unlike laminin-1 and laminin-2, does not stimulate neurite outgrowth although it is strongly adhesive *(44)*. It was originally identified by antibodies that stained the synaptic basal lamina at neuromuscular junctions, and sequence analysis revealed that it is a homolog of the $\beta1$ chain of laminin-1 *(45)*. The $\beta2$ chain of laminin-3 contains several sequences of a tripeptide, LRE, that is important for adhesion by laminin-3. Soluble LRE inhibits adhesion *(45)* and, LRE immobilized as substrate prevents neurite outgrowth, likely because it is so highly adhesive *(44)*. The adhesive properties and localization of laminin-3 at the neuromuscular junction strongly suggest that it plays an important role in the formation of synapses. Recent analysis of null mutant mice support this contention because there are structural and functional defects in their motor nerve terminals *(46)*.

Other forms of laminin have also been identified *(17,18)*, but their role in the development and maintenance of the nervous system is less clear than the first three forms. Laminin-5 is known to be enriched in the developing CNS *(47)* and elucidating the differences between this and the other new laminin forms for the growth and guidance of specific neuronal populations will be an exciting avenue for future research.

Although not laminins, the netrins are the last family of molecules that we mention because they share homology with laminin. Netrins are newly identified axon guidance molecules, found to be neurite growth promoting factors for commissural axons *(48)*. They have extensive homology to the amino terminal arm of the laminin $\gamma1$ chain, but they have completely divergent C-terminal sequences. These diffusible chemoattractant molecules are not considered as laminin isoforms but as relatives of laminin. A future challenge will be to determine the range and variability of such laminin-related molecules and to elucidate their receptors. Such information will allow us to determine the relationship between laminin family members and to discover the mechanisms whereby they stimulate neurite outgrowth.

Laminin as a Guidance Molecule

Because laminin promotes neurite outgrowth and is widely expressed in the developing nervous system, it is clearly an important permissive molecule for growing axons. However, does it also play a role in guiding axon growth to their appropriate target region? Ideas on this topic generated by in vitro experiments are coming full circle, and now both the in vitro and in vivo evidence suggest that laminin likely serves both permissive and instructive roles.

The early use of patterned laminin substrates first suggested that the adhesive properties of laminin could actually serve to steer

growing axons *(49,50)*. Thus, adhesive strength was believed to be an important criteria for pathway choice by axons. This idea lost favor when it became clear that guidance and substrate preference were not simply related to adhesiveness *(51,52)*. Moreover, the use of laminin gradients did not support substrate preference by adhesiveness *(53)*. However, recent studies that use patterned substrates to study the choices that axons make are providing new support for a guidance role for laminin. Patterned substrates force neurites to follow a specific growth pathway on active laminin *(50)*. However, when chick DRG neurons are plated on these substrates and treated with pertussis toxin, the neurites wander off the active laminin path, and surprisingly, growth cone movement and filopodia activity remain normal on inactivated laminin *(54)*. Therefore, pertussis toxin appears to prevent guidance by the laminin boundaries, but it does not affect the ability of laminin to promote growth, a finding that supports a guidance role for laminin.

Another recent series of experiments supports the contention that laminin is a guidance molecule by demonstrating that a single filopodial interaction with laminin can affect growth cone navigation *(55)*. Individual laminin-coated beads were positioned near elongating growth cones with laser tweezers, and interactions of filopodia with the beads were followed. Brief interactions changed the navigation of growth cones and elicited a long-lasting increase in the rate of growth cone advance. This finding is in keeping with the ability of soluble laminin to stimulate the growth rate *(56)*. Interestingly, no change in the elongation rate was observed when a laminin guidepost touched a neurite shaft. Therefore, the receptor molecules involved in guidance signaling are either located specifically on the growth cone or are only active on the growth cone *(55)*. High resolution studies of integrin localization on growth cones suggest the latter possibility is most likely *(57)*.

As illustrated by the laminin guidepost experiments, the rate of growth may be an important consideration for guidance by laminin. This might be especially true where laminin is present as a uniform substrate. A rapidly extending axon might be more likely to ignore irrelevant, less abundant guidance molecules when growing rapidly on a laminin substrate. In this case, the response to laminin might supersede other signals that are temporally or spatially inappropriate. Support for this idea comes from recent experiments that demonstrate laminin can override completely the action of the myelin-derived growth inhibitory molecules *(58)*. It is well known that certain proteins are present in myelin that block the extension of growing axons *(59,60)*. The reported experiments clearly show that laminin may abrogate the response of growth cones to molecules that do not favor growth, including such potent inhibitors as those present in myelin.

The hierarchy of growth substrate preference is likely of critical importance for axonal guidance in development where many different types of molecules are likely to play an important role. Many neurons grow well on cell surfaces that are rich in various types of cell adhesion molecules, and they may even prefer these to extracellular matrix proteins. For example, RGC prefer laminin as an extracellular matrix substrate *(31)*, but they have a small tendency to prefer L1 or N-cadherin when presented with a choice *(38)*. Further, they prefer Schwann cell surfaces compared to cell–free extracellular matrix *(61)*. Although the interactions of neurons with cell adhesion molecules are outside the scope of this article, they are important to consider when individual guidance pathways are investigated, and laminin could play a synergistic role in pathway choice in vivo where multiple guidance molecules are present.

The in vitro experiments discussed above provide strong evidence that laminin has the potential to act as a guidance molecule. The best evidence that laminin-related proteins are guidance molecules in vivo comes from the identification of genes required for the guidance of pioneer axons in *Caenorhabditis*

elegans. One of these genes, unc6, encodes for a laminin-related protein that is required for the migrations of pioneer growth cones *(62,63)*. This molecule is likely most related to the netrins (discussed above), but has been considered as strong evidence that laminin-like molecules are indeed guidance molecules. In the mammalian nervous system the temporal expression and location of laminin provides good circumstantial evidence that laminin is important in both axonal growth and guidance. When specific examples are studied in detail, these generalizations hold up.

In the visual system laminin is prominently present in the basal lamina (the inner limiting membrane) between the retinal ganglion fiber layer and the vitreous, and is present in the developing optic nerve *(40,64,65)*. In the retina, the RGC axons grow along the region of the basal lamina toward the central retina where the optic nerve forms. Cell surfaces, especially the end feet of the radial glia, may play the most important role in the actual promotion of RGC axon growth *(61,65)*, however, the localization of laminin and chondroitin sulfate proteoglycans (CSPGs), when considered together, suggest a guidance role, specifically to direct the axons toward the optic disk. This guidance role is suggested for the following reasons. CSPGs inhibit the growth of RGC axons, and they are present in the inner retina but are most abundant in the peripheral retina *(66,67)*. This distribution contrasts with laminin-1, which is more uniformly present along the entire inner limiting membrane *(40,68)*, and laminin-2, which has a reciprocal pattern to the CSPGs; laminin-2 is most immunoreactive in the central retina *(40)*. Therefore, if laminin can override the growth inhibition by the proteoglycans, as it can for other inhibitory proteins *(58)*, dual interactions with both laminin and CSPG might guide the axons toward the optic disk as they follow the pathway with an increasing ratio of favorable (laminin) to unfavorable (CSPG) substrate molecules. Once the axons enter the optic nerve they may be further guided by laminin, which is present in the appropriate temporal pattern to guide the first axons *(64)*, and the RGCs are known to be very responsive to laminin at these times *(69,70)*. Finally, laminin is expressed throughout life in goldfish optic nerve, where the RGCs show robust regeneration after injury *(71)*. Thus, the distribution and temporal expression of laminin in the retinotectal pathway is consistent with a guidance role for RGC axons.

Of course, laminin has been found to be quite widely expressed in development, such as in dorsal and ventral roots *(72)*, and in the pathways of trigeminal nerve *(73)* ciliary ganglion fibers *(74)*, but it is not clear that it has a guidance role in all instances. Other observations suggest that areas rich in laminin as guideposts exist because laminin aggregates occur in several regions of the mammalian CNS *(75)* and in injured brain regions *(75,76)*. A common finding in all of these studies is that high levels of laminin are present in sites where axons are growing. In the mammalian CNS, a clear drop in the laminin levels occurs at later stages of development after the period of growth *(64,69)*. However, laminin may remain in some regions of the adult brain. Most interesting is the presence of laminin in neurons of the adult mammalian CNS *(68,77,78)*. Even more surprising is the high mRNA levels for $\beta 1$ and $\gamma 1$ with little $\alpha 1$ detected *(68,78)*. These observations suggest the possibility that the cDNA probes may have hybridized to laminin-related molecules, such as the netrins, that might be produced as soluble factors by neurons. In either case, the function of the neuronal laminin is not known.

Neuronal Distribution of Integrins

A major task in elucidating the mechanisms by which molecules stimulate growth and guide axons is to understand how receptors in the plasma membrane influence the coordination and activity of intracellular growth cone components. As in nonneuronal cells, the integrin family of extracellular matrix receptors are the best understood receptors for lami-

nin. Other laminin-binding proteins have been described within the nervous system, but have not been as thoroughly investigated, and they will not be discussed here (reviewed in refs. *79,80*).

Integrin receptors recognize and bind various extracellular matrix molecules, such as laminin, collagen, and fibronectin. The specificity of ligand binding is determined by the large extracellular domain of αβ heterodimers that form the intact receptor *(7,81)*. There are many possible αβ combinations, but neuronal cells (at least those studied to date) use mainly β1 in combination with various α subunits *(20,41,43,74,82,83)*. Some heterodimers, such as α6β1 and α7β1, appear to bind exclusively laminin *(82,84)*, whereas others, such as α3β1, bind multiple ligands *(85,86)*. Therefore, different α subunits with the same β1 subunit determine the specificity of the receptor for the extracellular ligand.

The overall distribution of the different integrins in the nervous system has not been completely characterized, but some specific types of neurons and neuronal cell lines have been examined in detail. The picture that is beginning to emerge is that most neurons express several different types of integrins, and that in many cases the expression is developmentally regulated.

As with laminin in retinal development *(see above)*, the retinal integrins have been quite well studied. In the retina, α1β1, α3β1, and α6β1 integrins *(82,87)* are present. Antibody-blocking experiments indicate that α6β1 is one of the major functional receptors when RGCs grow on laminin-1, but that at least one of the other β1 integrin subtypes is important for the growth as well *(70)*. Therefore, RGCs have multiple functional laminin receptors, and, as a further complication, two different splice forms of α6 integrin are present in the retina. These two variants differ in their cytoplasmic domains and the differences are conserved between rodents and humans *(88)*. The α6B variant is uniformly expressed on all RGCs, whereas the alternative α6A form is restricted to RGCs at the disk region *(70)*. It is possible that the RGCs near the disk that express α6A are the pioneer RGCs that originate from the central retina. It will be of great interest to determine the functional significance of the alternative splicing and these interesting differences in expression.

Another aspect of β1α6 expression in RGCs is the developmental regulation of expression. Most retinal cells, including RGCs, lose their responsiveness to laminin-1 with increasing developmental age *(34,89)*. The decline in response is later with laminin-2 *(43)*, a result that raises the possibility that neurons may show a developmental switch in their laminin subtype preference. Bilateral ablation of the tectum prior to innervation prevents the decrease in responsiveness of RGCs to laminin-1 *(90)*, perhaps as a result of signaling molecules produced in the tectum and transported retrogradely by RGCs. However, removal of the tectum does not result in a change in the mRNA level for α6 integrin and significant α6 immunoreactivity can be detected on RGCs that have become less responsive to laminin *(70)*. These findings raise the possibility that α6β1 integrins on RGCs might not always be active and that the functional state of integrins on RGCs is regulated. Signaling molecules that might regulate integrin expression and function in RGCs are likely to be found in peripheral nerves because the replacement of the optic nerve with a peripheral nerve graft can induce again α6β1 expression in mature RGCs that regenerate *(91)*. These results are intriguing and suggest that likely candidates for such signals are the neurotrophins known to be produced by injured peripheral nerve.

The integrins of sensory neurons in DRG are also well characterized and these neurons express multiple integrins. Here, α1β1 *(41,87)*, and α6β1, and α3β1 *(92)* are among those known to be expressed. The well-characterized fibronectin receptor α5β1 is prominently expressed in the developing peripheral nerve, decreasing its level in the mature nerve *(93)*. The recent finding of α3β1 in sensory neurons as well as RGCs was surprising because in PC12 cells α3β1 is clearly a relatively weak

receptor compared to α1β1 *(20)* and it does not associate strongly with the cytoskeleton *(57)*. However, a careful analysis of receptor function through antibody blocking experiments indicates that α3β1 is one of several functional receptors when cells are plated on laminin-2 *(92)*. As discussed in the previous section, laminin-2 is present in both peripheral nerve and the developing visual system, and therefore multiple laminin receptors may be needed for accurate guidance on laminin-2.

One final example where integrin expression and function for a defined neuronal population has been carefully characterized deserves to be mentioned. A recent study of developing chick ciliary ganglion (CG) has shown that three different laminin receptors, α1β1, α3β1, and α6β1, are expressed by CG neurons *(80)*. Antibodies against the β1, α3, and α6 subunits interfere with growth on laminin-1. On laminin-2, however, only anti-β1 antibody interferes with growth, but it is not known if the α1β1 integrin or another β1-type integrin mediates growth on laminin-2. When compared to results with RGCs, these experiments show that a given receptor, α3β1, may in different cell types have different substrate requirements.

Point Contacts and Axonal Pathfinding

Although the evidence that links integrin function to the forward progression of growth cones comes from functional studies and from the patterns of integrin expression in development, the cellular mechanisms whereby integrins stimulate growth cone motility have yet to be worked out. One key to understanding the mechanisms will be to elucidate the proteins that interact with the cytoplasmic portion of integrins. In nonneuronal cells, protein colocalization studies have been invaluable in determining the cytoskeletal proteins that underlie the clusters of integrins to form a complex architectural arrangement. On growth cones, however, the intracellular structural relationship between the multiple integrins and the cytoskeleton is not clear, although the overall distribution of neuronal adhesion sites has been established. Immunocytochemical studies of DRG neurons demonstrated that, in general, integrins are rather uniformly distributed on the growth cone surface of DRG neurons *(94)* and close observations reveal a punctate pattern on all regions of the growth cone, including the filopodia extensions *(57,95)*. Observations of growth cones by interference reflection microscopy, which reveals sites of close apposition between the cell and the substrate, show that growth cones adhere to laminin at punctuate sites *(96)* that likely correspond to the clusters of integrins observed by immunocytochemistry. These sites are called point contacts because they differ in size and shape from the focal contacts *(83)* and they are likely analogous to structures, sometimes called podosomes, that have been observed in other cell types *(83,97–100)*.

Point contacts are the main adhesion structures on neuronal cells and growth cones (Fig. 2) and may be specialized for highly motile cells. They are particularly abundant in fibroblasts transformed by Rous Sarcoma virus (RSV), where they have been best studied. Integrins in fibroblasts are typically clustered as elongate foci called focal contacts, which are the termination sites for stress fibers (for review *see* refs. *99,101*). Focal contacts are regions of strong cell–substratum adhesion, and on the cytoplasmic side of focal contacts several different actin-binding proteins form a complex network that anchors the ends of the stress fibers to the membrane. Talin and α-actinin are cytoskeletal proteins that bind directly to β1 integrin, and other major structural proteins that form the adhesion complex are vinculin, paxillin, and tensin (*see* ref. *101*). Therefore, focal contacts are specialized regions of the cell through which traction is exerted from the extracellular matrix to the internal cytoskeleton. However, after RSV transformation, point contacts appear in addition to the focal contacts *(97–99)*. In these cells some cytoplasmic proteins that colocalize with focal contacts associate with point contacts and one speculation is

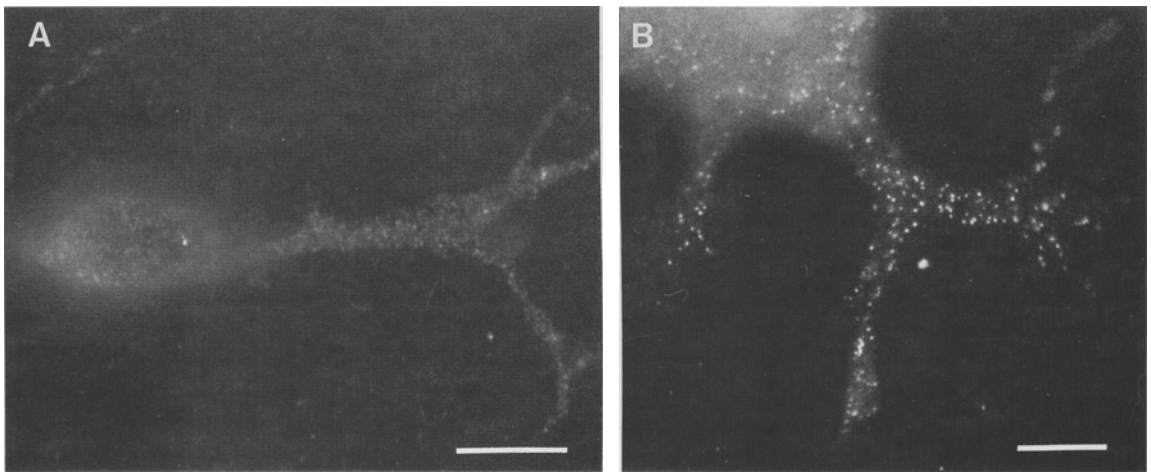

Fig. 2. Point contacts in neuroblastoma cells. **(A)** Localization of β1 integrin on a human neuroblastoma cell line, SY5Y. Integrins are distributed in a punctate pattern on both the cell body and neurites. Cells were grown on laminin-coated coverslips in DMEM medium with 10% fetal bovine serum and neurite formation was induced with 5 ng/mL bFGF and $1 \times 10^5 M$ retinoic acid. Immunocytochemical localization of integrins was with antihuman β1 antibody (UBI, Lake Placid, NY). **(B)** Distribution of integrins on NG108-15 cells revealed by immunoreaction with monoclonal anti-α1 antibody (monoclonal 3A3; ref. *57*). Point contacts are present on the cell body and neurites. NG108-15 cells were grown and differentiated as described *(60)*. Bars, 10 μm.

that they are initial immature contacts *(97)*. However, there are some additional unique aspects to point contacts in RSV-transformed cells. For example, some appear to lack association with vinculin *(100)*, and gelsolin has been observed as one component that may be involved in actin dynamics and motility *(98)*. Moreover, the use of epitope–specific antibodies revealed that tyrosine-phosphorylated integrins cluster at point contacts, not focal contacts *(102)*. This finding provides strong evidence that point contacts differ in signaling function from focal contacts.

In the few neuronal cell types examined to date (e.g., Figs. 2 and 3), point contacts are the major if not the only integrin-containing structure observed when cells are plated on laminin *(57,95)*. One might expect that neuronal point contacts will be found to be structurally similar to the point contacts observed in RSV-transformed cells, especially because the transforming protein of RSV, p60*src*, is a protein enriched in neuronal growth cones *(103)*. However, the structure of the neuronal point contact is just beginning to be worked out through protein colocalization studies *(57,104)*. A common focal contact component, talin, does not localize specifically to neuronal point contacts *(57)*. Vinculin, another major focal contact protein that is absent from the point contacts in astrocytes *(83)*, shows only a partial codistribution with point contacts in neurons *(57)*. The protein gelsolin, reported to be present in point contacts of RSV-transformed cells *(98)*, does not appear to localize in point contacts in neurons because the immunocytochemical localization more closely resembles that of actin *(105)*. In astrocytes, which have both focal contacts and point contacts, clathrin clusters at point contacts, where it might be needed to recycle receptors *(83)*. Finally, focal adhesion kinase (FAK), a protein tyrosine kinase concentrated at focal contact sites *(106)*, has been compared in astrocytes and PC12 cells by immunocytochemistry. Point contacts in both PC12 cells and astrocytes lack immunoreactivityto anti-FAK antibodies, whereas the focal contacts in astrocytes are strongly labeled *(57)*. These observations are puzzling because FAK is known to be expressed in the nervous sys-

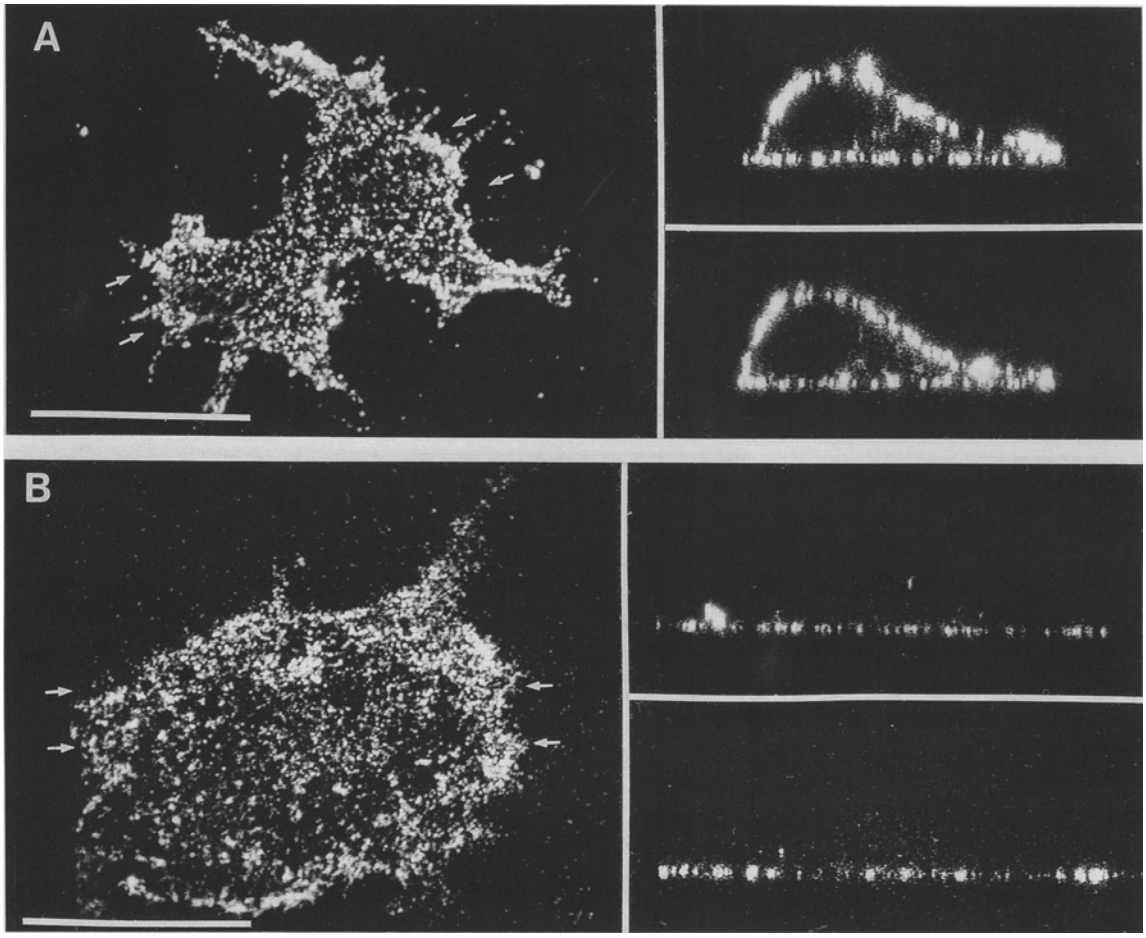

Fig. 3. Confocal images showing the localization of α1 in whole cells and cytoskeletal ghosts. **(A)** PC12 cells grown on laminin-1. **(B)** PC12 cells grown on laminin-1 then extracted with Triton-X in cytoskeletal stabilizing buffer *(57)*. **(Left)** Optical sections taken parallel to the substrate. **(Right)** Optical sections of the same cells taken perpendicular to the substratum (arrowheads indicate the positions). Note the punctate distribution of α1 immunoreactivity covers the entire surface of intact PC12 cells, but that after detergent extraction only the contacts at the lower cell surface remain. Bars, 20 μm. Reproduced from ref. *57*, with permission.

tem *(107)* and can be detected in PC12 cells by Western blots (Arregui and McKerracher, unpublished). Perhaps anti-FAK antibodies lack accessibility to FAK in point contacts. Further investigations of the structure of neuronal point contacts are needed to determine the composition of the clusters and to determine their role in motility.

Since function-blocking anti-integrin monoclonal antibodies disrupt attachment of PC12 cells *(108)* and integrins in PC12 cells are only clustered in point contacts, the point contacts are most likely to function in both cell attachment and growth cone motility. In PC12 cells two main integrins, α1β1 and α3β1, mediate PC12 cell attachment and neurite outgrowth on laminin *(20,108)*. Both integrins distribute in a dotted pattern all over the cell surface of PC12 cells, but only point contacts at the interface between the cell and the substrate are resistant to solubilization by gentle detergent treatment (Fig. 3). Therefore, in these cells only the ligand-bound point contacts are tightly linked to the cytoskeleton *(57)*. More-

over, in PC12 cells, only the α1 and β1, not the α3 subunits, are resistant to detergent extraction when cells are grown on laminin-1. It would be of interest to determine if the use of laminin-2 as substrate would lead to stronger linkages of α3β1 integrin with the cytoskeleton, because laminin-2 is a better substrate for α3β1-mediated growth *(92)*.

In nonneuronal cells, adhesion sites that differ from focal contacts are beginning to be characterized. In PDGF-activated fibroblasts small vinculin-containing structures are located at the cell margins *(109)*. These structures are regulated separately from focal contacts because injection of rac, a small regulatory GTP-binding protein, stimulates these point contact-like structures separately from the ρ-induced focal adhesions. These results raise the possibility that adhesion sites involved in cell attachment and those involved in motility might be differentially regulated. In PC12 cells the vinculin-containing point contacts are most numerous at the cell margins, and, together with the finding that they are resistant to detergent extraction, these findings suggest that the vinculin foci represent the strongest adhesion sites *(57,110)*. The role of vinculin in neural cells has recently been investigated in PC12 cells by an antisense approach *(111)*. In vinculin-deficient PC12 cells, filopodia and lamelopodia form normally but are less stable than those in control cells and neurite outgrowth was reduced. Taken together, these results suggest that vinculin plays an important role in stabilizing adhesive contacts as the growth cone moves forward.

Might some point contacts be both adhesive sites and transducers of force generation? Perhaps point contacts are uniquely expressed in highly motile cells because they are specialized for both functions. In rapidly extending growth cones the adhesion sites must be strong enough to transmit force, but must be able to rapidly detach as the growth cone advances. Focal contacts might be too strongly adhesive to efficiently accomplish this role, as suggested by the observation that they are ripped away from the cell membrane as a fibroblast moves forward *(112)*. It is anticipated that the neuronal integrins, which clearly form physical links between the extracellular matrix and the cytoskeleton *(57)*, will be found to be important signaling molecules as well, as they are in other types of cells *(9)*. Through such dual roles they are likely well suited to regulate both adhesion and motility in a coordinated way.

The Cytoplasmic Domain of Integrins

The structural part of the integrin molecule that transmits information to the inside of the cell is the cytoplasmic domain, and recent investigations at the molecular level are shedding light on the contribution of the various cytoplasmic regions to integrin localization and function. Generally, the cytoplasmic portion of a given integrin subunit is conserved between species, but cytoplasmic sequences diverge more among different integrins *(9,113)*. For example, both β1 and β8 are subunits of laminin receptors, but the β8 cytoplasmic domain shares little homology with the β1 cytoplasmic domain *(114,115)*, and each domain may have a specific function. The differences between the two are likely biologically important, and might best be elucidated through some of the molecular approaches discussed below.

The approach of truncating the cytoplasmic domain of an integrin subunit, or of forming chimeric molecules, has been very useful to analyze the roles of different integrin cytoplasmic domains (*see* Table 1). Many such studies have been carried out with mutant constructs introduced into nonneuronal cells by transection. They are reviewed here to illustrate the usefulness of the approach and to illustrate how the cytoplasmic domain contributes to receptor function and cell motility.

The β1 integrin was the first subunit to be studied by mutational analysis, and it remains the most intensively investigated. Total or partial truncation of the β1 cytoplasmic domain demonstrated that the cytoplasmic tail is required for localization to focal contacts (Table

Table 1
Mutational Analysis of Integrin Cytoplasmic Domains

Integrin subunit[a]	Cytoplasmic modification	Cell type	Dimerization αβ	Cell surface expression	Localization in FA	Binding to ECM[b]	Cell migration	References
β1	Total truncation	NIH3T3	Yes	Yes	No	Yes	ND	*116*
β1	Partial truncation	NIH3T3	Yes	Yes	No	Reduced[c]	ND	*118,152*
β1	Chimeric with Ex and Tm of IL2R	Human fibroblast	No	Yes	Yes	—	Decreased	*124,125*
β1	Chimeric with Ex of N-cadherin	NIH3T3 CHO	No	Yes	Yes	—	ND	*126*
α5	Chimeric with Ex and Tm of IL2R	Human fibroblast	No	Yes	No diffuse	—	ND	*124*
α5	Chimeric with Ex and Tm of α2	RD	Yes	Yes	ND	Yes[c]	Normal	*152*
α4	Chimeric with Ex and Tm of α2	RD	Yes	Yes	ND	Yes[c]	Increased	*152*
α1	Truncation after GFFKR	NIH3T3	Yes	Yes	Yes	Yes[c]	ND	*127*
α5	Partial truncation	CHO	Yes	Yes	ND	Yes[c]	Normal	*128*
α5	Total truncation	CHO	Yes	Reduced	ND	Slower[c]	Decreased	*128*
α6	Truncation after GFFKR	Mouse macrophage	Yes	Yes	ND	No[c]	ND	*129*
α4	Truncation after GFFKR	K562 MIP101 PMWK	Yes	Yes	ND	Decreased[c]	ND	*130*
α4	Truncation 4 AA after GFFKR	MIP101 CHO	Yes	Yes	ND	Yes[c]	ND	*131*

[a]Only β1 of the β subunits is listed.
[b]Abbreviations, FA, focal adhesion; ECM, extracellular matrix; ND, not done; Ex, extracellular domain; Tm, transmembrane domain; IL2R, interleukin-2 receptor, RD, rhabdomyosarcoma; K562, erythroleukemia; MIP101, colon carcinoma; PMWK, primary melanoma.
[c]Tested by cell adhesion assays.

1). This suggests that the cytoplasmic domain of the β1 subunit interacts directly with the cytoskeleton *(116–118)*. This interpretation is consistent with biochemical evidence that α-actinin binds to β1, an interaction that has been shown by affinity chromatography with a synthetic peptide corresponding to the cytoplasmic domain of β1 *(119,120)*. Also, talin is thought to bind directly to the β1 subunit because talin retards β1 mobility in gel filtration columns *(121)*. The region of interaction of β1 with talin has been mapped to a region that is phosphorylated on tyrosine in RSV-transformed fibroblasts *(120)*. When phosphorylated and nonphosphorylated integrins were compared for their ability to interact with talin, the tyrosine phosphorylated integrins showed a decreased ability to interact with talin *(122)*. In addition, because β1 integrin in point contacts is phosphorylated *(102)*, it is perhaps not surprising that in neurons talin does not colocalize to point contacts *(57)*. Moreover, mutations introduced in the region of the cytoplasmic domain that interacts with talin abolish the localization of β1 to focal contacts and reduce cell adhesion *(123)*. Together these results suggest that the point contacts may be less stable adhesion structures involved in motility. This region of the β1 cytoplasmic domain that interacts with talin might regulate whether focal contacts or point contacts are formed.

Experiments with chimeric molecules, where the β1 cytoplasmic domain is fused to the extracellular portion of another receptor, also demonstrate that the cytoplasmic domain of β1 integrin is necessary and sufficient for the localization of the integrin heterodimer to focal contacts *(124–126)*. Chimeric constructs, consisting of the extracellular and transmembrane domain of the interleukin 2 receptor fused to the β1 cytoplasmic domain, act as dominant negative mutants and are able to inhibit cell spreading. The cytoskeletal proteins likely are no longer available for binding to the endogenous integrins because of competition when the mutant form is expressed at high levels *(125)*. These chimeras also show decreased cell migration, but migration is not blocked, suggesting that the integrin α subunit may also interact with the cytoskeleton in association with motility.

In contrast to β1, the α integrin cytoplasmic domain is not sufficient to target integrins to focal contacts. Chimeras of the α5 integrin cytoplasmic domain fused to the extracellular and transmembrane portion of the interleukin 2 receptor are not able to cluster, and they are expressed diffusely on the cell surface *(124)*. When similar chimeras were made with the β1 cytoplasmic domain, they clustered together, even though they could not bind ligand, a surprising finding because β1 clustering is normally ligand-dependent. Together these results suggest that the α5 cytoplasmic domain might negatively regulate interactions of β1 with the cytoskeleton, depending on ligand binding *(124)*. The idea that the α subunit inhibits β1 clustering in the absence of ligand is supported by experiments with the cytoplasmic domain of the α1 subunit *(127)*. With deletion of the α cytoplasmic domain, the integrins will cluster when the cells are plated on nonligand substrates. Therefore, the α integrin cytoplasmic domain may act as an inhibitor to block interaction of β1 with the cytoskeleton in the absence of ligand.

The α subunits all share one conserved region, the GFFKR sequence, which is adjacent to the membrane. This sequence appears to be required for cells to adhere to their ligand substrate (Table 1). Bauer et al. *(128)* analyzed cells transfected with three different α5 constructs, one with a full-length cytoplasmic domain, the second with a truncation after the intact GFFKR region, and the third a complete truncation of the entire cytoplasmic domain. They found that only the total truncation of the cytoplasmic domain resulted in dramatic changes in cell adhesion and cell motility. Similar experiments have shown that the region just after this GFFKR region is necessary for the adhesive activity of the integrin receptor *(129,130)*. Addition of only four amino acids after the GFFKR sequence restores the adhesion properties of the integrin lost by deletion of the

cytoplasmic domain after the GFFKR region *(131)*. A known protein, calreticulin, binds to the GFFKR sequence, as shown by affinity chromatography *(132)*. Expression of an antisense calreticulin oligonucleotide can inhibit attachment of cells plated on extracellular matrix *(133)*, supporting the contention that this sequence is important for cell motility and adhesion. Studies of α1 deletions are also consistent with these findings because deletion after the GFFKR sequence did not prevent α1 from clustering with the endogenous α5β1 receptors and no affect on cell adhesion was observed. Therefore, the unique, nonconserved region of the α cytoplasmic domains does not appear to be required for integrin-mediated adhesion. This is of interest because many neurons express α1β1 integrins and, at least in PC12 cells, the α1β1 is known to be strongly linked to the detergent insoluble cytoskeleton (Fig. 3). One might speculate that the cytoplasmic domain of this subunit might have a more subtle role, such as a role in axonal guidance. Mutational approaches applied to neurons should provide answers to this and to related questions on integrin functions in guidance.

More recently, attempts are being made to identify the regions of the cytoplasmic domains that are required for binding to specific cytoskeletal proteins. For example, Lewis and Schwartz *(134)* have used β1 cytoplasmic domain mutants for mapping the interaction of the β1 integrin with different components of focal adhesions. They find that ability to colocalize FAK and talin rides in the same region near the C-terminal, and that α-actinin binds at a different site. Synthetic peptides of β1 fragments coupled to beads also have been used to MAP binding sites on β1 *(135)*. These studies indicate that FAK and paxillin bind to β1 near the transmembrane domain, with α-actinin further along. These recent studies substantiate direct linkage between these cytoskeletal proteins and integrins, and similar techniques should be useful to investigate β1 clusters of point contacts in neurons.

From such studies it seems certain that the cytoplasmic domain of neuronal integrins will be found to play a critical role in transmitting guidance cues from the extracellular environment to the neuronal growth cone. Both the β and α cytoplasmic domains likely will be found to be required in axonal guidance because of their unique roles in integrin clustering and motility.

Signaling Through Integrins

Integrins mediate cell adhesion to the extracellular matrix through direct physical linkage to the growth cone cytoskeleton, but also through an equally important role in receptor–mediated signaling. Intracellular signaling mediated by integrins effects a wide range of cellular events, from gene expression to cell shape and cell motility *(9)*. There are several general mechanisms whereby integrins mediate these effects through well-established signaling pathways.

Most recently, tyrosine phosphorylation has been found to be an important part of the integrin-mediated responses and such signaling results in the activation of many different cellular proteins (*see* refs. *9,136*, for reviews). The integrin-mediated phosphorylation cascade was first elucidated by the identification of substrates for the oncogene *src* in RSV-transformed cells. Monoclonal antibodies were raised to phosphotyrosine-containing proteins, and the identification of a protein recognized by one of the antibodies revealed that it was itself a type of protein tyrosine kinase *(106)*. Moreover, immunocytochemistry revealed that it was localized to focal contacts, and thus this protein was named FAK *(106)*. In vitro experiments indicate that the NH2 terminal of FAK can bind to the cytoplasmic domain of β1 *(135)* and the carboxy terminal to paxillin and talin *(137,138)*. Details of signaling through FAK have revealed that FAK is phosphorylated in response to integrin engagement *(139)* and the inhibition of phosphorylation blocks the formation of focal adhesions *(140)*. The phosphorylation of FAK stimulates its own tyrosine kinase activity and a number of different proteins, including the cytoskeletal proteins

paxillin and tensin, are known to be phosphorylated as part of the integrin signaling cascade *(9)*. Possibly the changes in these cytoskeletal proteins are important to regulate adhesion complex formation. In human SY5Y neuroblastoma cells, FAK phosphorylation accompanies integrin-mediated cell adhesion. Treatment of the cells with herbomycin to block FAK phosphorylation decreases FAK phosphorylation and also reduces neurite outgrowth *(141)*. However, phosphatase inhibitors are also known to block the growth of neurites in neuroblastoma cells *(142)*. These effects of inhibitors on neurite outgrowth are interesting because these cells have point contacts (Fig. 2), and in other neuronal cells FAK is not enriched in point contacts as it is in focal contacts *(57)*. At this point it is not known if the difference in FAK localization to focal contacts and point contacts is merely quantitative, if the accessibility of monoclonal anti-FAK antibodies to FAK differs, or if FAK is present but not clustered in point contacts. FAK will likely be an important signaling molecule in axonal growth because it is widely expressed in the nervous system *(107)*. Perhaps FAK association with point contacts is transient because the phosphorylation events in highly motile growth cones must be very dynamic.

As one further point on integrin signaling through protein tyrosine kinase activation, signaling through integrins can induce the activation of mitogen-activated protein (MAP) kinase and the integrity of the actin cytoskeleton is required for this activation *(142)*. Such activation may be an important link between signaling through integrins to regulate adhesion and signaling through neurotrophins to regulate other aspects of neurite growth. Because neurotrophins are also known to stimulate MAP kinase *(144)*, and MAP kinase is thought to play a key role in converging signals from multiple types of receptors, it seems likely that convergence of signaling mechanisms allows growth cones to integrate and respond to multiple growth promoting signals.

Another aspect of signaling through integrins likely of great relevance to neurons is the ability of integrin-mediated adhesion to modulate the intracellular free calcium level *(145–147)*. A comparison of integrin-transfected cells with nontransfected and nonresponsive cells implicate integrins in altering intracellular calcium, and demonstrates that calcium oscillations follow the phosphorylation of a FAK-like protein *(147)*. These results are interesting because both high and low levels of calcium can block growth cone extension *(148)*. Large increases in intracellular calcium can arrest growth or cause complete growth cone collapse *(148,149)*, whereas growth cones stop extending in calcium-free media *(150)*. Moreover, calcium is well known to affect the stability of many cytoskeletal proteins, and direct experimental evidence shows that calcium may regulate the stability of actin filaments in neurons *(150)*. It is likely that calcium regulates the motile dynamics of the cytoskeleton that generates the force for motility.

The idea that there might be interplay between calcium-mediated signaling and tyrosine protein phosphorylation is attractive because both calcium ions and tyrosine protein phosphorylation modulate growth cone motility. One speculation is that the calcium oscillations induced by integrins are required for the maintenance of tyrosine protein phosphorylation *(147)*. This idea might explain how dynamic attachment and detachment from the substrate, an absolute requirement for motility, might be regulated both by calcium and protein phosphorylation. It remains to be determined how integrins might be implicated in these processes, especially when neurons extend their axons in development or during axon regrowth in regeneration.

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