Substrate–Cytoskeletal Coupling as a Mechanism for the Regulation of Growth Cone Motility and Guidance

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ABSTRACT: **Growth cones are highly motile structures at the end of neuronal processes, capable of receiving multiple types of guidance cues and transducing them into directed axonal growth. Thus, to guide the axon toward the appropriate target cell, the growth cone carries out different functions: it acts as a sensor, signal transducer, and motility device. An increasing number of molecular components that mediate axon guidance have been characterized over the past years. The vast majority of these molecules include proteins that act as guidance cues and their respective receptors. In addition, more and more signaling and cytoskeleton-associated proteins have been localized to the growth cone. Furthermore, it has become evident that growth cone motility and guidance depends on a dynamic cytoskeleton that is regulated by incoming guidance information. Current and future research in the growth cone field will be focussed on how different guidance cues transmit their signals to the cytoskeleton and change its dynamic properties to affect the rate and direction of growth cone movement. In this review, we discuss recent evidence that cell adhesion molecules can regulate growth cone motility and guidance by a mechanism of substrate–cytoskeletal coupling.** © 2000 John Wiley & Sons, Inc. J Neurobiol 44: 97–113, 2000

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Although the detailed studies by Ramon y Cajal (1890) revealed the neuronal growth cone as an actively motile structure being important for axon outgrowth more than 100 years ago, it has been only during the last quarter of the 20th century that the growth cone has regained the attention of an increasing number of researchers. Of course, the renaissance of growth cone research was partially due to the development of modern molecular and cell biological techniques, but has also been driven by description of the unique organization of the growth cone cytoskeleton (Yamada et al., 1970, 1971). After these initial studies, some researchers investigated biophysical growth cone properties such as motility (Bray and Chapman, 1985) and tension (Bray, 1979, 1984; Joshi et al., 1985; Dennerll et al., 1988; Lamoureux et al., 1989), whereas others focussed on growth cone morphologies and pathfinding *in situ* (Caudy and Bentley, 1986a,b; Bovolenta and Mason, 1987). From a number of *in vitro* and *in vivo* experiments it became obvious that growth cone motility and guidance depends on a dynamic actin and microtubule cytoskeleton (Tosney and Wessells, 1983; Bentley and Toroian-Raymond, 1986; Letourneau et al., 1987; Dennerll et al., 1988; Forscher and Smith, 1988). An increasing number of reports have confirmed these initial findings and addressed the role of cytoskeletal dynamics in growth cone motility and guidance in detail. A comprehensive discussion of these studies can be found in several excellent reviews (Bray and Hollenbeck, 1988; Mitchison and Kirschner, 1988;

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Bentley and O'Connor, 1994; Lin et al., 1994; Tanaka and Sabry, 1995; Heidemann, 1996; Letourneau, 1996).

Growth cones are capable of responding to a variety of guidance cues resulting in different pathfinding behaviors such as outgrowth, retraction, stalling, turning, and fasciculation. Guidance cues include diffusible factors that can attract axons over long distances, as it has been shown for the netrins (Kennedy and Tessier-Lavigne, 1995). Diffusible guidance molecules may have repulsive as well as inhibitory effects, which can result in total growth cone collapse, as demonstrated for collapsin-1 (Luo et al., 1993). Growth cones can be also be guided by short-range, contact-mediated mechanisms involving cell adhesion molecules (Brummendorf and Rathjen, 1996; Takeichi et al., 1997; Walsh and Doherty, 1997) and extracellular matrix proteins (Reichardt and Tomaselli, 1991; Hynes and Lander, 1992), which in turn can be either attractive or repulsive. In summary, axon guidance molecules can be grouped into at least four categories according to their range and type of action (Goodman, 1996; Tessier-Lavigne and Goodman, 1996).

Subsequent to identification of the major families of guidance molecules, many laboratories are now turning their attention to the investigation of downstream effector mechanisms. Several signal transduction pathways and effector molecules (tyrosine kinases, serin-threonine kinases, Ca^{2+}) have been implicated in growth cone guidance and motility in recent years. However, how they affect cytoskeletal dynamics, and thereby motility remains speculative in most cases. This is partially because of the limited size and resolution of many vertebrate and invertebrate growth cones. In contrast, *Aplysia* bag cell neurons of the abdominal ganglion form large growth cones in culture, which have a well-defined cytoplasmic organization and provide an ideal system for analyzing cytoskeletal dynamics (Fig. 1). Using this system, we have recently provided evidence that cell adhesion molecules (CAMs) can promote growth and guidance by a mechanism of substrate–cytoskeletal coupling (Suter et al., 1998). Further evidence for this model arose from work on integrin receptors in mediating motility of fibroblast-like cells (Felsenfeld et al., 1996; Huttenlocher et al., 1996; Palecek et al., 1997).

After reviewing our current understanding of cytoskeletal dynamics in growth cones, we will focus in this review on recent findings from both the growth cone and fibroblast literature that relate to the substrate–cytoskeletal coupling model. Finally, we will discuss different parameters, such as tyrosine phosphorylation, that appear to regulate the strength of coupling between CAMs and the cytoskeleton.

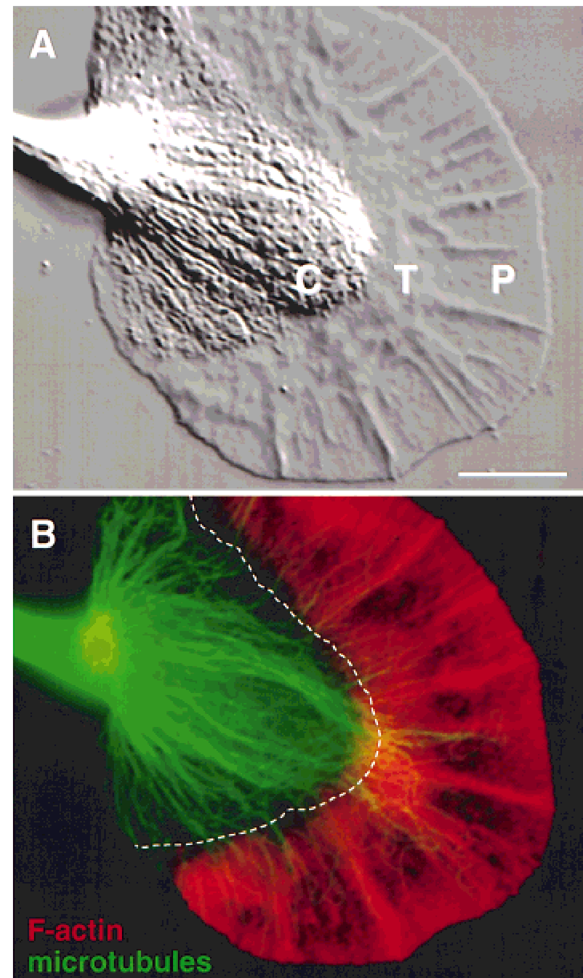

Figure 1 Organization of cytoplasmic domains and cytoskeletal structures in *Aplysia* growth cones. (A) *Aplysia* bag cell growth cone on poly-lysine substrate visualized by high-resolution DIC (differential interference contrast) optics. Different cytoplasmic domains are indicated: central domain (C), transition zone (T), and peripheral domain (P). Bar = 10 μm. (B) Growth cone in (A) was fixed, extracted with 1% Triton X-100, and stained with rhodamine-phalloidin for F-actin (red) and with a tubulin antibody for microtubules (green). A dashed line indicates the border between the central domain and the transition zone/peripheral domain.

GROWTH CONE CYTOSKELETAL DYNAMICS

It is important to consider key attributes of cytoskeletal protein dynamics first before delving into growth cone–substrate interactions that rely on these pro-

cesses. On the basis of morphology, one can distinguish three major cytoplasmic domains in neuronal growth cones: the peripheral domain consisting of lamellipodia and filopodia, the central domain containing organelles, and a transition zone characterized by actin-based ruffling activity that is located between the peripheral and central domains [Fig. 1(A)]. This organization can be observed very clearly in cultured *Aplysia* growth cones (Fig. 1; Goldberg and Burmeister, 1986; Forscher et al., 1987; Forscher and Smith, 1988). Although these domains are present in vertebrate growth cones too, the boundaries between them are typically less well defined compared with the *Aplysia* cones. The distribution of the two main cytoskeletal structures, filamentous (F)-actin and microtubules, parallels this domain organization to a high degree [Fig. 1(B)]: actin filaments are found predominantly in the peripheral domain, whereas microtubules are localized mainly in the central cytoplasmic domain (Gordon-Weeks, 1987; Forscher and Smith, 1988; Bridgman and Dailey, 1989). It is in the transition zone that many of the distal tips of microtubules reside. However, some microtubules can also extend through the transition zone into the F-actin-rich peripheral domain [Fig. 1(B)].

Actin Dynamics in Growth Cones

Detailed EM studies revealed two major populations of F-actin networks in the peripheral growth cone domain (Fig. 2): bundles of longer filaments with their plus ends toward the leading edge and more randomly oriented networks of shorter filaments between the bundles (Lewis and Bridgman, 1992). The filament bundles span the width of the lamellipodia and extend into filopodia. Both lamellipodia and filopodia are highly dynamic growth cone structures that continuously extend and retract (Bray and Chapman, 1985; Goldberg and Burmeister, 1986). This dynamic behavior enables the growth cone to explore the extracellular environment for potential guidance cues that mediate adhesion and motility. It is currently believed that the dynamic shape of the growth cone lamellipodia and filopodia is mainly determined by the underlying F-actin dynamics (Tanaka and Sabry, 1995; Letourneau, 1996). The dynamic properties of actin filaments in growth cones (and motile cells in general) can be described by three independent kinetic processes (Fig. 2): (1) assembly of actin filaments from G-actin monomers at the leading edge and tips of filopodia [Fig. 2(A), right inset; Forscher and Smith, 1988; Okabe and Hirokawa, 1991; Mallavarapu and Mitchison, 1999]; (2) constant retrograde flow of F-actin networks powered by myosin motors [Fig. 2(A), lower inset, Fig. 2(B); Forscher and Smith, 1988; Lin et al., 1996]; and (3) proximal recycling of F-actin in the transition zone, a necessary process not yet well characterized in growth cones [Fig. 2(A), lower inset]. Regulation of these dynamic properties occurs through an increasing number of actin-binding proteins, frequently characterized first in nonneuronal cells (Stossel, 1993; Welch et al., 1997c). Although many of these proteins have been localized in the peripheral domain of neuronal growth cones (Letourneau, 1996), studies addressing their functions as well as their ultrastructural localization in growth cones are still mostly lacking.

F-actin assembly at the leading edge [Fig. 2(A)] results from a combination of nucleation, polymerization, and annealing of short actin filaments. These processes are likely mediated by nucleation factors such as the Arp2/3 complex (Welch et al., 1997a,b), barbed-end capping proteins such as CapZ (Caldwell et al., 1989), actin monomer-binding proteins such as profilin and thymosin β4 (Pantaloni and Carlier, 1993), and proteins of the Ena/Mena/VASP family that enhance the polymerization rate by recruiting profilin to sites of actin assembly (Chakraborty et al., 1995; Gertler et al., 1996; Kang et al., 1997). Genetic studies have recently provided evidence that both profilin and Ena control motor axon outgrowth and guidance in the *Drosophila* embryo (Wills et al., 1999a,b). With respect to vertebrate neurons, Mena-deficient mice have defects in commissure formation, suggesting an important role for Mena in neuronal growth and pathfinding (Lanier et al., 1999). Furthermore, these authors showed localization of Mena right at the tip of growth cone filopodia, where actin polymerization takes place (Lanier et al., 1999). Much about the functional properties and binding interactions of the actin regulatory proteins mentioned above has been learned by studying the motility of intracellular bacterial pathogens such as *Listeria* and *Shigella.* Analysis of the mechanism by which these bacteria commandeer the host cell's actin machinery to propel themselves through the cytoplasm is likely to provide us with important insights into how cells regulate F-actin assembly under steady state condition as well as in response to extracellular signals (for recent excellent reviews see Beckerle, 1998; Carlier, 1998; Dramsi and Cossart, 1998).

After being assembled at the leading edge, F-actin bundles and networks are translocated toward the central domain by retrograde F-actin flow [Fig. 2(B); Forscher and Smith, 1988; Mitchison and Kirschner, 1988]. Evidence for retrograde F-actin flow arose from studies in which blocking of plus-end F-actin assembly with cytochalasin B resulted in a progres-

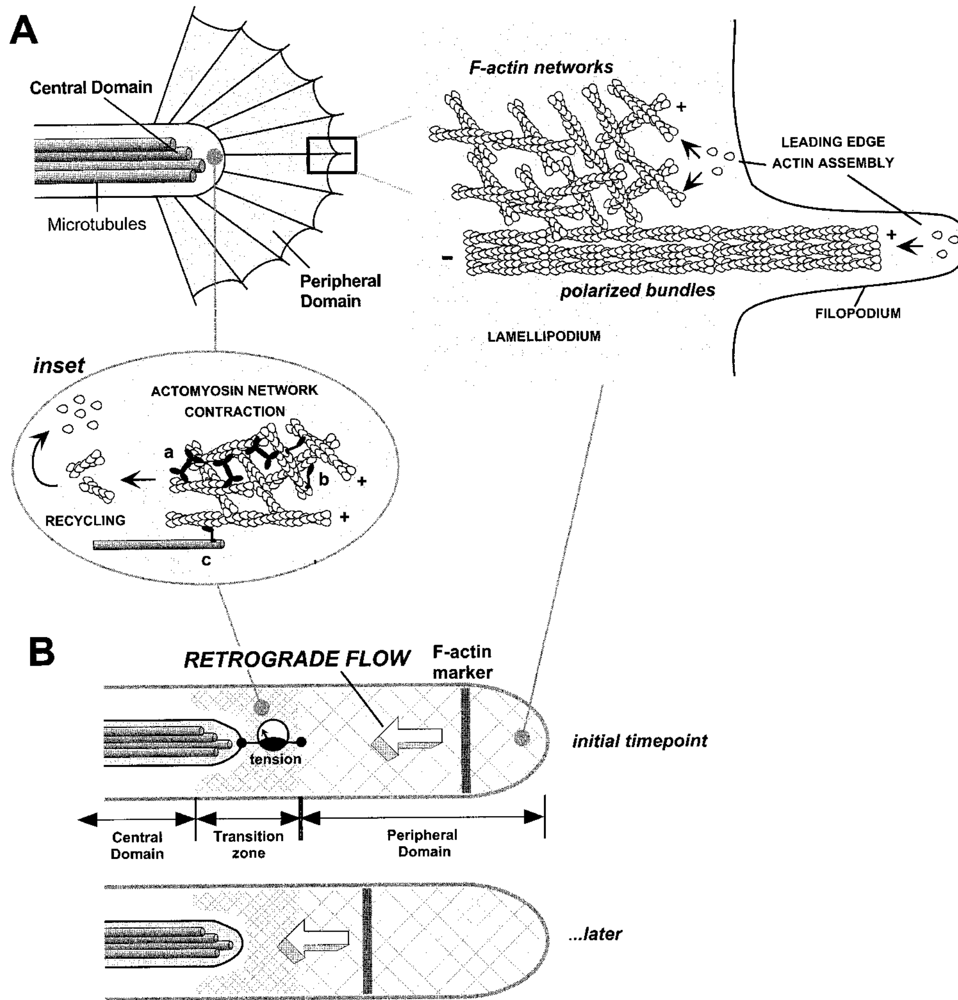

Figure 2 Cytoskeletal organization and actin dynamics in growth cones. (A) Distribution of the two major cytoskeletal components in neurites and growth cones. Microtubules are localized in the neurite and the central domain of the growth cone, whereas actin filaments are distributed in the peripheral domain. Blowup to right shows the organization of actin filaments in more detail. Filopodia contain bundles of filaments with their plus (barbed) ends oriented toward the leading edge. These bundles can span the whole width of the lamellipodia. A second population of actin filaments form less polarized networks in lamellipodia. Plus (barbed) end assembly occurs at the leading edge and at the tips of filopodia. (B) Cross section of a growth cone demonstrating the dynamic processes involved in actin-based growth cone motility. Retrograde F-actin flow is indicated by a marker (e.g., flow-coupled bead). Inset: retrograde translocation of actin filaments is driven by myosin motors that are likely in the transition zone. Possible actomyosin combinations for tension generation are shown in inset (a–c), double headed myosin II and single headed myosin I subtypes are depicted. Retrograde flow rate is high and tension is low if actin networks are not stabilized by substrate interactions (cf. Fig. 4). Filament recycling occurs by action of putative severing proteins in the transition zone such as gelsolin and/or factors such as ADF/cofilin. (Reprinted from Curr Opin Neurobiol, Suter and Forscher, An emerging link between cytoskeletal dynamics and cell adhesion molecules in growth cone guidance, 8:106–116, 1998, with permission from Elsevier Science).

sive clearance of actin structures in the peripheral domain beginning at the leading edge (Forscher and Smith, 1988). F-actin flow rates in *Aplysia* growth cones plated on poly-lysine substrates are 4–6 μm/min (Forscher and Smith, 1988; Lin and Forscher, 1995). Thus, in a growth cone with a 15- to 20-μm-wide peripheral domain, the entire F-actin cytoskeleton turns over every ~3 min. Studies from our labo-

ratory have provided evidence that retrograde F-actin flow in growth cones is powered by myosin motors (Lin et al., 1996). Myosin inhibition either by injection of *N*-ethyl maleimide (NEM)-inactivated myosin S1 fragments or by cell treatment with 2,3-butanedione-2-monoxime (BDM; an inhibitor of myosin ATPase) resulted in slowing of retrograde F-actin flow accompanied by protrusive growth of filopodia. This filopodial growth was due to persistent actin assembly during myosin inhibition, as evidenced by its high sensitivity to cytochalasin B (Lin et al., 1996). These results also suggest that actin assembly and translocation of F-actin structures by myosin are distinct processes that could be subject to independent regulation. Of all the myosins identified in growth cones so far, biochemical, genetic, immunocytological, and physiological evidence indicate myosin II and/or myosin I as conceivable candidates for the motors that drive retrograde flow (for review see Hasson and Mooseker, 1997).

Recycling of F-actin networks is likely to occur in the transition zone if a steady state flux is to be maintained [Fig. 2(A), lower inset]. It could be mediated by severing proteins such as gelsolin (Yin and Stossel, 1979) and/or depolymerizing factors of the ADF/cofilin family (Carlier et al., 1997; Lappalainen and Drubin, 1997; Rosenblatt et al., 1997). Growth cones from gelsolin knockout mice have more filopodia than control growth cones as a result of a delayed retraction rate (Lu et al., 1997). Impaired F-actin severing at the base of filopodia could account for delayed retraction rates observed. Signals that activate ADF/cofilin by dephosphorylation are associated with enhanced neurite growth (Meberg et al., 1998). On the other hand, overexpression of LIM-kinase, which inactivates cofilin through phosphorylation, leads to accumulation of F-actin in nonneuronal cells as well as in growth cones (Arber et al., 1998). Taken together, increased actin turnover could explain increased axonal growth rates after ADF/cofilin activation (Meberg et al., 1998). Finally, N-WASP, a neural homologue of WASP (Wiskott–Aldrich syndrome protein) could also contribute to actin recycling in growth cones. This PIP_2-binding protein has been shown to have actin-depolymerizing activity and is implicated in signal transduction between the receptor tyrosine kinases and the actin cytoskeleton (Miki et al., 1996). However, more recent studies have provided evidence that N-WASP is an important regulator of actin assembly by linking signals such as Cdc42 and lipids to the Arp2/3 complex (Ma et al., 1998; Machesky and Insall, 1998; Miki et al., 1998; Rohatgi et al., 1999).

Microtubule Dynamics in Growth Cones

Microtubules are the prominent cytoskeletal components in neuronal processes as well as in the central domain of the growth cone [Fig. 1(B)]. They provide both structural support for axon elongation and act as substrates for fast axonal transport of organelles into the growth cone. After entering the growth cone, microtubules typically splay out [Fig. 1(B)] and continuously probe the actin-rich peripheral domain by extension and retraction (Tanaka and Kirschner, 1991). This highly dynamic microtubule behavior is believed to occur by stochastic bouts of microtubule assembly and disassembly, a process referred to as dynamic instability, and/or by microtubule sliding, potentially mediated by the action of microtubule motors (Mitchison and Kirschner, 1988; Heidemann, 1996). Pharmacological studies using low concentrations of vinblastine, nocodazole, or taxol, which inhibit dynamic instability without causing marked microtubule loss, revealed that both axonal advance and growth cone guidance depend on dynamic microtubules (Tanaka et al., 1995; Tanaka and Kirschner, 1995; Rochlin et al., 1996; Williamson et al., 1996; Challacombe et al., 1997). A recent study also suggested that there is a significant sliding component to microtubule movements in growth cones (Dent et al., 1999). However, a detailed analysis of the mechanism of microtubule dynamics (analysis of treadmilling vs. sliding, for example) in growth cones has yet to be accomplished. Furthermore, research on proteins involved in regulation of microtubule dynamics has proceeded at a slower pace than related work in the actin dynamics field. An exception to this trend is the well-established role of microtubule-associated proteins (MAPs) in regulating microtubule stabilization (Heidemann, 1996).

SUBSTRATE–CYTOSKELETAL COUPLING IN GROWTH CONES

It is generally established that the regulation of growth cone and cell motility involves coordinated control of cytoskeletal protein distribution and dynamics (Mitchison and Kirschner, 1988; Stossel, 1993; Letourneau, 1996; Mitchison and Cramer, 1996) as well as cell–substrate adhesion (Hammarback et al., 1988; Payne et al., 1992; Gomez et al., 1996). A recently developed paradigm for growth cone and cell motility is the “substrate–cytoskeletal coupling” model (Mitchison and Kirschner, 1988; Lin et al., 1994; Suter and Forscher, 1998). According to this model, cells and growth cones can move forward

if they are capable of coupling intracellular actomyosin-based motility to a fixed (noncompliant) extracellular translocation substrate via cell surface receptors (see Fig. 4). These receptors must form a strong linkage between the substrate and the actin cytoskeleton, allowing actomyosin contractions to pull the growth cone forward. If a permissive translocation substrate is not available, myosin motors will drive retrograde flow, but the resulting F-actin flux does not effectively contribute to forward movement [Fig. 2(B)]. Two different lines of evidence provide the main support for this model: (1) Work from our laboratory focusing on the regulation of cytoskeletal dynamics in *Aplysia* bag cell growth cones by a cell adhesion molecule of the immunoglobulin (Ig) superfamily (that we will discuss predominantly in this chapter); and (2) Studies investigating integrin–cytoskeleton interactions in fibroblasts and growth cones (this will mainly be discussed in the section on regulation of receptor–cytoskeleton coupling).

Restrained Bead Interactions—An Assay to Study Substrate–Cytoskeletal Coupling

A number of *in vivo* and *in vitro* studies in the last few years have indicated that rapid rearrangement of the actin and microtubule cytoskeleton occurs when growth cones respond to attractive extracellular guidance cues (reviewed in Tanaka and Sabry, 1995). To summarize, during growth cone–target interactions microtubules reorient and extend toward interaction sites, and F-actin tends to accumulate distal to microtubule ends. Furthermore, from many studies it has been concluded that F-actin reorganization proceeds and in some manner guides microtubule reorganization and advance (Tanaka and Sabry, 1995). For example, studies from our laboratory demonstrated that microtubules specifically extend to sites of F-actin accumulation that occur when *Aplysia* growth cones undergo homophilic interactions in culture (Lin and Forscher, 1993). Analysis of retrograde F-actin flow during such *Aplysia* growth cone–target interactions revealed an inverse relationship between rates of central domain extension and retrograde flow (Lin and Forscher, 1995). Using Concanavalin A (Con A)-coated microbeads as markers for measuring retrograde F-actin flow rates, Lin and Forscher (1995) demonstrated that F-actin flow attenuates specifically along the target interaction axis at the same time the rate of central domain extension increases. These findings suggested the substrate–cytoskeletal coupling model; however, the putative receptors mediating coupling were unknown.

To test this model rigorously with respect to a specific cell adhesion receptor type, we developed a novel *in vitro* target interaction system, referred to as restrained bead interaction (RBI) assay (Suter et al., 1998). This assay utilizes relatively large silica beads (5 μm diameter) with amino-functional groups on their surface that permit covalent coupling of proteins of interest as target substrates. Beads coated with ligands to specific cell adhesion receptors can then be placed onto the peripheral domain of *Aplysia* bag cell growth cones to assess their effects. We were particularly interested in investigating ligands to apCAM, a member of the Ig superfamily and the *Aplysia* homologue of vertebrate NCAM (Mayford et al., 1992), inasmuch as apCAM had previously been shown to accumulate at interaction sites between growth cones in culture and to functionally interact with the F-actin cytoskeleton (Thompson et al., 1996). Ligands to apCAM in our RBI assay included a monoclonal antibody to an extracellular epitope of apCAM, as well as biochemically purified apCAM and Con A—a lectin determined to bind a multitude of neuronal cell surface proteins including apCAM (Thompson et al., 1996). Beads coated with apCAM ligands rapidly bind to the cell surface, couple to retrograde F-actin flow, and will normally be cleared from the peripheral domain in ~3 min (Thompson et al., 1996). However, in the RBI assay, beads are restrained with a microneedle to prevent retrograde translocation, the logic being that physiological substrates, like cells and extracellular matrices, are generally noncompliant.

The results of an RBI experiment are shown in Figure 3 (Suter et al., 1998). During the first 10 min of restraining an anti-apCAM–coated bead (referred to as the latency period), no obvious changes in growth cone structure and motility were observed [Fig. 3(A)]. However, the latency period abruptly ends when the central domain begins to extend toward the bead interaction site, and the leading edge begins to protrude distal to the bead [Fig. 3(B), arrow]. Significantly, central domain extension is accompanied by a marked increase in tension in the RBI axis as indicated by a progressive increase in needle bending during the interaction period [Fig. 3(F), red line]. These observations demonstrate that the growth cone exerts a pulling force on the dorsally placed bead substrate during the interaction. Major cytoskeletal reorganization including microtubule extension directly to the bead binding site and F-actin accumulation around the restrained bead underlies these structural changes [Fig. 3(C–E)]. Importantly, quantification of retrograde F-actin flow during the interaction revealed attenuation of flow along the target

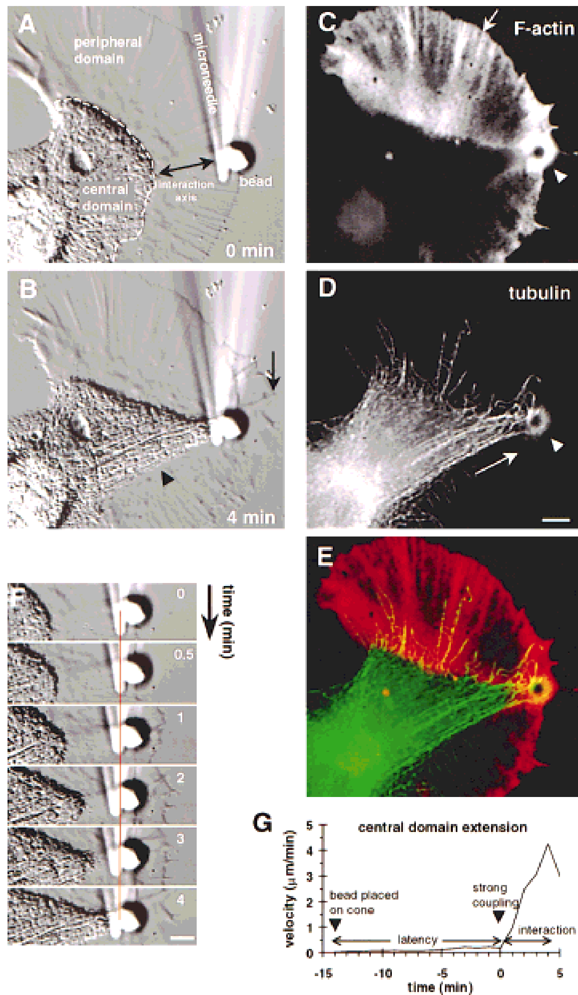

Figure 3 Crosslinking of apCAM triggers C domain extension, protrusive growth, and cytoskeletal remodeling. All images refer to the same growth cone. (A) Beads coated with the anti-apCAM antibody (4E8) were placed on the peripheral domain of an *Aplysia* bag cell growth cone near the leading edge and restrained from retrograde movement using a microneedle. This video-enhanced DIC image was recorded after the latency period (see G) at the start of central domain extension. The double-headed arrow indicates interaction axis between bead and central domain. (B) After 4 min, the central domain boundary extended to the bead (arrowhead marks initial boundary position). Arrow indicates new leading edge position. (C) Rhodamine-phalloidin labeling of F-actin and (D) β-tubulin immunofluorescence after fixation at the 5-min time point. Note F-actin accumulation around the bead (arrowhead in C) and typical control staining for actin bundles in adjacent areas (arrow in C). Microtubule extension (arrow in D) was directed toward the bead. (E) Pseudocolor overlay of F-actin (red) and tubulin (green) stainings. (F) DIC time course of this interaction. Line indicates needle position at time 0; note rearward displacement over time. (G) Central domain extension rates plotted as a function of time. Bead placement (arrowhead) followed by latency and interaction periods (double-headed arrows) are indicated. Bars = 5 μm. Video sequence to this experiment can be found on the Web site of *Journal of Neurobiology:* http://www.interscience.wiley.com/jpages/0022-3034/. (Reproduced from J Cell Biol, 1998, 141:227–240, by copyright permission of The Rockefeller University Press).

interaction axis but not in the adjacent lamellipodium (Suter et al., 1998). The structural and cytoskeletal changes we observe with the RBI assay (using a chemically defined substrate) faithfully mimic the changes previously reported for growth cone interactions with native cellular targets (Lin and Forscher, 1993, 1995). These results provided direct evidence for the substrate–cytoskeletal coupling model and demonstrated for the first time that adhesion molecules of the Ig CAM superfamily can act as force transducers between motile cytoskeletal networks and extracellular substrates.

Attenuation of Retrograde F-Actin Flow by Formation of a Molecular Clutch Complex

How could apCAM-clustering result in attenuation of retrograde flow? One intriguing possibility is that apCAM-clustering results in formation of a complex that acts like a molecular “clutch.” When engaged, this clutch supports tension between extracellular substrates and the contractile actomyosin networks embodying retrograde flow. In the context of an RBI experiment, during the latency period, retrograde flow rates along the RBI axis were indistinguishable from off-axis flow rates in the adjacent lamellipodium (Suter et al., 1998). We speculate that linkages between the bead substrate and the actin cytoskeleton are initially relatively weak and as a result there is “clutch slippage” at the receptor–actin interface during the latency period [Fig. 4(A)]. This conclusion is evidenced by continued retrograde F-actin flow in the target interaction axis, by the fact that target beads do move with the flow when needle restraint is removed, and also by the absence of significant tension build-up during the latency period. We hypothesize that over time, the number and/or state of apCAM/actin linkages changes promoting progressive strengthening of the F-actin–apCAM linkage to the extent that eventually the full force of the myosin driven retrograde flow can be restrained [Fig. 4(B), “linkage strengthening”]. Under this condition the actomyosin contractile system would be applying maximal pulling force on the substrate.

Note that if F-actin assembly rates remain unchanged as flow attenuation proceeds, lamellipodium protrusion distal to the point of flow restraint (the bead) is predicted, which is exactly what we observe [Fig. 3(B)], although we can not rule out other possible contributing factors such as site-directed actin assembly also triggered by apCAM clustering (Thompson et al., 1996). apCAM is the first Ig superfamily CAM (IgCAM) shown to couple to retrograde F-actin flow upon clustering (Thompson et al., 1996) and to transduce force (Suter et al., 1998). In addition, very recently it was shown that another IgCAM, NrCAM, associates to retrograde flow in growth cones after clustering through its ligand F3 (Faivre-Sarrailh et al., 1999). Thus, it appears that regulation of growth cone motility by substrate–cytoskeletal coupling is likely to be a conserved mechanism.

Force

It is well known that growth cones exert pulling forces and can advance in the direction of applied tension (Bray, 1984; Lamoureux et al., 1989). We observed an increase in tension between the bead substrate and the central domain during the interaction period of the RBI experiment [Fig. 3(F)] that is consistent with these studies. Thus, tension development parallels both retrograde F-actin flow attenuation and central domain advance. It seems reasonable to assume that the same myosin motors that drive retrograde F-actin flow under control conditions (Lin et al., 1996) are responsible for tension development during RBIs. Studies in migrating fish keratocytes strongly suggest that myosin II–based contractile networks located in the transition zone are responsible for generating retrograde flow and tension during translocation (Svitkina et al., 1997)—a similar situation may exist in growth cones because myosin II has been localized to the transition zone here too (Miller et al., 1992; Rochlin et al., 1995).

Microtubule Extension

When the central domain extends along the target interaction axis toward a bead during an RBI, underlying microtubules also extend [Fig. 3(D)]. The fact that central domain extension was accompanied by both flow attenuation and tension increase strongly suggests that a mechanical continuum develops over time between the apCAM bead substrate, the peripheral actin domain, and the central domain; however, a detailed description of the molecules involved in this central–peripheral domain linkage remains an important area for further studies. A related question that remains to be addressed is the mechanism of microtubule extension during directed growth (see also Suter et al., 1998; Suter and Forscher, 1998). There is evidence that F-actin and retrograde flow impose steric constraints to microtubule advance (i.e., peripheral F-actin acts as a physical barrier). In support of this idea, microtubules rapidly extend into peripheral domains depleted of F-actin subsequent to cytochalasin B treatment (Forscher and Smith, 1988), and a recent related study provides evidence that retrograde F-actin flow exerts inhibitory effects on both plus-end assembly and microtubule sliding (Waterman-Storer and Salmon, 1997). Note that simple steady state kinetics predicts that F-actin flow attenuation during a target interaction would result in a compensatory forward shift of the F-actin domain if one assumes unchanged recycling rates [Fig. 4(B)]. This could provide an opportunity for microtubules to grow into a region of decreased F-actin density (i.e., up the target interaction axis).

An alternative mechanism for microtubule extension could involve myosin-dependent pulling on microtubule ends. Myosins may be associated with microtubule ends that penetrate the transition zone [Fig. 4(A), left inset]. If the peripheral domain is restrained by interaction with a substrate, microtubule-associated myosins would tend to pull the microtubule domain forward toward the plus end of the actin filaments [Fig. 4(B)]. Although this model is intriguing, evidence that myosins provide a dynamic link between microtubules and actin filaments is largely missing. In summary, whatever mechanism(s) actually mediate microtubule extension during directed growth, they are likely to involve dynamic interactions with the contractile actin cytoskeleton.

CAM LINKAGE TO THE CYTOSKELETON

Do CAMs have the requisite properties to act as coupling agents between extracellular substrates and intracellular actomyosin networks during directed neuronal growth? Neuronal CAMs are important mediators of neurite growth and guidance involving a mechanism of contact attraction or contact repulsion (Goodman, 1996; Tessier-Lavigne and Goodman, 1996). They can represent both guidance cues and receptor molecules, and frequently, but not exclusively, bind to members of the same structural family. The classical neuronal CAMs belong to three distinct structural families: (1) integrins, (2) cadherins, and (3) IgCAMs. All of these cell adhesion molecules have been originally identified to mediate adhesive func-

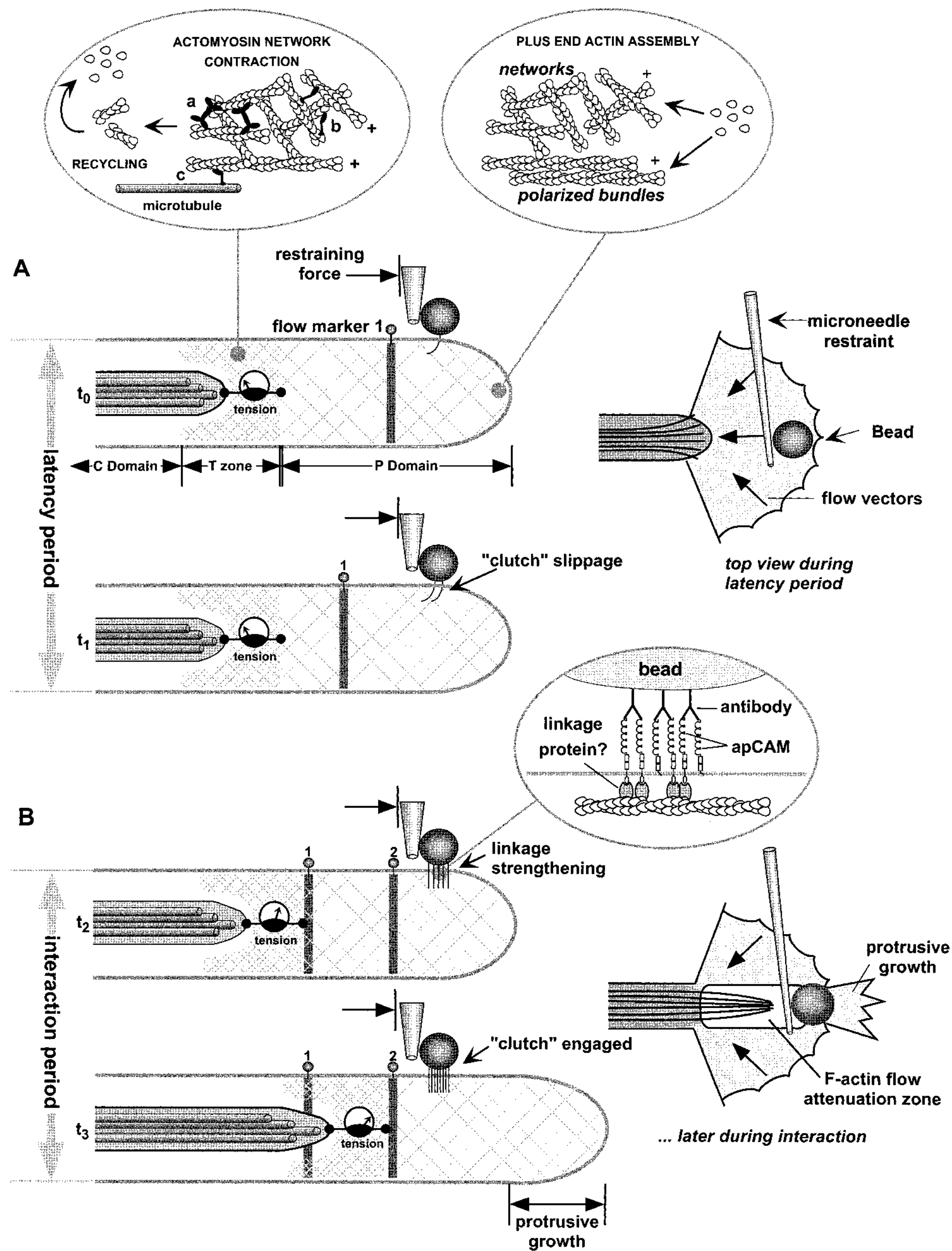

Figure 4 Substrate-cytoskeletal coupling model. Schematic cross sections through a growth cone demonstrate the cytoskeletal organization of the peripheral and central domain, as well as of the transition zone, at different stages of an RBI experiment. Left inset shows potential conventional (a), unconventional (b), and microtubule-associated (c) myosin localizations and details of actin recycling; right inset depicts actin filament organization in filopodia and lamellipodia as well as actin assembly. Inset in (B) shows details of a potential "molecular clutch." Cross section and top views of growth cones during RBIs are shown on left and right, respectively. (A) Latency period. The molecular "clutch" between receptor and actin cytoskeleton exhibits slippage at low levels of apCAM clustering early in the RBI. ApCAM-actin linkage is not strong enough to support significant central–peripheral domain tension or attenuate retrograde flow. Retrograde flow is maximal (cf. F-actin flow marker 1 displacement) and growth is slow. (B) Interaction period. When enough functional linkages are engaged by restrained beads, retrograde flow is attenuated (marker bead 2), central-peripheral domain tension increases and the central domain extends toward the restrained bead. Protrusive growth may result directly from continued actin assembly during F-actin flow attenuation. (Reproduced from J Cell Biology, 141:227–240 1998, by copyright permission of The Rockefeller University Press).

tions (e.g., neurite fasciculation), but later have also been shown to have morphogenic effects such as promoting axonal growth and to interact with signal transduction pathways as well. Many excellent reviews have been written on various aspects of these cell adhesion receptors (Reichardt and Tomaselli, 1991; Hynes and Lander, 1992; Brummendorf and Rathjen, 1995; Brummendorf and Rathjen, 1996; Takeichi et al., 1997; Walsh and Doherty, 1997). Therefore, we will introduce the three CAM families only briefly, and focus on the proteins that link these receptors to the actin cytoskeleton.

Integrins

Integrins are important heterodimeric receptors that interact with extracellular matrix molecules (ECM) as well with Ig superfamily molecules to mediate cell–cell adhesion (Hynes, 1992). Integrins are widely expressed in the nervous system and play a role in various developmental processes such as neuronal migration, axonal growth, and guidance by attaching to ECM proteins such as laminin, fibronectin, and tenascin (Reichardt and Tomaselli, 1991; Hynes and Lander, 1992). Initial studies addressing the role of integrins in neurite outgrowth involved the application of function-blocking antibodies to neurites growing on either glial cells or purified ECM proteins *in vitro* (e.g., Bozyczko and Horwitz, 1986; Neugebauer et al., 1988; Tomaselli et al., 1988). A more recent study showed enrichment of integrin receptors at the tip of growth cone filopodia, suggesting a role in axon guidance (Wu et al., 1996). Evidence for such a function arose from both *in vitro* studies using ECM proteins as molecular guideposts (Kuhn et al., 1995; Kuhn et al., 1998) and genetic analysis of integrins in *Drosophila* axon guidance (Hoang and Chiba, 1998).

With respect to cytoskeletal linkages, linkage regulation, and signaling, the integrins are by far the best-studied family of adhesion receptors. Much of our knowledge on the proteins that link integrins to the actin cytoskeleton stems from studies on focal adhesions formed by nonneuronal cells in culture when attaching to ECM substrates (Burridge and Chrzanowska-Wodnicka, 1996; Schoenwaelder and Burridge, 1999). Besides the immense number of cytoskeletal-associated (e.g., vinculin and talin) and signaling proteins (e.g., FAK, Rho, and Src) found in focal adhesions, the fact that integrins can engage in bidirectional signaling (Ginsberg et al., 1992; Schoenwaelder and Burridge, 1999) adds an additional level of complexity to integrin function.

Many of these focal adhesion proteins have been detected in growth cones (Cypher and Letourneau, 1991; Helmke and Pfenninger, 1995), suggesting a role for integrin-cytoskeletal coupling in growth cone motility and guidance. It is reasonable to assume that the molecular composition and architecture of integrin–cytoskeleton linkages of migrating growth cones depends on the substrate. Interference reflection microscopy (IRM) and immunofluorescence studies of growth cone interactions with ECM and CAM proteins support this assumption (Burden-Gulley and Lemmon, 1996; Gomez et al., 1996; Drazba et al., 1997). Specifically, it was found that growth cone contacts on fibronectin but not laminin resemble fibroblastic focal adhesions (Gomez et al., 1996). Recent functional studies involving protein inactivation methods such as microscale chromophore-assisted laser inactivation (CALI) or suppression of protein expression by antisense methods confirmed a role in cytoskeletal coupling in the case of talin and vinculin (Varnum-Finney and Reichardt, 1994; Sydor et al., 1996).

Cadherins

Cadherins are Ca^{2+}-dependent homophilic CAMs that play important roles in morphogenesis in both neuronal and nonneuronal systems (Takeichi et al., 1997). N-cadherin is widely expressed in the developing central nervous system (Hatta et al., 1987). Several *in vitro* studies have demonstrated the function of N-cadherin in promoting neurite outgrowth, either when presented as purified protein substrate or when expressed on the surface of cells (Neugebauer et al., 1988; Tomaselli et al., 1988; Bixby and Zhang, 1990; Payne et al., 1992). Expression of a dominant negative N-cadherin mutant lacking a large portion of the extracellular domain in *Xenopus* revealed that N-cadherin function is necessary for axonal and dendritic outgrowth from retinal ganglion cells *in vivo* (Riehl et al., 1996).

The linkage between cadherins and the actin cytoskeleton is well described. The general structure of this linkage involves either β-catenin or γ-catenin binding to the cytoplasmic domain of cadherin as well as to α-catenin, which in turn is thought to link the cadherin/catenin complex to the actin cytoskeleton (Aberle et al., 1996). Although cadherin/catenin interactions are required for cell adhesion, they do not appear to be critical for axonogenesis of retinal ganglion cells. This has been demonstrated in *Xenopus* by overexpression of the cytoplasmic N-cadherin tail containing mainly the catenin-binding region (Riehl et al., 1996).

IgCAMs

The Ig superfamily is the largest family of structurally related proteins, with well over 100 members, all characterized by the presence of at least one Ig domain (Williams and Barclay, 1988). The first neuronal member of the IgCAMs characterized in mediating cell adhesion of retina cells is NCAM (Hoffman et al., 1982). In recent years, the number of neuronal IgCAMs expanded significantly, and members can be grouped according to their domain organization, amino acid sequence similarity, and type of membrane anchorage (Brummendorf and Rathjen, 1996). Using a variety of different protein-binding approaches, several neuronal IgCAMs have been demonstrated to undergo multiple homophilic and heterophilic interactions, which are Ca^{2+}-independent (in contrast to cadherins) and occur both in *trans*- and *cis*-configurations (for review see Brummendorf and Rathjen, 1996). Such multiple protein interactions give rise to a high level of complexity that is important for the various pathfinding decisions during nervous system development. Regulating the expression of different interacting CAMs provides a mechanism for growth cone pathfinding involving a limited number of molecules. For example, results from *in vivo* antibody perturbation studies suggested such a mechanism for axonin-1, NgCAM, and NrCAM during commissural axon guidance to and at the midline (Stoeckli and Landmesser, 1995). Functional cooperation of axonin-1 and NgCAM during growth cone–substrate interactions and fasciculation has further been demonstrated by a combination of immunolocalization, neurite outgrowth, heterologous expression, and biochemical crosslinking studies (Buchstaller et al., 1996; Kunz et al., 1996, 1998; Stoeckli et al., 1996).

Despite recent progress on how neuronal IgCAMs may activate signal transduction pathways (Doherty and Walsh, 1996), molecular details of IgCAM–cytoskeleton linkages are far less characterized relative to the integrins and cadherins. Most is known about ankyrin and its role in linking L1 family members to the spectrin/actin cytoskeleton (Davis and Bennett, 1994). Recruitment of ankyrin to cell contact sites induced by homophilic interactions of the *Drosophila* L1 homologue neuroglian suggests that neuroglian clustering promotes ankyrin/IgCAM association (Dubreuil et al., 1996). Evidence for a functional cooperation between ankyrinB and L1 *in vivo* is given by the observations that ankyrinB (−/−) mice exhibit similar nervous system defects as L1 (−/−) mice, such as dilated optic nerve axons (Scotland et al., 1998).

Interestingly, when neuroglian was expressed with a glycosylphosphatidylinositol (GPI) anchor, it was unable to recruit ankyrin (Dubreuil et al., 1996). Many IgCAMs are inserted into the plasma membrane via a GPI anchor (Brummendorf and Rathjen, 1996). How do these molecules transmit their signals and associate with the underlying cytoskeleton? Can they transduce force? One possibility involves *cis*-interactions with membrane-spanning binding partners. For example, the GPI-linked IgCAM axonin-1/TAG-1 has been demonstrated to interact with its transmembrane binding partner NgCAM/L1 only in *cis* (e.g., in the plane of the same membrane), but not in *trans* (Buchstaller et al., 1996; Kunz et al., 1998; Malhotra et al., 1998). In support of the assumption mentioned above, Malhotra et al. (1998) have shown that during homophilic TAG-1–mediated cell adhesion, a *cis*-interaction between TAG-1 and L1 promotes ankyrin recruitment to cell contact sites. However, clearly more work is needed to better understand how GPI-linked CAMs transmit guidance information and to find out if they can transduce force.

REGULATION OF CAM–CYTOSKELETON COUPLING

How are linkages between CAMs and the underlying actin cytoskeleton regulated? This question is particularly important with respect to motile cells and growth cones, which have to rapidly form and disassemble substrate–cytoskeletal linkages for migration to occur. In addition, the cell has to be able to regulate the speed of migration. Supporting this concept, it has been shown that the speed of integrin-mediated cell migration is highest at intermediate levels of adhesiveness, which is determined by a combination of different parameters such as ligand concentration, receptor density, and affinity, as well as the presence of a functionally active cytoplasmic receptor domain (Huttenlocher et al., 1996; Palecek et al., 1997). In addition, these studies from the Horwitz laboratory have shown that by varying the above parameters the maximum migration speed attainable remains unchanged (Palecek et al., 1997). Such findings are completely in line with our results on the substrate-dependent correlation of retrograde F-actin flow and advance rates of *Aplysia* growth cones (Lin and Forscher, 1995; Suter et al., 1998), where maximum growth rate on a permissive substrate was never found to exceed the retrograde flow rate on a nonpermissive substrate.

A very recent study on focal adhesion motility in stationary and migrating fibroblasts provides further

evidence that migration speed is regulated by a molecular clutch between extracellular substrates and the actin cytoskeleton (Smilenov et al., 1999). Using green fluorescent protein (GFP)-tagged β_1-integrin, Smilenov and coworkers showed that focal adhesions move retrogradely in association with actin fibers in stationary fibroblasts, whereas little focal adhesion movement was observed in migrating cells, suggesting a clutch-like mechanism between integrins and ECM proteins. Interestingly, again the rate of focal adhesion movement in stationary cells was similar to the rate of fibroblast cell migration. Taken together, all these studies suggest that the migration speed of cells and growth cones is most likely to be regulated at the clutch level (e.g., somewhere at the substrate–CAM–cytoskeleton interface).

Several parameters have been demonstrated to regulate CAM–cytoskeleton linkages involved in cell migration. These include ligand binding, receptor clustering, force, and tyrosine phosphorylation. Most of the corresponding studies have focused on integrin-mediated cell adhesion and migration of fibroblast-like cells. However, there is increasing evidence that the above parameters (e.g., clustering and tyrosine phosphorylation) also modulate cadherin and IgCAM linkages regulating growth cone motility. For example, apCAM–actin interactions depend on clustering density (Thompson et al., 1996). At lower levels of apCAM clustering, this IgCAM associates with retrograde flow, whereas at higher density it can promote *de novo* site-directed F-actin assembly.

Many studies addressing the functional interaction of integrin receptors with the actin cytoskeleton have used ligand-coated microbeads to study recruitment of focal adhesion proteins (Miyamoto et al., 1995) or to analyze biophysical parameters such as receptor localization and mobility (Schmidt et al., 1995), cytoskeletal coupling (Felsenfeld et al., 1996), and linkage strengthening (Choquet et al., 1997). Interestingly, there appears to be a hierarchy of events in the assembly of integrin–cytoskeletal complexes. An extensive study in fibroblasts revealed that cytoskeletal and signaling proteins can be grouped depending on their recruitment to fibronectin-coated beads in response to the following parameters: ligand occupancy, integrin clustering, and the presence of tyrosine phosphorylation activity (Miyamoto et al., 1995). Using beads coated with low levels of integrin antibodies, the addition of an RGD-containing peptide induced the coupling of such beads to the retrograde moving F-actin cytoskeleton (Felsenfeld et al., 1996). These findings indicated that ligand binding promotes the association of integrin receptors with the cytoskeleton. Furthermore, in both fibroblasts and neuronal growth cones, integrin linkages were found to be stronger at the leading edge when compared to positions closer to the cell body (Schmidt et al., 1993, 1995). In addition to ligand occupancy, receptor clustering, and localization, the application of external forces increases the strength of integrin–cytoskeleton interactions, suggesting that cells can sense and respond to the rigidity of the ECM (Choquet et al., 1997). Consistent with a role for force in regulating integrin–cytoskeletal linkages are the findings that contractility stimulated by the small GTPase Rho promotes focal adhesion assembly and integrin clustering (Burridge and Chrzanowska-Wodnicka, 1996; Chrzanowska-Wodnicka and Burridge, 1996).

An important regulator of CAM–cytoskeleton linkages not only for integrins but also cadherins and IgCAMs is tyrosine phosphorylation. Tyrosine phosphorylation events may occur on the level of the adhesion receptors (cytoplasmic domain of integrins, cadherins, and L1) but also of the cytoskeletal linkage (e.g., vinculin, paxillin, and catenin) and signaling proteins (e.g., FAK and $p130^{cas}$). Furthermore, tyrosine phosphorylation may either enhance or weaken receptor–cytoskeleton coupling, depending on the molecular interaction. In the case of focal adhesions, integrin clustering and ligand binding promotes an overall increase in tyrosine phosphorylation and inhibiting tyrosine kinase activity blocks focal adhesion formation (Miyamoto et al., 1995; Burridge and Chrzanowska-Wodnicka, 1996). However, details on the series of events involving tyrosine phosphorylation during focal adhesion assembly are not completely understood (Burridge and Chrzanowska-Wodnicka, 1996; Schoenwaelder and Burridge, 1999). Recent studies from our laboratory have shown increased phosphotyrosine levels at restrained apCAM–bead interaction sites but not around unrestrained beads (D. M. Suter and P. Forscher, unpublished results). Furthermore, the application of the tyrosine kinase inhibitor genistein blocked restrained bead interactions without completely inhibiting weaker apCAM–actin interactions required for retrograde bead translocation. In summary, these observations suggest that total phosphotyrosine levels are generally higher at sites where strong apCAM–actin linkages occur.

Specific tyrosine phosphorylation events have also been found to uncouple receptor-linkage protein interactions in the case of all three families of CAMs. Tyrosine phosphorylation of β-catenin was found to result in reduced cadherin-mediated cell adhesion (Matsuyoshi et al., 1992). Similarly, it was demonstrated that tyrosine phosphorylation of the cytoplasmic tail of neurofascin at a site highly conserved among members of the L1-family abolished the bind-

ing of neurofascin to ankyrin and resulted in an increased lateral mobility of neurofascin (Garver et al., 1997). Finally, the nonreceptor tyrosine kinase Src has been shown to negatively regulate the strengthening of linkages between the cytoskeleton and the integrin vitronectin, but not fibronectin receptor (Felsenfeld et al., 1999).

CONCLUDING REMARKS

Functional studies with cell adhesion proteins from different structural families suggest substrate–cytoskeletal coupling as a mechanism for regulating growth cone and cell motility. Furthermore, many reports provide evidence that an important site for the regulation of forward movement is the receptor–actin as opposed to the myosin–actin interface. Besides acting as force transducing agents, cell adhesion receptors may also promote growth by stimulating *de novo* F-actin assembly, as it has been shown in the case of apCAM (Thompson et al., 1996). However, increased F-actin assembly rates can only lead to enhanced long-term growth rates, if the whole peripheral actin domain shifts forward (e.g., by coupling to a permissive substrate). With respect to receptors regulating F-actin assembly, an interesting connection from the membrane to the cytoskeleton has been recently established for *Drosophila* motor axon guidance. Genetic and biochemical studies have revealed that the receptor phosphatase Dlar may transmit a guidance signal to the actin cytoskeleton via interaction with Ena, which in turn regulates actin assembly via interaction with profilin (Wills et al., 1999a,b). Furthermore, both Ena and Dlar appear to be negatively regulated by the nonreceptor tyrosine kinase abl (Wills et al., 1999a).

Many intriguing questions remain with respect to mechanisms of substrate–cytoskeletal coupling in growth cone motility and guidance; for example, (1) What is the detailed series of events during the establishment of a functional receptor–cytoskeleton linkage? (2) How are signaling complexes recruited to growth cone target interaction sites? (3) Which myosins produce force during substrate-mediated growth and where are the myosins localized? (4) How does force affect the strength of CAM–cytoskeletal coupling linkages? (5) What is the mechanism of microtubule extension during directed neuronal growth? The rapidly evolving power of molecular and live cell imaging techniques will be important to approach these interesting questions in the future.

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