Microtubule and Rac 1-dependent F-actin in growth cones

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Summary

Extracellular cues control the rate and direction of growth of neuronal processes in large part by regulating the cytoskeleton of the growth cone. The actin filament network of the peripheral region is thought to be the primary target for these cues, with consequences for the advance and organization of microtubules. Binding of laminin to integrin receptors is a cue that accelerates the growth of processes from many types of neurons. It was applied acutely to sympathetic neurons in culture to study its effects on the cytoskeleton of the growth cone. Microtubules advance to the edge of the growth cone and bundle in response to laminin, and it was found that small veils of membrane appear near the ends of some of those microtubules. To examine more clearly the relationship between the microtubules and the appearance of actin-rich structures at the periphery, a low dose of cytochalasin D was used to deplete the peripheral region of the growth cone of pre-existing F-actin. The subsequent addition of laminin resulted in the bundling of ends of dynamic (tyrosinated) microtubules at the distal edge of the growth cone, most of which were associated with foci of F-actin. Observations of labeled actin within living growth cones confirmed that these foci formed in response to laminin. Suppression of microtubule dynamics with drugs eliminated the actin foci; washout of drug restored them. Rac 1 did not co-concentrate with F-actin in the peripheral region of the growth cone in the absence of laminin, but did co-concentrate with the foci of F-actin that formed in response to laminin. Inhibition of Rac 1 functioning prevented the formation of the foci and also inhibited laminin-induced neurite growth with or without cytochalasin. These results indicate that extracellular cues can affect actin in the growth cone via microtubules, as well as affect microtubules via actin. They also point to the mediation of microtubule-dependent accumulation of F-actin at the front of the growth cone as a role of Rac 1 in neurite growth.

Key words: Growth cone, Microtubules, F-actin, Rac 1

Introduction

The growth cone is the specialized ending of a growing neuronal process. Its activity and interaction with environmental cues control the rate and direction of neurite outgrowth. The peripheral (P) region of the growth cone is rich in actin filaments (Letourneau, 1983; Tosney and Wessells, 1983; Lewis and Bridgman, 1992). Longitudinally arrayed microtubules, comprising the core of the neurite, are the other major cytoskeletal elements of the growth cone. The actin is thought to play the leading role in elongating the neurite: coupling of the peripheral actin network of the growth cone to the substrate is thought to facilitate the advance of microtubules and other cytoplasmic constituents (Lin and Forscher, 1995; Heidemann, 1996). The peripheral actin network is also thought to be the primary target for environmental cues (Challacombe et al., 1996; Suter and Forscher, 1998; Dickson, 2001; Song and Poo, 2001). For example, certain inhibitory cues cause a rapid loss of peripheral F-actin, resulting in collapse of the P region and, sometimes, retraction of the microtubule-rich neurite (Fan et al., 1993; Jurney et al., 2002). Coupling of substrate-bound stimulatory cues to the motile peripheral F-actin facilitates the advance of microtubules (Suter et al., 1998). Yet, there is evidence in growth cones and in non-neuronal motile cells of interdependence of actin and microtubules. Actin-rich digitate 'intrapodia' are apparently stimulated by microtubules to grow from the dorsal surface of the growth cone (Rochlin et al., 1999) and interdependence of actin filaments and microtubules at axon branch points has been described (Dent and Kalil, 2001; Gibney and Zheng, 2003). Actin polymerization seems to be stimulated by the ends of growing microtubules in motile fibroblasts (Waterman-Storer et al., 1999).

Laminin-1 elicits rapid growth from many types of neurons in culture (Lander, 1987) and fosters the differentiation of axons (Lein et al., 1992). Laminins are probably also important for the growth of axons in vivo, when present either on the surfaces of cells along which the axons grow or as components of the extracellular matrix (Bixby et al., 1988; Werner et al., 2000). Laminins are major constituents of the basal lamina that surrounds the axons of a peripheral nerve; retention of this lamina after injury is probably important for successful regeneration (Ide, 1996). Laminin-1 (hereafter laminin) promotes growth mainly by binding to integrins (Tomaselli et al., 1990; Rivas et al., 1992). Processes on laminin not only grow rapidly, but also tend to be thin with more tightly aligned microtubules. Reorganization of microtubules occurs within minutes of exposure of a growth cone to laminin (Burden-Gulley and Lemmon, 1996; Tang and Goldberg, 2000). We

report here that at least some of the effect of laminin on actin in the growth cone is mediated by these microtubules. This indicates that extracellular cues might affect actin in the growth cone via microtubules, as well as affect microtubules via actin. We also find that this effect is mediated by Rac 1, which might be one pathway by which this signaling molecule could contribute to neurite growth.

Materials and Methods

Materials

Fibronectin from bovine plasma, cytochalasin D, taxol (paclitaxel), nocodazole, tubulin mAb (JDR8) and dimethylpolysiloxane were obtained from Sigma Chemical (St Louis, MO). Laminin 1 (from mouse EHS sarcoma) was from Roche Diagnostics (Basel, Switzerland), nerve growth factor (NGF) from Alomone Labs (Jeruselam, Israel), oxyrase from Bioworld (Dublin, OH) and Alexa-Fluor594-phalloidin, Alexa-Fluor488-actin and Alexa-Fluor-conjugated secondary antibodies from Molecular Probes (Eugene, OR). Rac 1, Rho and cdc42 mAbs were from BD Transduction Laboratories (San Diego, CA), and wild-type (wt) Rac 1-GST fusion protein and dominant-negative (N17) Rac 1-GST fusion protein were from Cytoskeleton (Denver, CO). G2 anti-actin pAb was a generous gift from J. C. Bulinski (Columbia University). W2 pAb against tyrosinated α-tubulin was a generous gift from G. Gundersen (Columbia University).

Cell culture

Sympathetic ganglia were dissected from E10-E13 chick embryos, washed in Hank's buffer, and dissociated by using 0.25% trypsin for 10 minutes at 37°C. After pelleting, cells were plated onto coverslips glued to the bottom of dishes with a hole cut in them. Coverslips were prepared by treatment with concentrated HCl, rinsed and then coated with polyamine (a mixture of poly-D-lysine and poly-L-ornithine). Coverslips were preincubated with RPMI medium containing 4% heat-inactivated horse serum for 2 hours at 37°C, and cells were incubated overnight in L15 medium supplemented with 2% fetal bovine serum and 4% heat-inactivated horse serum, 0.2 mg/ml glutamine and 50 ng/ml NGF. For most experiments, neurons were cultured overnight in the presence of serum and then replated in serum-free defined medium (L15 supplemented with 2% BSA, 0.2 mg/ml glutamine, 10 μg/ml transferrin, 5 μg/ml insulin, 5 ng/ml sodium selenite, 100 mM sodium pyruvate) and 50 ng/ml NGF. Cells were triturated with a fire-polished pipette and plated onto either a polyamine substrate, or a polyamine substrate coated with either fibronectin or laminin. Coating of extracellular matrix proteins was achieved by incubation of each at a concentration of 20 mg/ml in L15 medium for 30 minutes at 37°C. For the microinjection experiments, cells were cultured overnight as above and then washed several times with serum-free defined medium and 50 ng/ml NGF.

Immunocytochemistry

Two fixation procedures were used. For tubulin and actin staining (without GTPases), cells were fixed by a procedure developed previously (Lewis and Bridgman, 1996). Cultures were treated with 5 ml of cacodylate buffer (134 mM sodium cacodylate, 10 mM $MgCl_2$, 5 mM $CaCl_2$, pH 7.4) containing 0.25% glutaraldehyde for 5 minutes, followed by three rinses with buffer and then permeabilization by three 5 minute treatments with 0.5% Triton X-100 in PBS. For experiments involving GTPase staining, cells were fixed by treatment with PBS containing 4% paraformaldehyde, 250 mM sucrose and 0.1% glutaraldehyde for just 30 seconds at 37°C, followed by one rinse and three 5 minute washes in PBS containing a reduced concentration (0.15%) of Triton X-100. For triple stains, Alexa-Fluor594-phalloidin staining for F-actin was carried out by incubation in a 1:40 dilution in PBS containing 1% normal goat serum for 15 minutes after the appropriate fixation and permeabilization. After observation and recording (see below), phalloidin staining was quenched by incubation in 1% OsO_4 for 5 minutes at 4°C followed by seven washes in PBS. Preparations were then reduced by incubation in 5% β-mercaptoethanol in PBS for 30 minutes at room temperature (rt) followed by seven washes in PBS (Lewis and Bridgman, 1996) before incubation with primary antibodies.

Nonspecific binding sites were blocked by incubation in 10% normal goat serum (NGS) in PBS for 30 minutes at rt. Preparations were then incubated in primary antibody diluted in PBS/1% NGS, 0.015% Triton X-100. Incubation times were either 1 hour at rt or overnight at 4°C. For double staining, primary antibodies were applied consecutively. Cells were then washed in PBS and incubated in Alexa-Fluor-conjugated secondary antibody diluted 1:1000 in PBS/1% NGS for 1 hour at rt. After a final wash in PBS, cells were mounted in 20 mg/ml propyl gallate in 90% glycerol/10% PBS. Stains were visualized using a Nikon TE 200 microscope with a 40× neofluor/1.3 NA objective and a 100× plan Apo/1.4 NA objective. Images were captured using a cooled CCD camera (see below).

Microinjection

Dishes of cultured neurons containing 5 ml of serum-free medium were placed at rt on the motorized stage of a Nikon TE 200 microscope and cells were located using differential interference contrast (DIC) optics with a 40× neofluor/1.3 objective. Metamorph software (Universal Imaging, West Chester, PA) was used for cell location. Alexa-Fluor488-actin (3-5 mg/ml) in 5 mM Tris (pH 8.1), 0.2 mM $CaCl_2$, 0.2 mM dithiothreitol, 0.2 mM ATP or wt and dominant-negative Rac 1 fusion proteins (1 mg/ml) in 2 mM Tris (pH 7.5), 0.4 mM $MgCl_2$, 0.4% sucrose, 0.1% dextran, 0.25 mM rhodamine-dextran, were back loaded into Eppendorf femto II tips (Brinkman, Westbury, NY) and attached to a hydraulic micromanipulator (Narishige, Tokyo, Japan). Injection pressure was set at 1-2 PSI using a femtojet picospritzer (Eppendorf) to produce a steady stream of injectant and then cells were injected manually by quickly piercing the cell soma (0.1-0.2 seconds). Cultures were placed into an incubator at 37°C for 1 hour to recover. Cells that were labeled were detected by visualizing either fluorescent actin or fluorescent dextran; of these, 40-50% were viable as demonstrated by normal morphology and dynamics.

Image acquisition and analysis

Dishes were placed back onto the motorized stage heated to 37°C. Oxyrase (1/100, to lower free oxygen radicals, which are injurious to neurons) was added to the medium and 1 ml of dimethylpolysiloxane was layered on top to prevent cooling, evaporation and excess oxygenation. Cells were observed with a 40× neofluor/1.3 NA objective and images captured using a cooled CCD camera (Princeton Instruments, Trenton, NJ). Frequency of imaging varied with experimental demands. Time-lapse recording was achieved by capturing DIC images at 10 second intervals. Epifluorescence images were acquired with 500-1000 msecond exposures as controlled by a programmable Uniblitz shutter (Vincent Associates, Rochester, NY). Epifluorescence illumination was reduced by filtration through neutral density filters, and the images collected were binned to allow reduced illumination.

Analyses were carried out on digitized images using Metamorph software. To determine the presence of microtubule accumulations and F-actin foci, the following criteria were applied: (1) a staining intensity greater than 15% of the total gray scale range above the background-subtracted basal level, and (2) an area greater than 1 μm. All statistical tests of significance were carried out using the Student's *t* test or, for multiple comparisons, ANOVA with Bonferroni's modified *t* test.

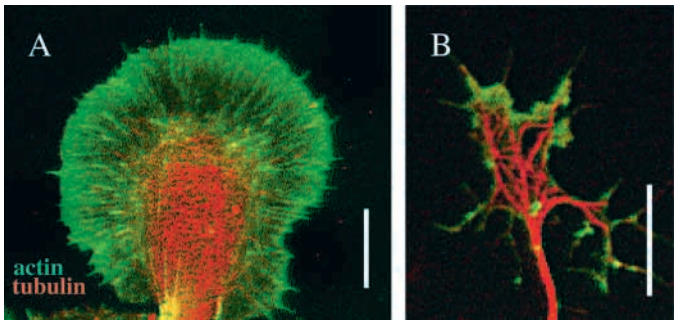

Fig. 1. Morphology and cytoskeleton of growth cones on different substrates. (A) A large growth cone on a polyamine substrate has a peripheral ribbed F-actin (green) network with microtubules (red) occupying the central region. (B) Growth cones on a laminin substrate are smaller and microtubules penetrate closer to the leading edge. Bars, 10 μm.

Results

Neurites that emerge from sympathetic neurons in culture in defined, serum-free medium on a substrate coated with polyamine or fibronectin often have a large growth cone, with F-actin filling a broad P region and microtubules largely confined to the central (C) region several microns back from the edge (Fig. 1A). By contrast, neurites on a substrate coated with laminin typically have small growth cones with microtubules projecting close to the distal edge (Fig. 1B). In the first several hours after placement of the cells in culture, neurite growth on laminin is about threefold and tenfold faster than on fibronectin and polyamine, respectively. Effects of laminin on the cytoskeleton can be conveniently studied by adding it to the culture medium of cells on a polyamine or fibronectin substrate (Chamak and Prochiantz, 1989; Rivas et al., 1992; DiTella et al., 1996; Kuhn et al., 1998); acute addition to the culture medium is the standard technique for studying the effects of inhibitory molecules on the growth cone (Fan et al., 1993; Kuhn et al., 1999; Jurney et al., 2002). When so added, laminin rapidly sticks to the substrate and affects the growth cone. Most or all of the effects are due to substrate-bound laminin because, if the substrate is pre-coated with large amounts of laminin that is then inactivated by UV irradiation (Hammarback et al., 1988), the growth cones of subsequently cultured cells do not respond to new laminin added to the medium (data not shown).

Acute addition of laminin to neurons causes, within 20 minutes, microtubules to advance to the edge of the growth cone and to bundle (Rivas et al., 1992; Tang and Goldberg, 2000). We found that, after microtubules and organelles had reached the edge of the growth cone, veils of membrane frequently appeared at the edge, often on the shafts of filopodia that were thickening with the entry of microtubules and organelles, and thin neurites with small growth cones then advanced rapidly (Fig. 2). These features of advance were also seen when cells were cultured on a substrate pre-coated with laminin rather than responding to laminin added acutely. Outgrowth on a pre-coated laminin substrate was often accompanied by ‘splashes’ of veil at the distal ends of fused filopodia or along the sides of filopodia. The frequency of veils on filopodial shafts was found to be almost threefold higher on laminin (4.1±0.5 veils/10 minutes, n=19 growth cones, total observation time=300 minutes) than on fibronectin (1.4±0.3 veils/10 minutes, n=18 growth cones, total observation time=187 minutes). The percentage of filopodia per growth cone that contained microtubules was sixfold higher on laminin than on fibronectin (18% versus 3%, n=112 growth cones).

The close association of veils with the ends of microtubules in the presence of laminin suggested the possibility of a causal relationship. There is evidence of stimulation of actin polymerization by the ends of microtubules in both growth cones and motile fibroblasts (Rochlin et al., 1999; Waterman-Storer et al., 1999; Dent and Kalil, 2001). In fibroblasts, this appears to be mediated by the monomeric GTPase Rac (Waterman-Storer et al., 1999), which promotes the formation of meshworks of F-actin (Hall, 1994), such as found in veils. We therefore assessed by immunocytochemistry the distribution of Rac 1 in the absence or presence of laminin. Rac 1 did not noticeably colocalize with F-actin in the P region of large growth cones on fibronectin or polyamine (Fig. 3A,B). Some colocalization was seen in concentrations in the C region of the growth cone, which is heavily populated with microtubules. By contrast, numerous growth cones on a laminin substrate had peripheral concentrations of Rac (Fig. 3C,D). The ratio of Rac 1 to F-actin in growth cones on a laminin substrate was fourfold higher than on fibronectin (n [laminin]=74; n [fibronectin]=30). Furthermore, peripheral concentrations of Rac 1 were often associated with

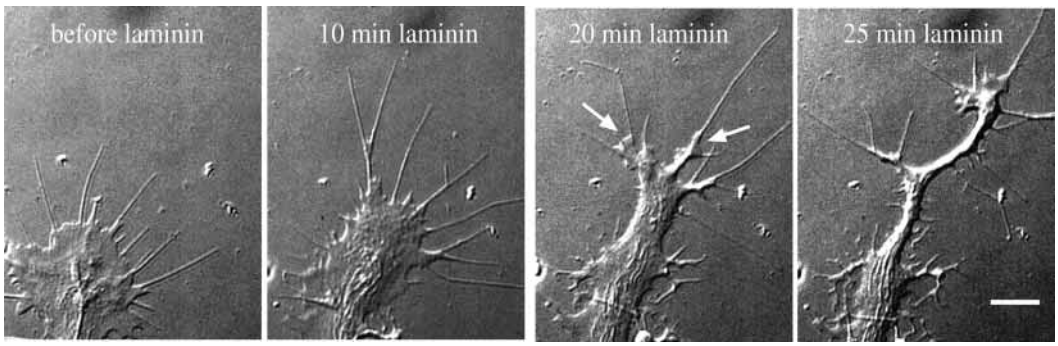

Fig. 2. Induction of rapid growth by the acute addition of laminin. DIC recording of growth from a neuron plated on a fibronectin substrate that was then exposed to laminin. By 20 minutes after the addition of laminin, veils of membrane have appeared on the thickened shafts of filopodia (arrows). By 25 minutes, the large-spread growth cone has given rise to a thin process with a smaller growth cone. Bar, 10 μm.

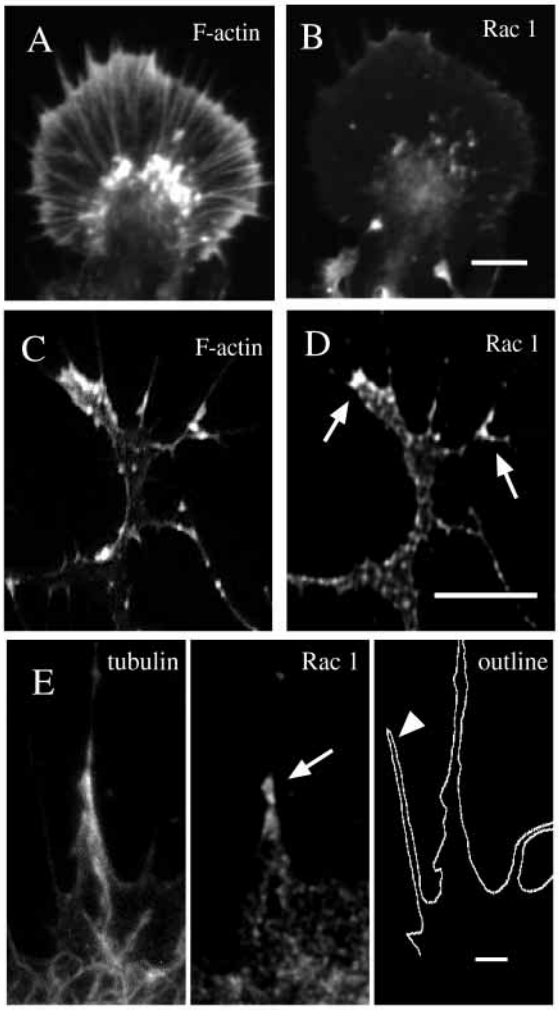

Fig. 3. Distribution of Rac 1. (A,B) Localization of Rac 1 in a growth cone cultured on a polyamine substrate. There is an extensive ribbed network of F-actin (A). Rac 1 in the same growth cone does not substantially localize to this peripheral network, but some colocalization is seen in the C region of the growth cone (B). Bar, 10 μm. (C,D) On a laminin substrate, Rac 1 is often colocalized with F-actin at the leading edge of the growth cone (arrows). Bar, 10 μm. (E) Detail of a part of a growth cone on a polyamine substrate that was acutely exposed to laminin. At the site of outgrowth of a streamlined growth cone, microtubules advance into a filopodium and colocalize with Rac 1 (arrow). A filopodium lacking microtubules does not contain Rac 1 (arrowhead). Bar, 2 μm.

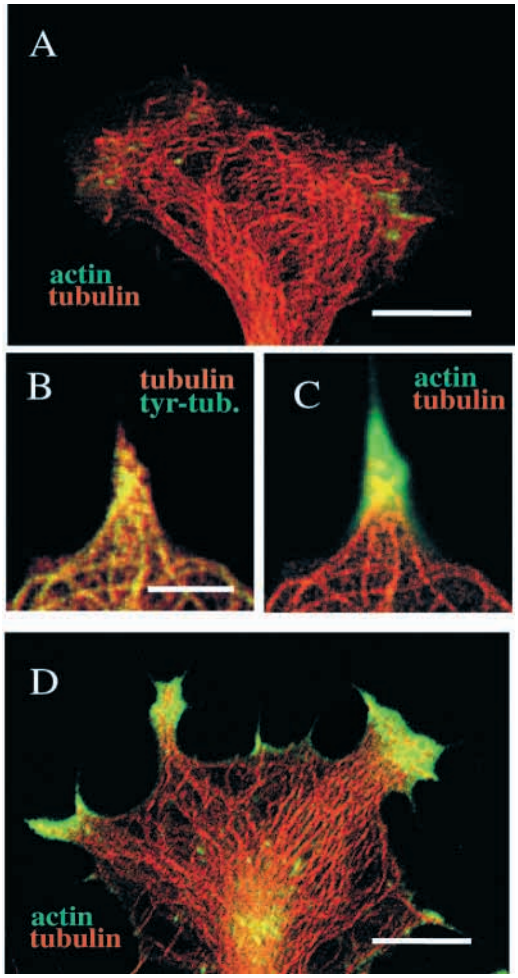

Fig. 4. Bundled dynamic microtubules associate with F-actin during outgrowth in the presence of CD. (A) After treatment with 125 nM CD for 20 minutes, a growth cone on a polyamine substrate shows an almost complete loss of F-actin (green), with microtubules (red) filling the growth cone. Bar, 10 μm. (B-D) 25 minutes after the addition of laminin to such a growth cone, microtubules have bundled at peripheral sites where protrusions form and then develop into neurites. (B) Merged image of tyrosinated tubulin (green) and total tubulin (red) in a protrusion shows an enrichment of tyrosinated tubulin. Bar, 2 μm. (C) An F-actin focus (green) is intimately associated with an accumulation of microtubules (red). Bar, 2 μm. (D) All outgrowths show F-actin foci associated with bundling microtubules. Bar, 10 μm.

microtubules that had advanced deep into the peripheral region either in growth cones growing on a laminin substrate or in growth cones that had been treated acutely with laminin (Fig. 3E).

We wanted to examine more clearly the relationship between effects of laminin on microtubules and the formation of peripheral actin-rich structures. We were able to do so by using cytochalasin D (CD). Low concentrations of CD inhibit the polymerization of actin by binding to the barbed end of the actin filament. Cytochalasins rapidly deplete F-actin from the P region of the growth cone and allow microtubules to advance to the edge of the growth cone (Forscher and Smith, 1988). We found that 125 nM CD did this to growth cones on polyamine or fibronectin (Fig. 4A) and completely halted neurite growth. Thus, pre-treatment with the low dose of CD had the advantage of removing most of the pre-existing F-actin from the P region and thereby allowing the appearance of new actin to be more clearly visualized. When laminin was added to these growth cones, microtubule ends bundled at the edge of the growth cone (Fig. 4B,D); we had previously found that laminin could stimulate bundling of microtubules in the presence of CD (Tang and Goldberg, 2000). Laminin increased the number of peripheral microtubule accumulations per growth cone (see Materials and Methods for criteria for accumulations) by more than threefold, from 0.64±0.13 (n=50) before the addition of laminin to 2.16±0.28 (n=42; P<0.00001) 25 minutes after

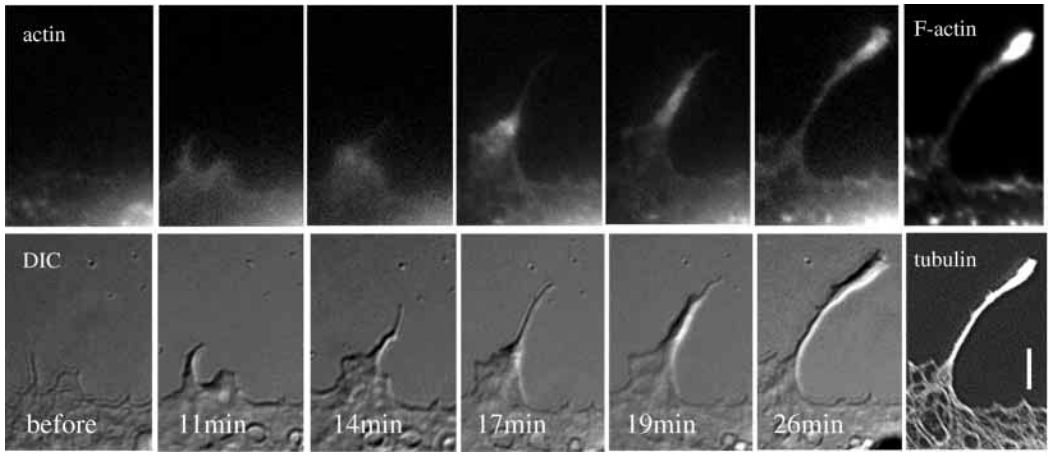

Fig. 5. Emergence of a process induced by the acute addition of laminin in the presence of CD is associated with actin. A growth cone labeled with fluorescent actin and growing on a polyamine substrate was treated with 125 nM CD for 15 minutes before exposure to laminin. Shown is a portion of the growth cone just before, and at indicated times after, the addition of laminin. Fluorescent actin (top row) and DIC images (bottom row) show the appearance of protrusions, which fuse and sprout a single filopodium. An intense focus of actin precedes a rapid elongation of the new process. Last panels show F-actin (phalloidin stain) and tubulin (immunocytochemical stain) in the same structure that was fixed at 27 minutes. Live actin staining is similar to F-actin staining. Tubulin is present and bundled in the shaft. Bar, 5 μm.

laminin. Fibronectin did not significantly increase the number of microtubule accumulations (0.75±0.17 [*n*=33]). (The responses to laminin of growth cones on polyamine and fibronectin substrates were similar. In this and some other sets of experiments, cells were cultured on polyamine so that fibronectin could be added acutely as a control for acute addition of laminin.) These bundled microtubule ends were enriched in tyrosinated α-tubulin, which marks more dynamic microtubules (Fig. 4B). They typically invested membrane protrusions that formed in response to laminin. These protrusions developed into neurites that grew out; that is, laminin restarted neurite growth in the presence of CD (see below).

Most (83%) of the peripheral microtubule accumulations were intimately associated with foci of F-actin; F-actin sometimes appeared to encircle the end of the microtubule accumulation (Fig. 4C,D). There was an increase in the number of F-actin foci per growth cone from 0.68±0.15 (*n*=38 growth cones) on a polyamine substrate to 2.35±0.36 (*n*=44; significantly different from control, $P<0.00001$) after addition of laminin. Since only about half of the growth cones respond to laminin (with or without CD present), this value is probably an underestimate of the strength of the effect. Fibronectin added instead of laminin did not induce the formation of F-actin foci (0.71±0.17, *n*=55).

We examined the formation of these foci by injecting fluorescent G-actin into neurons, which were then exposed to 125 nM CD. Fig. 5 shows the emergence of a process in response to the addition of laminin. Before the addition of laminin, there is hardly any detectable actin in the region from which a neurite later emerges. By 11 minutes after the addition of laminin, diffuse actin has appeared in membrane protrusions. These protrusions fuse to form one structure from which sprouts a filopodium. By 17 minutes after the addition of laminin, actin is highly concentrated at the base of the filopodium; this precedes a rapid saltatory elongation of the process (17-26 minutes). Four such process outgrowths were observed. Although residual and transient patches of actin were sometimes seen in growth cones treated with CD, all observed growth cones showed the appearance of new actin foci at places

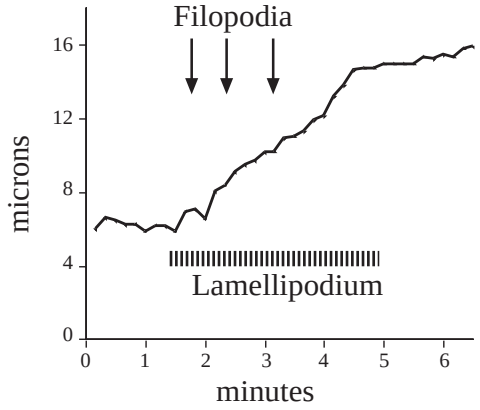

Fig. 6. Outgrowth in the presence of CD and laminin is correlated with the presence of actin-based protrusions. The advance of a single process during a spurt of growth was recorded live by DIC video microscopy. The advance and appearance of actin-based structures were quantitated. A small area of lamellipodium appears immediately prior to advance and is present throughout advance. Single filopodia (three in all) extend during the growth spurt.

at the periphery of the growth cone from which outgrowths would emerge. These foci intensified immediately before outgrowth. Subsequent growth in the presence of CD was discontinuous, but was rapid and always coincided with the presence of a filopodium or small lamellipodium at the tip of the process (Fig. 6); that is, growth proceeded only when there was an actin-based protrusion.

Thus, laminin not only caused microtubule ends to bundle, but it stimulated the formation of intense foci of F-actin in the presence of CD. These foci were not only closely associated with the microtubules, but were dependent on them for their formation. We used three drugs that all reduce microtubule dynamics, but probably by different mechanisms (Jordan and Wilson, 1998; Goold et al., 1999). Nocodazole (33 nM), taxol (30 nM), and Li^+ (20 mM, with 20 mM myoinositol to prevent depletion of inositol in the cell) were added to separate cultures that had been treated with CD and then induced to form

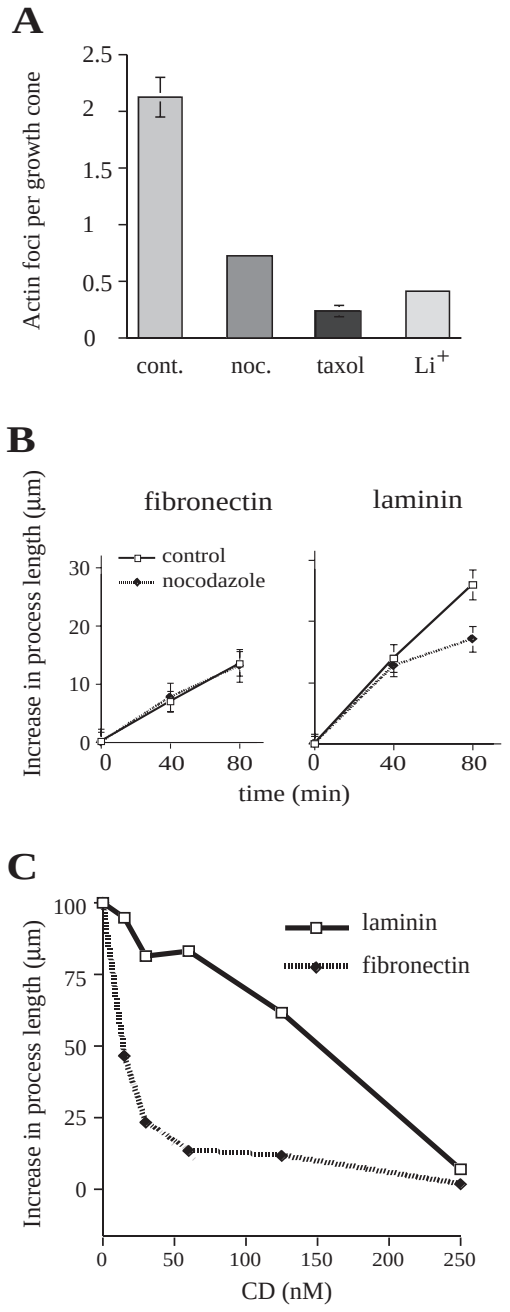

Fig. 7. Microtubule dynamics are necessary for F-actin foci and rapid outgrowth on laminin. (A) Suppression of laminin-induced F-actin foci by nocodazole, taxol and Li^+. Growth cones were induced to form F-actin foci by treatment with CD for 15 minutes followed by laminin for 30 minutes. Each drug was then added to cultures for 15 minutes before fixation and staining for F-actin. All three drugs significantly (P<0.00001) reduced the frequency of foci. Error bars for nocodazole and Li^+ are too small to see. (B) Nocodazole inhibits the rapid growth of processes on laminin. Neurons were plated onto laminin or fibronectin and then exposed to 33 nM nocodazole. Recordings of the same growth cones over time were made to determine the increase in process length. Only the rapid outgrowth on laminin is substantially inhibited by this low concentration of nocodazole. (C) Dose-response curves for process outgrowth from neurons plated onto laminin or fibronectin and then exposed to CD. Cells were treated with CD for either 4 hours (fibronectin) or 2 hours (laminin) and the increase in process length measured. Error (s.e.m.) bars are too small to see. Results are representative of three separate experiments.

processes by treatment with laminin for 25 minutes. Each drug greatly reduced the number of actin foci (Fig. 7A). Growth cones appeared similar to those treated with CD alone (flat, without many actin foci, associated microtubule bundles or nascent outgrowths), indicating that reducing the dynamics of microtubules reversed the effects of laminin. Removal of nocodazole (in the maintained presence of CD) resulted in the reappearance of F-actin foci at the periphery. The number of F-actin foci per growth cone increased from 0.32±0.06 (n=144) to 1.55±0.16 (n=137; P<0.0001), indicating that changing the activity or organization of microtubules is sufficient to induce the formation of F-actin foci in the presence of CD and laminin.

The foregoing results demonstrate that laminin reduces the ability of CD to inhibit actin polymerization in the growth cone, and does so by a microtubule-dependent mechanism. If this microtubule-dependent actin contributes to normal neurite growth on laminin, the low concentration of nocodazole that prevents formation of the actin foci in the presence of CD should also inhibit neurite growth in its absence. This was indeed the case: neurite growth on laminin, but not on fibronectin, was slowed by nocodazole (Fig. 7B). One would also expect that neurite growth on a pre-coated laminin substrate is less sensitive to inhibition by CD than is growth on a fibronectin substrate. This also was true: a 50% decrease in growth rate required about a tenfold higher concentration of CD on laminin (150 nM) than on fibronectin (15 nM) (Fig. 7C).

Experiments in the presence of CD additionally provided clear evidence that Rac 1 mediates the microtubule-dependent formation of F-actin foci. Rac 1 did not noticeably colocalize with the dense network of F-actin in the P region of growth cones in the absence of laminin and CD (Fig. 3A). Addition of CD alone did not result in the appearance of new concentrations of Rac 1 (data not shown). However, the subsequent addition of laminin resulted in the appearance of peripheral accumulations of Rac 1 colocalized with the foci of F-actin associated with microtubule accumulations (Fig. 8A). By contrast, Rho and cdc42 showed only a slightly increased concentration in only a few of the new F-actin foci (Fig. 8A). Staining of Rac 1 together with microtubules shows the close relationship between bundled microtubules and Rac 1 at the sites of outgrowth (Fig. 8B).

We examined the importance of Rac 1 to the effects of laminin by microinjecting wt Rac 1-GST fusion protein or dominant-negative (N17) Rac 1-GST fusion protein into neurons that were then exposed to CD and laminin. Vehicle solution of buffer and fluorescein dextran marker was either injected alone or with wt or dominant-negative Rac 1 (1 mg/ml) into cells growing on a polyamine substrate. After a 30-40 minute recovery period, cells were screened for viability (normal appearance for these culture conditions) and the presence of dextran marker. Loading was estimated at 10% of the cell volume, though some variation in fluorescence intensity was observed. Cells were then treated with 125 nM CD for 15 minutes followed by laminin for 1 hour. The number

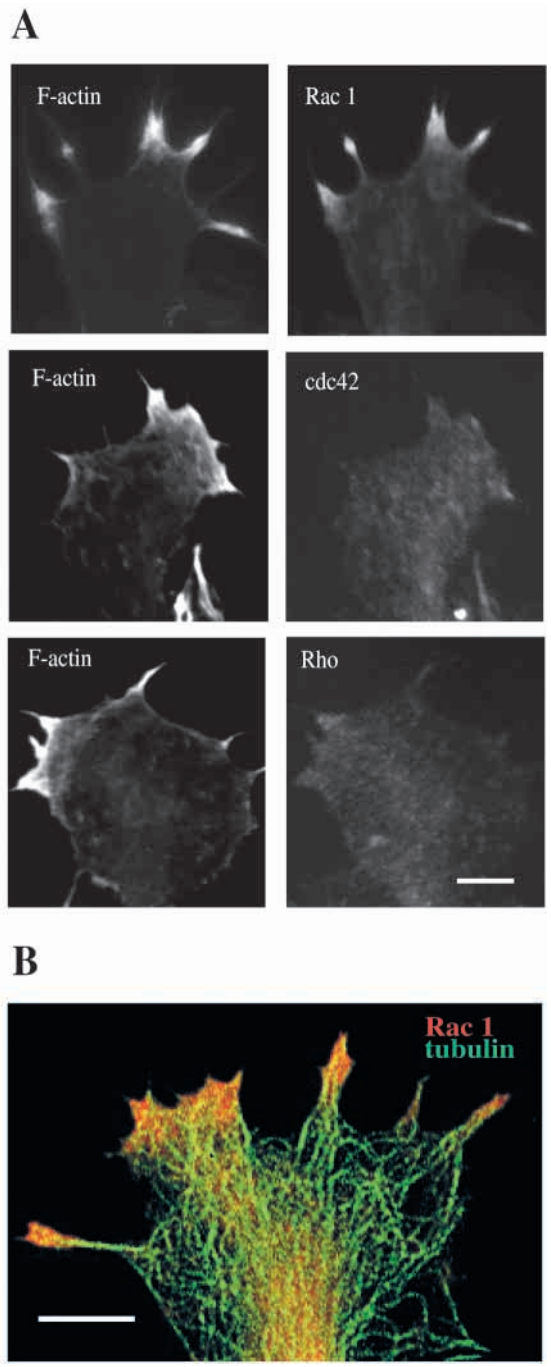

Fig. 8. Association of Rac 1 with laminin-induced outgrowth in the presence of CD. (A) Localization of Rho family proteins in growth cones treated with CD for 15 minutes followed by laminin for 25 minutes. Cultures were fixed and double stained for each GTPase and actin with either phalloidin and antibody to Rac 1, or an antibody to actin together with antibodies to Rho and cdc42. Only Rac 1 co-concentrates with actin. Bar, 10 μm. (B) Rac 1 is intimately associated with bundled microtubules. Rac 1 (red) colocalizes with bundled microtubules (tubulin, green) at the leading edge of neurites induced by laminin. Bar, 10 μm.

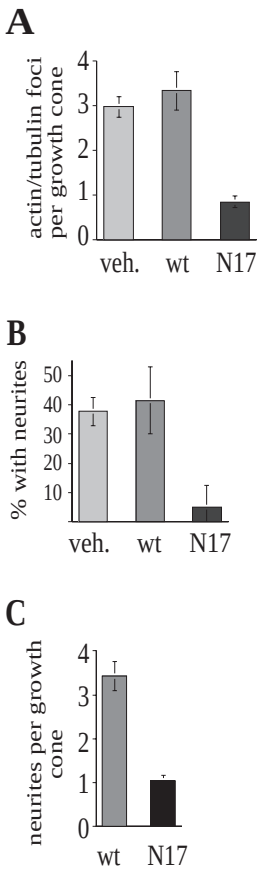

Fig. 9. Effects of laminin are dependent on Rac 1. Cells growing on a polyamine substrate were injected with either vehicle (veh.), wild-type Rac 1 (wt) or dominant-negative Rac 1 (N17). After determining which cells were alive and labeled, CD was added to the cultures for 15 minutes followed by treatment with laminin for 1 hour. CD was then washed out of the cultures and further observations made after 1 hour. (A) 25 minutes after the addition of laminin, F-actin foci have formed in cells injected with wt Rac 1 but typically not in cells injected with N17 Rac 1. (B) 1 hour after the addition of laminin, N17 Rac 1 has also inhibited neurite outgrowth. (C) After washout of CD, N17 Rac 1 continues to inhibit outgrowth compared to the wt control.

of F-actin foci with associated microtubule bundles, as determined after cell fixation, was reduced by dominant-negative Rac 1 to less than a third of the number seen with vehicle or wt Rac 1 (Fig. 9A). Also, dominant-negative Rac 1 inhibited outgrowth compared with wt Rac 1 or vehicle alone: the percentage of growth cones giving rise to outgrowths longer than 5 μm was reduced almost tenfold from that seen with vehicle or wt protein (Fig. 9B).

To confirm the dependence of laminin-induced outgrowth on Rac 1, we removed the CD from cultures of microinjected cells in order to examine the effect of dominant-negative Rac 1 in the absence of the F-actin-disrupting drug. When CD is removed from these sympathetic neuron cultures, the growth cones typically recover their network of F-actin completely. The nature of the restored F-actin depends on the culture conditions; in cells that had previously been exposed to laminin, we expected the nascent outgrowths to transform into the rapidly extending streamlined processes seen in the absence of CD. This was indeed the case for cells injected with vehicle or wt Rac 1; we observed an average of nearly four outgrowths per growth cone (Fig. 9C). In the case of cells injected with the dominant-negative Rac 1, the growth cones that recovered actin-based structures (filopodia and sometimes lamellipodia) showed a 77% reduction in the frequency of rapidly extending processes. Thus, Rac 1 is necessary for the formation of the microtubule-associated F-actin that facilitates rapid process outgrowth.

Discussion

We show here that an important extracellular cue, laminin, can regulate actin in the growth cone through a microtubule-

dependent mechanism. This effect on actin is mediated by the small GTPase, Rac 1. Rac/microtubule-dependent actin polymerization can contribute to neurite growth.

Laminin affects F-actin by a microtubule- and Rac 1-dependent mechanism

It is known that extracellular cues that regulate the rate and direction of neurite growth act on the growth cone, but the intracellular mechanisms of action are only beginning to be delineated. The cytoskeleton is thought to be an important target, particularly the actin filaments of the P region. Semaphorin 3A and ephrin A2 inhibit growth by triggering a loss of F-actin from the P region of the growth cone, resulting in its collapse (Fan et al., 1993; Jurney et al., 2002). The effect on the actin network seems, in the case of semaphorin 3A, to be mediated via LIM kinase and cofilin (Aizawa et al., 2001). Many inhibitory cues work by activating Rho, which is thought to target the actin network of the growth cone (Patel and Van Vactor, 2002). Substrate-bound ApCAM (an invertebrate homologue of vertebrate NCAM) facilitates the advance of the neurite by linking the substrate to the F-actin network that continually flows rearward in the P region (Suter et al., 1998). This induced linkage, which has also been demonstrated for extracellular matrix ligands that bind to integrin (Felsenfeld et al., 1996), facilitates the forward movement of microtubules and, thereby, the growth of the neurite. In fact, this coupling of the peripheral motile F-actin network to the substrate has been suggested as a basic mechanism for neurite growth (Mitchison and Kirschner, 1988; Lin and Forscher, 1995; Heidemann, 1996), similar to the crawling of motile non-neuronal cells.

We present here the first evidence of a role for microtubules in mediating an action of an extracellular cue on the F-actin of the growth cone. Laminin induces advance and bundling of microtubules, and actin-based protrusive structures (e.g. veils) form near the ends of the microtubules. We used a precise low dose of CD (125 nM, which inhibits growth on fibronectin but not on laminin) to reveal a cytoskeletal mechanism of growth specific to laminin. By eliminating most of the pre-existing F-actin in the P region, we were more clearly able to see laminin induce the de novo formation of foci of F-actin around the bundled ends of dynamic microtubules at the periphery of the growth cone. These foci disappeared if microtubule dynamics were suppressed by drugs and they reappeared upon drug washout, indicating that the foci not only depend on microtubules but are stimulated to form by them. The presence of microtubules at the distal end of the growth cone was not sufficient to induce foci of actin, as microtubules advanced to the edge without producing foci when CD (rather than laminin) was added to growth cones on polyamine or fibronectin. Changes induced by laminin were required. We favor the idea that a change in the organization or activity of the microtubules induced by laminin is critical. Alternatively, some other change caused by laminin creates conditions that allow dynamic microtubules to induce the formation of peripheral foci of F-actin. Whatever the mechanism, laminin was able to induce actin polymerization in the presence of CD. We do not know if the sensitivity to CD was altered and polymerization occurred by the barbed end assembly of F-actin or if laminin stimulates polymerization by another mechanism. In any case, we believe that this subset of F-actin in growth cones might contribute to the rapid growth on laminin in the absence of CD (see below).

The microtubule-dependent formation of foci of F-actin in the presence of laminin required Rac 1 activity. Rac 1 concentrated in the foci, whereas it did not co-concentrate with F-actin in the P region in the absence of laminin. Inhibition of Rac 1 by a dominant-negative construct greatly inhibited the formation of F-actin foci in response to laminin. Microtubule growth has been shown to activate Rac 1 and to induce the formation of actin-based protrusions at the periphery of motile fibroblasts by a Rac 1-dependent mechanism (Waterman-Storer et al., 1999). Active Rac 1 stimulates the formation of meshworks of F-actin, as in veils and lamellipodia (Hall, 1994). In the growth cone, the foci of F-actin were preferentially associated with dynamic microtubules. Thus, it seems simplest to suggest that microtubules elicit the foci by activating Rac 1. One attractive possibility is that laminin, perhaps through a signal transduction pathway, induces the bundling of the ends of dynamic microtubules, producing a focus of activated Rac 1 that, in turn, focally stimulates the accumulation of F-actin. Recently, Rac has been shown to be capable of regulating microtubules as well as actin. Rac causes phosphorylation (and, thus, inactivation) of the microtubule catastrophe factor stathmin, which could enhance polymerization or stabilization of microtubules (Daub et al., 2001). Also, Rac increases the interaction of IQGAP with CLIP-170, which binds to the polymerizing end of microtubules; this might capture microtubule ends at the plasma membrane (Fukata et al., 2002). Activation of integrin can recruit activated Rac to the plasma membrane (Del Pozo et al., 2000). Thus, another possibility is that binding of laminin to integrin recruits activated Rac 1 to the membrane, which captures dynamic microtubules. The dynamic microtubules could in turn increase the local concentration of Rac 1 in a positive-feedback cycle, with the concentrating of active Rac 1 producing a focus of F-actin, as above. Any explanation must account for the dependence of actin accumulation on both dynamic microtubules and active Rac 1; activation of Rac 1 by ligand-bound integrin alone is insufficient.

Contribution of a Rac/microtubule mechanism to neurite growth

Clearly, laminin-induced growth in the presence of 125 nM CD entails the Rac-dependent formation of small protrusions of F-actin at the ends of bundling, dynamic microtubules that have penetrated deep into the P region of the growth cone. Evidence points to this mechanism also contributing to growth on laminin without CD. Neurite growth from sympathetic neurons newly plated on laminin differs from growth on polyamine or fibronectin in showing: (1) an increased frequency of veils on filopodia; (2) more filopodia containing newly polymerized microtubules; (3) a greater sensitivity of growth to the inhibition of microtubule dynamics by nocodazole, (4) an increased concentration of Rac 1 in peripheral veils and filopodia; and (5) an inhibition of growth by knockout of Rac 1 (see also Kuhn et al., 1998).

We suggest that Rac/microtubule-dependent actin polymerization provides an additional mechanism of advancing the leading edge of the growth cone. In a growth

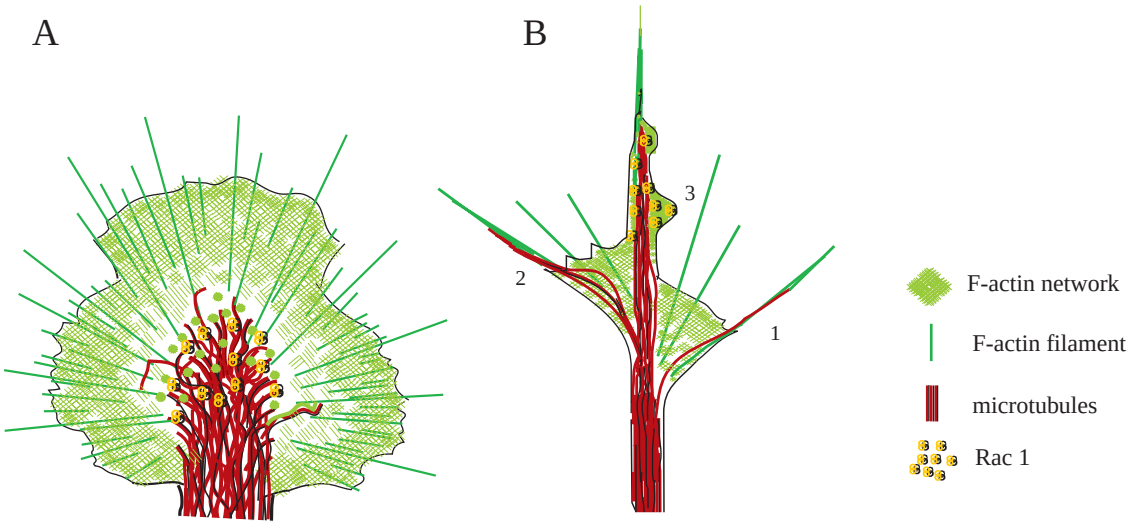

Fig. 10. How microtubule and Rac 1-dependent actin might contribute to rapid growth on laminin. (A) On a polyamine or fibronectin substrate, there is an incremental advance of the leading edge of the growth cone by polymerization onto the existing actin network, well ahead of the microtubules. (B) An additional mechanism that could contribute to normal growth on laminin. Microtubules penetrate filopodia (1). Multiple microtubules penetrate filopodia and peripheral regions (2). Rac 1-dependent F-actin colocalized with bundled microtubules produces 'splashes' of veil at the distal edge (3) and facilitates rapid advancement.

cone with a large P region striated with prominent bundles of F-actin (such as seen shortly after plating of sympathetic neurons on a polyamine or fibronectin substrate), the leading edge of the growth cone mainly advances incrementally, by polymerization onto the existing F-actin network (Fig. 10A). This advance of the leading edge occurs well ahead (several micrometers) of, and is not dependent on, dynamic microtubules or Rac. Alternatively, dynamic microtubules penetrating close to the leading edge of the growth cone stimulate polymerization of actin at their ends via activated Rac 1, thereby advancing the leading edge (Fig. 10B). These two modes of advance of the leading edge presumably coexist, but we suggest that, because of the effects of laminin on the microtubules, the Rac 1/microtubule-dependent mode of advance of the peripheral F-actin network contributes more significantly to overall neurite growth on laminin than on substrates such as polyamine or fibronectin. Since neurite growth requires both F-actin based protrusion and microtubule advance, the intimacy of the two processes in the Rac-dependent mechanism might make it particularly efficient and could contribute to the high speed of growth on laminin.

Several studies have implicated Rac in axonal growth, branching and guidance (Luo et al., 1994; Luo et al., 1996; Kaufmann et al., 1998; Kuhn et al., 1998; Kunda et al., 2001; Ng et al., 2002). For example, interfering with the functioning of Rac 1 inhibits growth in vivo of axons of *Drosophila* peripheral neurons (Luo et al., 1994) and mouse central neurons (Luo et al., 1996). Suppression of Tiam 1, a guanine nucleotide exchange factor for Rac 1, inhibits the formation of axons in cultured hippocampal neurons, whereas overexpression stimulates their formation (Kunda et al., 2001). However, the specific mechanism by which Rac affects dynamic changes in the growth cone cytoskeleton and neurite growth remains unclear. Our results suggest the mediation of microtubule-dependent actin polymerization in the peripheral region of the growth cone as one way in which Rac contributes to neurite growth.

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