Association of $\beta 1$ Integrin with Phosphotyrosine in Growth Cone Filopodia

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Filopodia of growth cones are key elements in the transduction of extracellular cues that guide axon growth during development. How they are specialized to carry out this role is poorly understood. We previously had found tyrosine phosphorylated protein to be heavily concentrated at the tips of many filopodia of *Aplysia* growth cones in certain culturing conditions, suggesting that tyrosine phosphorylation might be involved in filopodial specialization. Immunocytochemistry was used to analyze the protein composition of the tip aggregates to determine whether there was an association of the tip phosphorylation with any important extracellular cue. $\beta 1$ integrin, a subunit of the receptor for laminin-type neurite growth promoters, coconcentrated with phosphotyrosine at filopodial tips of both *Aplysia* and mouse growth cones. Several observations indicated that the association of $\beta 1$ integrin with phosphotyrosine

is close. $\beta 1$ integrin and phosphotyrosine are known to colocalize at focal contacts, sites of adhesion of cells to the extracellular matrix, but the composition and behavior of the tip aggregates mark them as distinct structures. Also found in the tip aggregates was a member of the ezrin–radixin–moesin family of proteins, which are thought to link membrane proteins to submembranous bundles of actin filaments. Use of an inhibitor of protein–tyrosine kinases to deplete tip phosphotyrosine also caused disappearance of $\beta 1$ integrin from the tip, suggesting a role for tyrosine phosphorylation in facilitating interaction of growth cones with certain environmental cues by fostering the aggregation of receptors in filopodia.

Key words: growth cone; filopodium; integrin; tyrosine kinase; phosphorylation; laminin; actin

Regulated axon growth during nervous system development is essential in establishing correct neuronal networks. Extracellular cues, both soluble and bound, regulate the speed and direction of growth. Most important in the detection and transduction of these cues is the growth cone, particularly its digitate filopodia. Axons deprived of filopodia grow waywardly *in situ* (Bentley and Toroian-Raymond, 1986; Chien et al., 1993), and contact of single filopodia with substrate- or cell-bound cues can change the direction of growth (Caudy and Bentley, 1986; Hammarback and Letourneau, 1986; Bandtlow et al., 1990; O'Connor et al., 1990). Yet we know little about the ways in which filopodia are specialized to detect or transduce cues.

Our recent finding that phosphotyrosine could concentrate at the tips of *Aplysia* filopodia suggests the involvement of protein–tyrosine phosphorylation in this specialization (Wu and Goldberg, 1993). Protein–tyrosine kinases (PTKs) are involved in the transduction of cues for neurite elongation and pathfinding both as surface receptors and more distally in signaling pathways. Examples of the former are the *trk* receptors for neurotrophins such as nerve growth factor (NGF) (Lamballe et al., 1991; Loeb et al., 1991; Squinto et al., 1991); Eph PTKs, which function in creating

the topographic projection of retinal ganglion axons on the optic tectum (Cheng et al., 1995; Drescher et al., 1995); and the Drl PTK, which is important in axon pathfinding in *Drosophila* (Callahan et al., 1995). *c-src* and *fyn*, related nonreceptor PTKs, are expressed at especially high levels in the developing nervous system (Maness et al., 1988; Bare et al., 1993). *c-src* seems to be involved in mediating the promotion of neurite growth by NGF (Alemá et al., 1985) and L1, a molecule of the immunoglobulin superfamily (Ignelzi et al., 1994). *fyn* seems to be important for neurite growth promoted by neural-cell adhesion molecule (N-CAM), another member of that superfamily (Beggs et al., 1994). Deletion of the nonreceptor PTK *abl* contributes to disoriented growth by certain axons in *Drosophila* (Elkins et al., 1990). The activation of focal adhesion kinase (FAK), as well as *c-src*, has been implicated in transducing signals from extracellular adhesion molecules in non-neuronal cells (Burrage et al., 1992). Some of these molecules, such as laminin, act as promoters of neurite growth (Sanes, 1989).

We sought to examine in this study the protein composition of the filopodial tip aggregates of phosphotyrosine to gain some insight into their organization and function. Our finding that $\beta 1$ integrin, a subunit of the receptor for laminin and certain other substrate-bound promoters of neurite growth, is closely associated with the phosphotyrosine at filopodial tips supports the idea that the filopodial phosphorylation is involved in certain interactions with environmental cues. Our results suggest receptor aggregation in filopodia as a specific role for the tyrosine phosphorylation. So, in addition to participating in signaling pathways as described previously, tyrosine phosphorylation may facilitate interactions with cues by influencing the distribution of certain receptors in the growth cone.

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MATERIALS AND METHODS

Cell culture. Neurons of the sea hare *Aplysia californica* were cultured according to published procedures (Goldberg, 1991). Individual medium-sized and large neurons were removed from the buccal ganglia of 2–5 gm *Aplysia* and placed on a glass substrate pre-exposed to poly-D-lysine. The culture medium was L15 supplemented with salts and antibiotics and was protein-free.

Embryos at embryonic day 14–15 (E14–E15) were retrieved from pregnant C57/BL6 mice that had been anesthetized with ketamine/xylazine (50 and 10 mg/kg, respectively). The embryos were placed in cold F12-DMEM medium (Gibco, Grand Island, NY) with 15 mM HEPES. A small orienting incision was made at the ventral point of the diamond-shaped opening of the eye cup formed by the pigment epithelium. After removing the pigment epithelium, lens, and vitreous, together with the blood vessels, the whole retina was isolated and flattened. Wide strips of peripheral dorsal retina (250 μ m) were removed and cultured as explants, ganglion side down, in serum-free F12-DMEM medium [supplemented with 1% bovine serum albumin, 5 mg/l insulin, 5 mg/l transferrin, 5 μ g/l sodium selenite (Sigma medium supplement cat. no. I-1884, Sigma, St. Louis, MO) and 20 U/ml penicillin/streptomycin] with 0.4% methylcellulose. The latter was to ensure adhesion of the explant to the substrate. Substrates were exposed to 100 μ g/ml polylysine (Sigma) overnight and then either 7 or 25 μ g/ml mouse EHS laminin (Gibco) for 1–3 hr before addition of explants.

Immunocytochemistry. Monoclonal antibody to phosphotyrosine (clone 4G10) was obtained from Upstate Biotechnology (Lake Placid, NY). Polyclonal antibody to phosphotyrosine was obtained from Zymed Laboratories (San Francisco, CA). Anti-talin monoclonal antibody (clone 8d4) and anti-vinculin monoclonal antibody (clone 11–5) were obtained from Sigma. The following antibodies were gifts from the scientists indicated: polyclonals to the $\beta 1$ subunit of integrin (Dr. C. Buck, Dr. E. Marcantonio), monoclonal to ezrin-radixin-moesin (ERM) proteins (clone 13H9, Dr. F. Solomon), monoclonal to cortactin (clone 4F11, Dr. T. Parsons), and monoclonals to paxillin and tensin (Dr. K. Burridge).

The monoclonal antibody was used for single-label immunostaining for phosphotyrosine; double-staining used either the monoclonal or the polyclonal antibody, depending on what was available for the other antigen. We fixed vertebrate cells using either 0.12 M sucrose, 100 mM cacodylate, and 0.25% glutaraldehyde or 4% *p*-formaldehyde, 0.1% glutaraldehyde, and 0.12 M sucrose in PBS. We fixed *Aplysia* neurons using either 0.25% glutaraldehyde, as described previously (Burmeister et al., 1991), or 4% *p*-formaldehyde/0.1% glutaraldehyde/400 mM sucrose/artificial seawater.

For anti-phosphotyrosine staining, cells were rinsed three times with PBS containing 0.1% Triton X-100 (TX), blocked with 20% normal goat serum (NGS) in PBS containing 0.1% TX for 30 min, and incubated with 1:100 diluted 4G10 in PBS/5% NGS/0.1% TX for 1 hr at room temperature. After 3 rinses with PBS/0.1% TX, the culture was incubated for 1 hr with either fluorescein- or rhodamine-conjugated goat anti-mouse IgG diluted 1:100 in PBS/5% NGS/0.1% TX. Cells were rinsed three times with PBS and observed promptly on a Zeiss IM-35 inverted microscope (Thornwood, NY). For double staining, anti-integrin was used to stain growth cones with the same procedure after completing the staining of phosphotyrosine, except rhodamine-conjugated goat anti-rabbit was used as the secondary antibody. Double staining of *Aplysia* growth cones for phosphotyrosine and $\beta 1$ integrin involved the use of Texas Red-conjugated goat anti-mouse and fluorescein-conjugated goat anti-rabbit secondary antibodies. Double staining for phosphotyrosine and antigens other than integrin involved overnight incubation at 4°C with the antibody for the other antigen. Control experiments were performed for each antibody by (1) going through the entire procedure but omitting the first antibody, and (2) replacing goat serum, goat anti-mouse, and goat anti-rabbit with sheep serum, sheep anti-mouse, and sheep anti-rabbit (all from Cappel/Organon Teknika, Durham, NC), respectively. An additional control was done in which sheep anti-rabbit was used to replace goat anti-rabbit for rabbit antibodies when doing double staining, and goat anti-mouse was used for monoclonal antibodies. Images were recorded either with a Dage 66 SIT camera (Michigan City, IN) onto optical disks or with a 35 mm camera.

Analysis of neurite growth and filopodial tip staining in mouse retinal explants. Neurites of half of each of six explants were measured on enlarged photographic prints. We measured the straight line distance from growth cones to the explant, because individual neurites often fasciculated and thus were not discernible. Therefore, our measurement of neurite length is approximate, but the difference between low- and

high-laminin conditions was considerable. Mean neurite length was determined for each of six explants and the mean $\pm$ SEM of those six means was determined. Statistical assessment of the significance of the difference in means between high- and low-laminin conditions was done using Student's *t* tests. In four separate dishes at each of the two laminin concentrations, as well as at the low concentration with additional laminin added acutely 1 d after culturing, the percentages of growth cones having at least one filopodium with concentrated phosphotyrosine at the tip and of total filopodia with concentrated tip phosphotyrosine were determined. Only filopodia of growth cones that apparently were not contacting other neurites or growth cones were assessed. Statistical assessment of the significance of the differences among the three percentages (high laminin, low laminin, and low laminin plus acutely added laminin) for each of the two parameters (percent of growth cones with tip phosphorylation and percent of total filopodia with tip phosphorylation) was done using a χ^2 test.

RESULTS

$\beta 1$ integrin coconcentrates with phosphotyrosine at filopodial tips of *Aplysia*

Because it is not feasible to obtain the tips of growth cone filopodia in the purity or quantity needed to do biochemical analyses of their composition, we used double-labeling immunocytochemistry for phosphotyrosine and proteins of interest. We wanted to determine whether any receptor for extracellular cues was found in the phosphotyrosine aggregates. The $\beta 1$ subunit of integrin is part of the heterodimeric receptor for laminin, fibronectin, and collagen, proteins of the extracellular matrix that can promote neurite growth (Bozyczko and Horwitz, 1986; Tomaselli et al., 1990; Reichardt and Tomaselli, 1991). It previously has been found to concentrate with phosphotyrosine in focal contacts, sites of tight adhesion of cells to the substrate (Maher et al., 1985). It also has been reported to concentrate at the tips of chick growth cone filopodia (Letourneau and Shattuck, 1989). Therefore, we doubly stained *Aplysia* growth cones with a polyclonal antibody to $\beta 1$ integrin and a monoclonal antibody to phosphotyrosine to see whether they coconcentrate in filopodial tips. The polyclonal antibody had been raised against a conserved cytoplasmic sequence of $\beta 1$ integrin and had been shown to recognize invertebrate, as well as vertebrate, integrins (Marcantonio and Hynes, 1988). Immunoblotting showed this antibody to recognize predominantly a single band from *Aplysia* ganglia, the apparent molecular weight of which was similar to other invertebrate β integrins (Marcantonio and Hynes, 1988) and gel mobility of which differed between reducing and nonreducing gel conditions, as is characteristic of β integrin (data not shown). Double staining of *Aplysia* growth cones on polylysine with this antibody and with anti-phosphotyrosine antibody showed integrin and phosphotyrosine staining to be coconcentrated at the tips of numerous filopodia (Fig. 1*A,B*). In nine growth cones assessed quantitatively, all 31 of the filopodial tips that had concentrated integrin also showed concentrated phosphotyrosine, and an additional three tips showed concentrations of phosphotyrosine without clear concentrations of integrin.

There was specificity in this coconcentration. We showed previously that concentrations of phosphotyrosine at filopodial tips do not simply reflect general protein concentration (Goldberg and Wu, 1994). Here, we assessed the distribution of another membrane molecule involved in interaction of the growth cone with adhesive ligands that promote or direct growth: ApCAM, the *Aplysia* homolog of vertebrate N-CAM. Blockage of ApCAM with antibody alters the pattern of growth of *Aplysia* neurites in culture (Keller and Schacher, 1990; Zhu et al., 1994). Immunocytochemical visualization of ApCAM in fixed growth cones on a polylysine substrate did not reveal strong concentrations at filopodial tips

Figure 1. The $\beta 1$ subunit of integrin, but not ApCAM, coconcentrates with phosphotyrosine at the tips of *Aplysia* filopodia. *A*, Several filopodia of this *Aplysia* growth cone show enhanced staining with anti-phosphotyrosine antibody (arrowheads). *B*, These tips also show elevated staining with anti- $\beta 1$ integrin antibody at their tips. *C*, Bright staining with anti-phosphotyrosine antibody is present at the tips of several filopodia (arrowheads) of a fixed growth cone. *D*, The filopodia (arrows) that displayed elevated tip staining for phosphotyrosine did not show elevated tip staining with anti-ApCAM antibody when the same growth cone was viewed very shortly before fixation. Staining is low overall, because low concentrations of anti-ApCAM must be used on living growth cones. Scale bars, 5 μ m.

(data not shown). Although we expected phosphotyrosine to be concentrated at most of those tips (Wu and Goldberg, 1993), we could not determine whether it was, because ApCAM is extracted by the fixation procedures used to visualize phosphotyrosine. So we visualized immunocytochemically ApCAM in living growth cones (Zhu et al., 1995) and then fixed those cells and processed them for visualization of phosphotyrosine. ApCAM was not concentrated in filopodial tips, which after fixation could be seen to have concentrated phosphotyrosine (Fig. 1*C,D*).

Aggregates of $\beta 1$ integrin at filopodial tips are closely associated with phosphotyrosine

The finding that tip concentrations of $\beta 1$ integrin always were associated with concentrations of phosphotyrosine suggested a close association between the two. We therefore assessed the effects on integrin at filopodial tips of two treatments that deplete phosphotyrosine from the tips. Application of cytochalasins, fungal metabolites that specifically bind to and block one end of actin filaments, causes a rapid distoproximal withdrawal of the network of actin filaments which invests the peripheral region of the *Aplysia* growth cone (Forscher and Smith, 1988). Phosphotyrosine withdraws from filopodial tips simultaneously, presumably because it depends on actin filaments for its localization (Wu and Goldberg, 1993). We found that 1 μ M cytochalasin D also caused

$\beta 1$ integrin to disappear rapidly from filopodial tips. Some filopodia of growth cones fixed 2 min after application of cytochalasin had proximal coconcentrations of phosphotyrosine and integrin (Fig. 2*A,B*), indicating that phosphotyrosine and integrin remained colocalized even as they withdrew from the tips. We also fixed *Aplysia* growth cones 15, 30, and 45 min after washout of cytochalasin. Bright staining for integrin began to reappear at filopodial tips between 15 and 30 min after washout and was common by 45 min. In this time window during which integrin aggregates were just reappearing at filopodial tips, we never detected an integrin aggregate appearing without a phosphotyrosine aggregate. All 22 of the filopodial tips from the seven growth cones observed at these three recovery times that had concentrations of integrin also had concentrations of phosphotyrosine (Fig. 2*C-F*).

We also depleted phosphotyrosine from *Aplysia* filopodial tips by treating growth cones with genistein, a broad specificity inhibitor of PTKs. We previously had used 100 μ M genistein (Wu and Goldberg, 1993), but we recently have found that this causes disaggregation of actin filaments in the growth cone (Goldberg and Wu, 1995) and, therefore, we were concerned that any observed effect on integrin at filopodial tips could be caused by this effect rather than directly by a reduction in phosphotyrosine. We

Figure 2. $\beta 1$ integrin withdraws with phosphotyrosine from filopodial tips during cytochalasin treatment and reappears with phosphotyrosine during washout. *A, B*, Each show areas of two neighboring growth cones fixed 2 min after the application of 1 μ M cytochalasin D, a time at which the network of actin filaments is withdrawing from the peripheral region of the growth cone. All the filopodia of the growth cone on the *left* lack bright tip staining for $\beta 1$ integrin and phosphotyrosine. One of the filopodia has two bright, spread patches of $\beta 1$ integrin more proximally (*B*, arrowheads), which colocalize with patches of phosphotyrosine (*A*, arrowheads). Two of the filopodia of the growth cone on the *right* have bright staining for $\beta 1$ integrin near their tips (*B*, arrowheads). The staining is spread in both filopodia and, in one, is slightly withdrawn from the tip. These patches of $\beta 1$ integrin are matched by patches of phosphotyrosine (*A*, arrowheads). Relatively few filopodial tips have bright staining for phosphotyrosine (*C*) or $\beta 1$ integrin (*D*) 30 min after washout of cytochalasin. All tips that have bright staining for $\beta 1$ integrin also have bright staining for phosphotyrosine (arrowheads). Several filopodial tips have bright staining for phosphotyrosine (*E*) and $\beta 1$ integrin (*F*) 45 min after washout of cytochalasin. All those with bright $\beta 1$ integrin staining also have bright phosphotyrosine staining (arrowheads). Scale bar, 5 μ m.

found here that 2–3 hr of treatment with 50 μ M genistein greatly decreased phosphotyrosine in filopodial tips without apparent effects on actin, at least in some growth cones. We chose for analysis only those growth cones that did not show evidence of actin disaggregation in response to genistein; that is, growth cones

that retained relatively short filopodia and large expanses of flat lamellipodium with numerous bundles of actin filaments. Fixation of these and double staining with anti-phosphotyrosine and anti-integrin antibodies showed an absence of concentrations of phosphotyrosine and integrin from the large majority of filopodial tips

Figure 3. Inhibition of protein-tyrosine kinases causes loss of $\beta 1$ integrin (**B**) as well as phosphotyrosine (**A**) from filopodial tips. The growth cone was treated for 2.5 hr with 50 μ M genistein, a broad-specificity inhibitor, before fixation. This concentration of drug did not cause the effects on actin in the peripheral region of the growth cone typically seen with 100 μ M genistein, as evidenced here by the relatively short filopodia and the large lamellipodium striated with ribs of F-actin (seen as dark stripes running centrally from the bases of some filopodia). Scale bar, 5 μ m.

of seven growth cones (Fig. 3). No tips showed a retention of concentrated integrin without concentrated phosphotyrosine.

Substrate-sensitive phosphotyrosine coconcentrates with $\beta 1$ integrin at filopodial tips of mouse growth cones

To examine further the composition of the filopodial tip aggregates, we had to use vertebrate neurons because most of the available antibodies for proteins of interest did not have the necessary specificity for the target antigen in *Aplysia* nervous tissue, as assessed by immunoblotting. We used explants from retinæ of embryonic mice to minimize heterogeneity in our growth cone population; only retinal ganglion cells should send out long neurites. In addition, we took explants only from the peripheral dorsal region of the retina, because growth cones from retinal ganglion cells in different regions can show different responses to extracellular cues. We cultured explants on a substrate pre-exposed to polylysine and then pre-exposed to 7 μ g/ml lami-

Figure 4. The $\beta 1$ subunit of integrin coconcentrates with phosphotyrosine at the tips of embryonic mouse retinal ganglion cell filopodia. **A**, A growth cone has bright staining with anti- $\beta 1$ integrin antibody at the tips of one short and one long filopodium (arrowheads). **B**, The same filopodial tips are brightly stained with anti-phosphotyrosine antibody. Scale bar, 4 μ m.

nin, the lowest concentration that supported significant outgrowth from the explants in serum-free culture medium.

As in *Aplysia* growth cones, $\beta 1$ integrin and phosphotyrosine colocalized at mouse filopodial tips (Fig. 4). In the five growth cones assessed quantitatively, 14 tips showed concentrations of both $\beta 1$ integrin and phosphotyrosine, whereas only 1 tip displayed concentrated $\beta 1$ integrin without a discernible concentration of phosphotyrosine; 1 tip showed concentrated phosphotyrosine but not $\beta 1$ integrin.

Also similar to *Aplysia* tip phosphorylation was the sensitivity of mouse tip phosphorylation to the composition of the substrate. A much higher percentage of *Aplysia* filopodial tips had concentrated phosphotyrosine on a substrate on which growth is slower than on a substrate coated with growth-promoting material (Wu and Goldberg, 1993). Thus, we compared tip phosphorylation in mouse growth cones on substrates pre-exposed to 7 μ g/ml and 25 μ g/ml laminin in serum-free medium. Neurite growth was much enhanced by the higher concentration of laminin; mean length was 568 ± 17 μ m (mean of mean lengths of six separate cultures $\pm$ SEM) after 1 d in culture compared with 347 ± 20 μ m (significantly different, $p < 0.0001$) on the lower concentration of laminin. As with *Aplysia* growth cones, tip phosphorylation was more common in the slower-growth culture condition. Of the growth cones on the lower concentration of laminin, 82 of 141 (58%) (four separate dishes) had at least one filopodial tip showing concentrated phosphotyrosine compared with 44 of 156 (28%) on the higher concentration. Of the total filopodia on the lower concentration, 160 of 838 (19%) showed concentrated phosphotyrosine compared with 78 of 869 (9%) on the higher concentration of laminin. Analysis of separate growth cones showed that $\beta 1$ integrin also was concentrated less often at filopodial tips in the fast-growth culturing condition. Of the growth cones assessed on

Figure 5. Certain actin-associated proteins which are substrates for tyrosine phosphorylation do not concentrate at the tips of growth cone filopodia. *A*, Bright staining with anti-paxillin antibody in streaks and patches is seen in spread areas around a mouse retinal explant, but very little staining was detected in growth cones (not shown). *B*, Considerable staining with anti-vinculin antibody is seen in a growth cone, but filopodia are not strongly stained and their tips do not show significant concentrations. *C*, A mouse growth cone shows concentrated phosphotyrosine at the tips of three short filopodia (arrowheads). *D*, Bright anti-cortactin staining is not seen in these filopodial tips (arrowheads). Scale bar, 2 μ m.

the lower concentration of laminin, 11 of 17 (65%) had at least one filopodial tip showing concentrated $\beta 1$ integrin compared with 11 of 50 (22%) on the higher concentration. Of the total filopodia on the lower concentration, 19 of 59 (32%) had bright tip staining for $\beta 1$ integrin compared with 30 of 447 (7%) on the higher concentration of laminin.

It is possible that different populations of neurons sent out neurites from the explant in the two culture conditions, although we think this is unlikely because of the restricted provenance of the explants, as explained above. To confirm that the difference in tip phosphorylation was not caused by different populations of neurons, we assessed growth cones on the 7 μ g substrate that were exposed to 20 μ g/ml laminin added to the culture medium for 1 hr before fixation. Thus, we should have been assessing essentially the same population of growth cones as in the previous low-laminin (7 μ g) dishes. Acute addition of growth-promoting material to *Aplysia* growth cones on a polylysine substrate causes a rapid disappearance of phosphotyrosine from filopodial tips (Wu and Goldberg, 1993). Of the mouse growth cones fixed 1 hr after addition of laminin to the medium, 12 of 67 (18%) had at least one filopodial tip showing concentrated phosphotyrosine, and 17 of 196 (9%) of the total filopodia showed concentrated tip phosphotyrosine [significant difference among the three conditions (7 μ g/ml laminin, 7 μ g/ml laminin plus acutely added laminin, 25 μ g/ml laminin) $p < 0.001$ for each percentage]. These numbers are close to those of growth cones on the high-laminin substrate (25 μ g/ml), but much lower than those on the low-laminin substrate (7 μ g/ml). Thus, the aggregates of phosphotyrosine at filopodial tips of embryonic mouse retinal ganglion cell growth

cones are like those of *Aplysia* neurons in containing $\beta 1$ integrin and in being sensitive to the substrate.

ERM protein, but not other actin-associated proteins that are substrates for tyrosine phosphorylation, coconcentrates with phosphotyrosine at filopodial tips

Because it appears that the tyrosine-phosphorylated protein at the tips of filopodia interacts or associates with actin filaments (Wu and Goldberg, 1993), we performed immunocytochemistry using antibodies directed against six different actin-associated proteins known to be substrates for tyrosine phosphorylation. Antibodies that showed bright stainings at the tips of filopodia then were used together with anti-phosphotyrosine to doubly stain growth cones.

We stained growth cones of embryonic mouse retinal explants cultured on the low-laminin substrate, which has the higher frequency of tip concentration of phosphotyrosine. Antibodies to paxillin (Fig. 5*A*), tensin, and talin (data not shown, but similar to paxillin) all showed strong staining of spread regions close to the cell bodies of the explants, in streaks reminiscent of focal contacts. None of them gave substantial staining of growth cones, however, suggesting that these proteins are present in small amounts, if at all, in these growth cones.

Vinculin- and cortactin-specific antibodies produced substantial staining of growth cones, but neither showed concentrated staining of filopodial tips. Anti-vinculin staining was diffuse and not enhanced in the peripheral actin-rich region; it tended to be lowest in distal regions of the filopodia (Fig. 5*B*). Anti-cortactin antibody produced strong staining of the peripheral region. Filopodial staining was present but, in contrast to anti-

Figure 6. ERM protein coconcentrates with phosphotyrosine at the tips of many filopodia. *A*, Five filopodia (two indicated by arrowheads) of this mouse retinal growth cone show bright staining with anti-phosphotyrosine antibody at their tips. *B*, Four of those five tips show bright staining with anti-ERM protein antibody, although the pattern of staining in the lamellipodium differs substantially from that of anti-phosphotyrosine antibody. Scale bar, 2 μ m.

phosphotyrosine (Fig. 5C), was not concentrated at the tips (Fig. 5D).

One of the six actin-associated proteins examined was coconcentrated at filopodial tips with phosphotyrosine. We used a monoclonal antibody that recognizes all members of the ERM family of closely related proteins (Winckler et al., 1994) and that previously has been shown to stain actin-rich regions of vertebrate growth cones (Birgbauer et al., 1991). The antibody produced bright tip staining in some filopodia of singly stained mouse retinal ganglion cell growth cones, so we doubly stained, along with the anti-phosphotyrosine antibody, numerous mouse retinal growth cones. Of the 50 tips that showed bright anti-phosphotyrosine staining, 42 also showed bright anti-ERM staining. An example of coconcentration of phosphotyrosine and ERM immunoreactivities at the tips of filopodia is shown in Figure 6. Although we did not assess it quantitatively, it appeared that, like phosphotyrosine and $\beta 1$ integrin, ERM protein was more likely to be concentrated at filopodial tips in growth cones on a low-laminin substrate than on a higher-laminin substrate.

DISCUSSION

The localization of aggregates of phosphotyrosine at the tips of growth cone filopodia suggests that tyrosine phosphorylation is involved in facilitating or mediating certain interactions of filopodia with environmental cues. The finding here that $\beta 1$ integrin, a subunit of the receptor for growth-promoting proteins of the extracellular matrix, is a constituent of the aggregates supports that suggestion.

Several observations, in addition to that of their coconcentration at the tip, indicate that the phosphotyrosine and $\beta 1$ integrin in filopodia are closely associated. $\beta 1$ integrin was almost never concentrated at tips lacking concentrated phosphotyrosine (1/46 filopodia in *Aplysia* and mouse). It disappeared from *Aplysia* filopodial tips as rapidly as did phosphotyrosine in response to cytochalasin; in fact, aggregates of $\beta 1$ integrin apparently with-

drawing from tips could be found colocalized with aggregates of phosphotyrosine. In addition, the reappearance of concentrated $\beta 1$ integrin at tips after removal of cytochalasin was, at least approximately, contemporaneous with the reappearance of concentrated phosphotyrosine. We never found aggregates of $\beta 1$ integrin to precede concentrated phosphotyrosine at filopodial tips during recovery from cytochalasin. Finally, reduction of phosphotyrosine at tips by treatment with an inhibitor of PTKs was associated with a loss of the tip aggregates of $\beta 1$ integrin.

Filopodial tip aggregate differs from the focal contact

$\beta 1$ integrin previously has been found to coconcentrate with phosphotyrosine at focal contacts, specialized sites of tight adhesion between a cell and the substrate (Burrage et al., 1988). Yet our present immunocytochemical results and our previous morphological results demonstrate that the filopodial tip aggregates are not focal contacts. We showed previously, by VEC-DIC and confocal interference reflection microscopy, that the tips were not attached focally to the substrate (Wu and Goldberg, 1993). Our use of vertebrate neurons in this study has allowed us to assess the composition of the tip aggregates by immunocytochemistry using antibodies that react selectively with proteins of interest. Because our previous work had provided evidence that the tyrosine-phosphorylated protein interacts with the actin filament network (Wu and Goldberg, 1993), we assessed the distribution of actin-binding proteins that are known to be substrates for tyrosine phosphorylation. Vinculin, tensin, talin, and paxillin were not detectable in substantial amounts at filopodial tips, although they were detectable in substantial amounts elsewhere in the cells. These all are constituents of the focal contact (Burrage et al., 1988), so their absence from the phosphotyrosine aggregates at the filopodial tips further distinguishes these aggregates from the focal contact. In lacking talin and vinculin but having aggregated $\beta 1$ integrin, the filopodial tip aggregates resemble point contacts, which may be more common than focal contacts in growth cones (Arregui et al., 1994), but the tip aggregates are distinct in not being points of attachment to the substrate (Wu and Goldberg, 1993). These morphological and biochemical properties, plus the finding that the presence of extracellular ligand decreases, rather than increases, tyrosine phosphorylation (see below), stamp the tip aggregates as novel assemblages.

The only protein other than $\beta 1$ integrin that we found to coconcentrate consistently with phosphotyrosine in filopodial tips was a member of the ERM family. These proteins have been found to associate with motile protrusions of non-neuronal cells (Berryman et al., 1993) and of neuronal growth cones (Birgbauer et al., 1991), as well as with focal contacts and cell-cell adherens junctions (Takeuchi et al., 1994; Tsukita et al., 1994). ERM protein often has been found to be enriched in filopodia-like structures (Berryman et al., 1993). In intestinal brush border, where it was first described, ERM protein is found all along the length of the microvillus, in a juxtamembranous location, but not at the membrane between adjacent microvilli (Berryman et al., 1993). It has not been described before to concentrate at the tips of filopodia or like structures. The amino acid sequence of the ERM family members, as well as their ultrastructural localization, implies that they serve as linkers between plasma membrane proteins and the actin cytoskeleton, particularly bundles of actin filaments (Algrain et al., 1993; Berryman et al., 1993). Coconcentration of ERM protein and the plasma membrane glycoprotein CD44 and evidence of their physical linkage recently have been found in kidney cells (Tsukita et al., 1994). ERM protein may

participate in localizing integrin to growth cone filopodia by linking it to the core bundle of actin filaments.

Functional relationship between tyrosine phosphorylation and $\beta 1$ integrin

Our results suggest that tyrosine phosphorylation is important for the aggregation of $\beta 1$ integrin at filopodial tips. Reduction of tip phosphorylation through the use of the PTK inhibitor genistein was associated with a loss of most of the tip aggregates of $\beta 1$ integrin. The few that remained were associated with elevated phosphotyrosine, indicating that the drug was not completely effective. Because we used a modest concentration of genistein, which did not have the morphological effects on the growth cone seen with higher concentrations (Wu and Goldberg, 1993; Goldberg and Wu, 1995), we think the loss of $\beta 1$ integrin aggregates from filopodial tips was directly caused by the reduction in phosphotyrosine rather than being a secondary consequence of another effect of reduced phosphotyrosine. However, we cannot exclude this latter possibility. To do so, it will be necessary to identify the tyrosine-phosphorylated protein(s) in the filopodial tip aggregates and determine the consequences of elimination or mutation of its (their) phosphorylation site(s). $\beta 1$ integrin itself might be the tyrosine-phosphorylated protein. Its tyrosine phosphorylation affects its localization in the cell (Johansson et al., 1994) and its ability to link to the actin cytoskeleton (Schmidt et al., 1993). ERM protein also can be tyrosine-phosphorylated; its phosphorylation is associated temporally with its redistribution to actin-rich, motile areas of transformed epidermal cells stimulated with epidermal growth factor (Bretscher, 1989). Or as yet unidentified proteins could carry the phosphotyrosine in the filopodial tips.

The idea that tyrosine phosphorylation plays a role in aggregating certain receptors in the growth cone is consistent with other data reported in the past few years. There is evidence that tyrosine phosphorylation of the nicotinic acetylcholine receptor facilitates its clustering at postsynaptic sites of the neuromuscular junction (Wallace, 1994). Also, there is an indication that tyrosine phosphorylation is important in assembling focal contacts as well as in signaling through them (Burrage et al., 1992). Finally, it is clear that binding of phosphorylated tyrosine residues to specific (SH2) domains is essential for the recruitment of intracellular effectors to certain activated receptors, such as the neurotrophin receptors (Koch et al., 1991).

Although the present data point to a role for tyrosine phosphorylation in causing aggregation of integrin at filopodial tips, they do not rule out a role in mediating signaling from integrins. However, it is likely that the phosphotyrosine we detect at filopodial tips is distinct from the FAK-mediated tyrosine phosphorylation involved in signaling from integrin at focal contacts. Phosphorylation of FAK and its immediate targets at nascent focal contacts is triggered by adhesion of cells to substrate-bound ligand, such as fibronectin or laminin; adhesion to polylysine-coated substrate does not elicit the phosphorylation (Bockholt and Burrage, 1993). In contrast, we see the highest levels of tip phosphorylation when *Aplysia* neurons are cultured in protein-free conditions on a polylysine-coated substrate (Wu and Goldberg, 1993), and increased laminin on the substrate reduces tip phosphorylation in mouse growth cones. Also, PTK inhibitors, such as genistein, have been found not to suppress integrin-mediated neurite outgrowth (Bixby and Jhabvala, 1992; Miller et al., 1993; Williams et al., 1994), suggesting a lack of obligatory

involvement of tyrosine phosphorylation in promotion of overall neurite growth through integrins.

Thus, the filopodial tyrosine phosphorylation we have detected may function in more restricted interactions of growth cones with environmental cues. It may foster interaction with certain cues by promoting the localization and accumulation of receptors for those cues in filopodia, especially the tips. Tyrosine phosphorylation could be involved in assembling receptors before contact with a cue, essentially priming the filopodium. For example, we found relatively slow neurite growth from mouse retinal explants (on low-laminin substrates) to be associated with relatively high numbers of filopodia displaying tip aggregates of phosphotyrosine. It has been observed that growth cones slow or pause at decision regions in developing nervous systems (Godement et al., 1994; Halloran and Kalil, 1994). If this also is associated with accumulation of receptors at the tips of filopodia, it could specialize those filopodia for the detection of cues required in that region. Or tyrosine phosphorylation could promote recruitment of receptors to sites of contact of filopodia with cell-bound cues to facilitate the interaction.

It now should be interesting to determine whether changes in tyrosine phosphorylation in filopodia are associated with interactions of growth cones with cell-bound cues used for pathfinding or synapse formation. Whether changes in phosphorylation are associated with only certain interactions and certain changes in filopodial or overall growth cone behavior should shed more light on the role of tyrosine phosphorylation in filopodia.

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