Chick Sensory Neuronal Growth Cones Distinguish Fibronectin from Laminin by Making Substratum Contacts that Resemble Focal Contacts

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SUMMARY

The adhesive interactions of nerve growth cones stabilize elongating nerve fibers and mediate transmembrane signaling to regulate growth cone behaviors. We used interference reflection microscopy and immunocytochemistry to examine the dynamics and composition of substratum contacts that growth cones of chick sensory neurons make with extracellular adhesive glycoproteins, fibronectin and laminin. Interference reflection microscopy indicated that sensory neuronal growth cones on fibronectin-treated substrata, but not on laminin, make contacts that have the appearance and immobility of fibroblastic focal contacts. Interference reflection microscopy and subsequent immunocytochemical staining showed that β1 integrin and phosphotyrosine residues were concentrated at growth cone sites that resemble focal contacts. Two other components of focal contacts, paxillin and zyxin, were also co-localized with concentrated phosphotyrosine residues at sites that resemble focal contacts. Such staining patterns were not observed on laminin-treated substrata. Growth cone migration on fibronectin-treated substrata was inhibited by herbimycin A, a tyrosine kinase inhibitor. We conclude that sensory neuronal growth cones distinguish fibronectin from laminin by making contacts with distinct organization and regulation of cytoskeletal components at the adhesive sites. This finding suggests that growth cone interactions with different adhesive molecules lead to distinctive transmembrane organization and signaling to regulate nerve fiber elongation. © 1996 John Wiley & Sons, Inc.

Keywords: **growth cone, fibronectin, focal contact, phosphotyrosine, integrin.**

INTRODUCTION

Nerve growth cones are guided to their targets by interactions with cues in the tissues through which they migrate. Among the factors that guide growth cones, adhesive molecules on cells and in extracellular matrices (ECM) are recognized by receptors on growth cones (Hynes and Lander, 1992; Doherty and Walsh, 1994; Letourneau et al., 1994a). The interactions of surface receptors with adhesive ligands provide anchorage to stabilize a growth cone, its filopodia and lamellipodia, permitting further neuritic elongation (Letourneau, 1989; Heidemann et al., 1991). In addition, binding of adhesive ligands to receptors triggers transmembrane signals that can also regulate growth cone migration (Lankford and Letourneau, 1989, 1991; Kater and Mills, 1991; Letourneau and Cypher, 1991; Bixby and Jhabvala, 1993; Juliano and Haskill, 1993; Doherty and Walsh, 1994; Letourneau et al., 1994a).

Fibronectin (FN) and laminin (LM) are adhesive ECM molecules that promote neurite elongation by chick embryo sensory neurons. However, these molecules have distinct distributions *in vivo* (Rogers et al., 1986, 1989; Letourneau et al., 1994b), and they may have different functions in regulating axonal pathfinding. In three different experimental systems that offered a choice between FN- and LM-treated surfaces, growth cones of chick sensory neurons recognized that FN and LM

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are different, and they changed their migratory behaviors (Gundersen, 1987; Gomez and Letourneau, 1994; Kuhn et al., 1995). Filopodia have an important role in making this discrimination and in changing growth cone behavior (Gomez and Letourneau, 1994; Kuhn et al., 1995). In this article we provide evidence that sensory growth cones distinguish FN from LM by the formation of substratum contacts with distinctive concentrations of cytoskeletal components and signal transduction activities.

Many cells bind adhesive ECM components via receptors of the integrin family (Hynes, 1992). In fibroblasts the engagement of integrins with FN leads to assembly of complex structures, called focal contacts, which contain a characteristic array of cytoskeletal and membrane proteins (Romer et al., 1992; Sastry and Horwitz, 1993; Schaller and Parsons, 1994). Interference reflection microscopy (IRM) has been used to observe that fibroblasts form focal contacts beneath lamellipodia and filopodia that remain stationary, as a cell migrates, until individual focal contacts disappear beneath the cell body or trailing margin (Izzard and Lochner, 1976, 1980; Heath and Dunn, 1978; DePasquale and Izzard, 1987). Focal contacts may have several functions: to provide anchorage against which a migrating cell can pull forward, to stabilize the shape of stationary cells, and, in light of recent evidence, to be involved in regulating gene expression. This last function is suggested because focal contacts contain such proteins as $ppFAK_{125}$, a protein tyrosine kinase, and zyxin. $ppFAK_{125}$ is activated by cell–substratum adhesion (Burridge et al., 1992; Lipfert et al., 1992; Schaller et al., 1992; Schaller and Parsons, 1994), and zyxin contains zinc binding LIM domains, which are implicated in protein–protein interactions of transcription factors (Crawford and Beckerle, 1991; Crawford et al., 1992; Sadler et al., 1992).

IRM has been used to examine cell–substratum interactions of nerve growth cones (Letourneau, 1979; Gundersen, 1988; Letourneau et al., 1988; Wu and Goldberg, 1993; Gomez and Letourneau, 1994; Zheng et al., 1994). Substratum-specific differences have been noted, and correlations have been made between the closeness of contact, as assessed by IRM, and growth cone–substratum adhesion. Although some growth cone–substratum contacts in static images resemble fibroblastic focal contacts, there are no detailed descriptions of the composition or dynamics of contacts beneath migrating growth cones.

In this article we used IRM and found that migrating growth cones of chick sensory neurons make cell–substratum contacts on FN with several significant spatial and dynamic characteristics of focal contacts. We obtained immunocytochemical evidence that these contacts contain components of focal contacts, high levels of phosphotyrosine, and the proteins $\beta 1$ integrin, paxillin, and zyxin. Similar contacts were not seen beneath growth cones migrating on LM, and neither were there accumulations of phosphotyrosine, $\beta 1$ integrin, paxillin, or zyxin. These results support the hypothesis that growth cones distinguish FN from LM via specific transmembrane signaling and different regulation of the cytoskeletal machinery for growth cone migration.

MATERIALS AND METHODS

Cell Culture

Dorsal root ganglia (DRG) were removed from embryonic day 9 to 11 (E9 to E11) chicken embryos, dissociated with trypsin (Letourneau, 1975), and were plated in serum-free medium, supplemented as described previously (Letourneau et al., 1990). For immunocytochemistry, approximately 25,000 cells were plated onto 22 mm acid-washed coverslips that were coated for 4 h with 20 μg/ml FN or LM (generously provided by Dr. James McCarthy, University of Minnesota) in phosphate-buffered saline (PBS). The coverslips and cells were placed in 35 mm Petri dishes (Falcon 1008). For IRM or phase contrast microscopy of living growth cones, cells were plated on FN-treated coverslips that were mounted over 22 mm holes drilled into the bottom of 50 mm Petri dishes (Falcon 1006). The coverslips were blocked with 10 mg/ml hemoglobin (Sigma Chemical Co., St. Louis, MO) in PBS for 2 to 3 h. Patterned substrata of FN and LM stripes were prepared, as described in Gomez and Letourneau (1994). Dishes with cells were incubated in a humidified chamber at 40°C for 6 to 24 h.

Videomicroscopy

Following incubation, a culture dish with a coverslip insert was placed on an inverted microscope (IM-35, Carl Zeiss, Thornwood, NY) under an air curtain incubator (ASI 400, Carl Zeiss) at 40°C. Growth cones were viewed by phase-contrast optics, using a Newvicon video camera (NC-65, Dage-MTI, Michigan City, IN) or by IRM, using a SIT camera (SIT-66, Dage-MTI). A Zeiss × 63 phase 3 planapochromat objective was used for phase contrast, and a Zeiss ×63 antiflex objective was used for IRM. IRM involved use of a 50 W HBO epi-illuminator, 546 ± 2 nm precision filter, H-D reflector, and pre-objective and postobjective polarizers. To prevent photo-

damage, growth cones were illuminated intermittently using a shutter (Uniblitz D122, Vincent Associates, Rochester, NY). Image enhancement was performed using Image 1 software (Universal Imaging, West Chester, PA) run on a 486/33 computer system (Gateway 2000, Sioux City, SD). Images were recorded with an optical disc recorder (OMDR; Pansonic TQ-2026F, Panasonic Industrial Corp., Secaucas, NJ).

Averaged images of series of growth cone IRM images were generated from consecutive OMDR frames that were recorded at intervals of 30 s for 13 to 16 min. Using the Image 1 software (Universal Imaging Corp.), successive images were averaged (that is, 1 + 2, 3 + 4, etc.) to generate 16 image averages. The same protocol was then repeated until a single image average was created that equally represented all images of each series.

To determine the effects of herbimycin A (Calbiochem) on growth cone migration, DRG explants were cultured 24 or 48 h in 35 mm plastic dishes coated overnight with 20 μg/ml FN or LM. A dish was placed on a warmed microscope stage and images of a microscope field containing several growth cones were recorded once per minute for at least 45 min before and 45 min after the addition of herbimycin A, dissolved in dimethyl sulfoxide (DMSO). The concentration of DMSO did not exceed 4 μl/m. The video records were reviewed, and growth cones that were advancing at the time of addition of herbimycin A were scored as either advancing, stopped, or retracting after the addition of herbimycin A.

Fixation and Immunocytochemistry

After 24 h, the cells were fixed in Ca^{+2} and Mg^{+2}-free PBS (CMF-PBS), containing 4% paraformaldehyde and 0.01% glutaraldehyde. After 15 min, the fixative was washed out and unreacted aldehyde groups were inactivated by 15-min incubation in CMF-PBS containing 1 mg/ml sodium borohydride. Finally, each coverslip was incubated for 15 min in a soaking solution of CMF-PBS containing 5 mg/ml bovine serum albumin (BSA; Sigma Chemical Co.). Paxillin and the phosphotyrosine epitope (PY20) were detected with monoclonal antibodies purchased from Transduction Laboratories (Lexington, KY). A polyclonal antibody against phosphotyrosine (Transduction Laboratories) was used to double label growth cones for paxillin and phosphotyrosine. Polyclonal antibodies against avian β1 integrin and avian zyxin were generously provided by Drs. Alan F. Horwitz, University of Illinois, and Mary Beckerle, University of Utah. Actin filaments were labeled with fluorescein-conjugated phalloidin (Molecular Probes, Eugene, OR). All antibodies were diluted in the soak solution. Primary antibodies were applied for 45 min at the following dilutions: anti-β1 integrin, 30 μg/ml; anti-zyxin, 1 μg/ml; anti-paxillin, 10 μg/ml, monoclonal antiphosphotyrosine, 5 μg/ml; and polyclonal antiphosphotyrosine, 2.5 μg/ml. After rinsing out unbound antibodies and soaking the coverslips for 15 min, secondary antibodies were applied for 45 min. Fluorescein- and rhodamine-conjugated, purified goat immunoglobulin G (IgG) directed against whole rabbit and mouse IgG were purchased from Cappel Research Products (Durham, NC) and were used at 10 μg/ml. After incubation, the coverslips were rinsed, soaked, and mounted on glass slides in a solution containing polyvinyl alcohol and glycerol.

Confocal Microscopy

Immunofluorescent staining of growth cones was viewed and recorded with a BioRad MRC 600 confocal microscope equipped with an Olympus BH-2 microscope and a Nikon apochromatic ×60 1.4 numeric aperature objective. Illumination was provided by a krypton/argon laser tuned to 488, 568, and 647 nm (Brelje et al., 1993). To ensure that the images of the two fluorochromes were in the greatest possible register, the images were acquired by the same dual dichroic mirror. A long pass dichroic mirror was placed in the optical path to direct the green and red fluorescence into separate detectors. To achieve the best spectral separation of fluorochromes, fluorescence signals were acquired by sequential imaging of fluorescein first, then rhodamine. Digital images were transferred to Adobe Photoshop 2.5 for reproduction.

Western Blot Analysis

Forebrain and DRGs from 9 to 13-day old chick embryos were lysed by boiling in lysis solution [1% sodium dodecyl sulfate (SDS), 10 m*M* Tris, pH 7.4] for 5 min. The lysate was centrifuged, and protein concentration was determined using the bicinchonic acid (BCA) method (Pierce). Ten μl of a 1 mg/ml brain or DRG lysate solution was added to 10 μl sample buffer (188 m*M* Tris, pH 6.8, 30% glycerol, 2% SDS, bromophenol blue), or 20 μl of a 400 μg/ml chick embryo fibroblast lysate (Transduction Laboratories) was loaded into each well of a 10% polyacrylamide gel. Samples were run for approximately 1 hour, and the gel was blotted onto nitrocellulose (Towbin et al., 1979). The nitrocellulose was blocked with 1% BSA in 100 m*M* Tris base, 1.5 *M* sodium chloride (NaCl), 0.5% Tween-20 (TBST) for 1 h at 25°C or overnight at 4°C, and reacted with primary antibody diluted in 1% BSA in TBST for 1 h. Concentrations were as follows: anti-paxillin, 1 μg/ml; anti-zyxin, 1 μg/ml; anti-β_1 integrin, 3 μg/ml. Nitrocellulose was reacted for 1 h with a 1:20,000 dilution of alkaline-phosphatase conjugated anti-mouse or anti-rabbit secondary antibody (Pierce no. 31322 and 31345) in 5% Carnation dry milk in TBST, and visualized in a solution of 15 μg/ml 5-bromo-4-chloro-3-indolyl-phosphate and 30 μg/ml nitro blue tetrazolium (Promega Corp.) in alkaline phosphatase buffer (100 m*M* Tris base, 100 m*M* NaCl, 5 m*M* magnesium chloride). The color reaction was stopped by an equal volume of stop/storage buffer (200 m*M* Tris base, 50 m*M* sodium ethylene diaminetetraacetic acid). Air-dried blots were scanned on a Hewlett-Packard Scan-

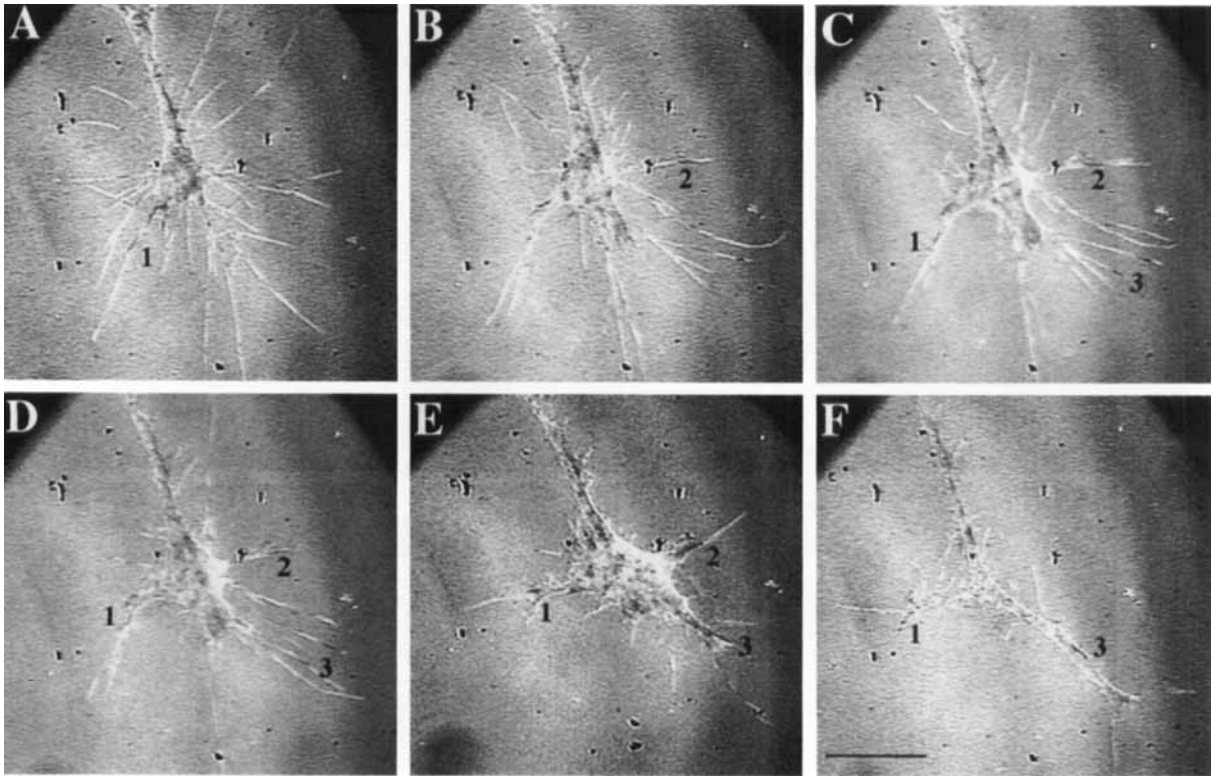

Figure 1 IRM video images from a 55-min sequence of a growth cone migrating on a FN-treated coverslip. Putative focal contacts are marked by numbers 1 to 3. Contact 1 was present beneath the proximal region of a filopodium in the first frame (A), and the other contacts were first seen as dark areas beneath the tip of a filopodium. Time after frame A for each subsequent frame: B, 9 min; C, 12 min; D, 21 min; E, 42 min; F, 55 min. Scale bar = 10 μm.

Jet Plus, and the images were transferred to Adobe Photoshop 2.5 for reproduction.

RESULTS

Interference Reflection Microscopy

Figures 1 to 3 present three series of IRM images of DRG growth cones migrating on a FN-treated substratum. These sequences were recorded during a study of growth cone behaviors at borders between substratum-bound FN and LM (Gomez and Letourneau, 1994). As reported in Gomez and Letourneau (1994), the mean IRM pixel intensities of growth cone–substratum contacts indicated that DRG growth cones were generally closer to a FN-treated substratum than a LM-treated substratum. Figures 1 to 3 show that the cell–substratum associations of growth cones on FN included the formation of small dark contacts that first appeared near the distal ends of filopodia and then were observed to remain stationary as a growth cone migrated. It is these persistent growth cone–substratum contacts that resemble focal contacts.

Figure 1 shows IRM images from a 55-min sequence of a growth cone migrating on FN. The first frame shows a dark contact already visible beneath a filopodium (1). In subsequent frames two other dark contacts appeared beneath filopodial tips (2 and 3). These contacts remained stationary, as the filopodia elongated, and as the growth cone advanced, these contacts came to lie beneath the growth cone margin. These contacts appeared as narrow streaks or bands, and their shape changed little. For the growth cone shown in Figure 1, the stationary contact labeled (1) remained visible at the end of a short branched process after the growth cone had turned toward the lower right corner of the field.

Figure 2 shows IRM images from a 36-min sequence of a growth cone that has just crossed a border from LM onto FN (the border is the line of black dots at the bottom of the field). In this series four dark contacts are labeled. Each contact ap-

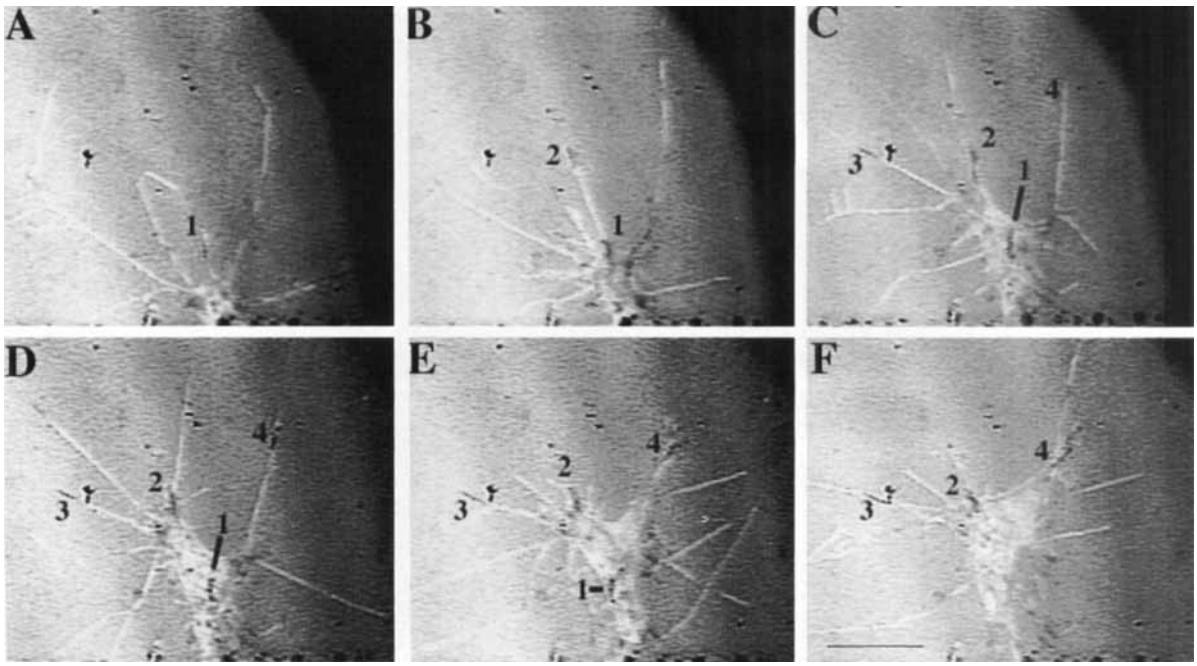

Figure 2 IRM video images from a 36-min sequence of a growth cone that had crossed a border from a LM-treated substratum to a FN-treated substratum. The border is visible as the row of black dots at the bottom of the images. In frame C a spot is marked with a 4 where focal contact 4 was seen in the next frame. Time after frame A for each subsequent frame: B, 8 min; C, 19 min; D, 22 min; E, 30 min; F, 36 min. Scale bar = 10 μm.

peared first at a filopodial tip and then remained stationary as the filopodia elongated and the growth cone advanced. The contact labeled 1 remained visible as the growth cone migrated, until the contact disappeared where the base of the growth cone narrowed to form the neurite.

Figure 3 shows IRM images from a 45-min sequence of another growth cone crossing from LM onto FN (the border is marked by a white line). Initially, gray areas of contact with LM were seen, but these contacts never lasted longer than a few minutes. Unlike the dark contacts beneath growth cones on FN, contacts with LM were never distinct and well defined. However, as soon as this growth cone contacted the FN-treated surface, dark contacts appeared beneath several filopodia [Fig. 3(1 to 4)].

Static images of these putative focal contacts are not as convincing as looking at time lapse replays of IRM sequences. To provide additional evidence that these contacts remained stationary as a growth cone migrated, series of IRM images of the growth cones pictured in Figures 1 to 3 were averaged over 13 to 16 minutes. We reasoned that any constant features of a changing image should be sharp in images that are the arithmetic mean of the intensity at each pixel, averaged over a series of images. On the other hand, an averaged image should be blurred in regions where the image intensity was changing over the duration of the image series. Figure 4 shows that the putative focal contacts formed on FN-treated substrata were prominent in the averaged images, whereas averaged images of growth cones on LM did not show dark contact areas that were stable beneath a migrating growth cone. It was noted that the rates of migration of these growth cones were not substantially different on FN versus on LM (also see Letourneau et al., 1988).

[Fig. 4(A)] is the arithmetic average of 32 images recorded over 16 min between frames B and E of Figure 1. The three contacts labeled in Figure 1 are labeled in [Fig. 4(A)], and several other dark contacts are also seen. [Fig. 4(B)] shows the average of 32 images recorded over 16 min between frames B and F of Figure 2. The contacts labeled 1 to 3 in Figure 2 are clearly evident, although contact 4 was not present in enough of the 32 images to appear clearly in the averaged image. [Fig. 4(C)] shows the average of 26 images recorded over 13 min between frames C and F of Figure 3. The contacts labeled 1 to 3 in Figure 3 are visible in [Fig. 4(C)]. [Fig. 4(D)] is the average of 32 images re-

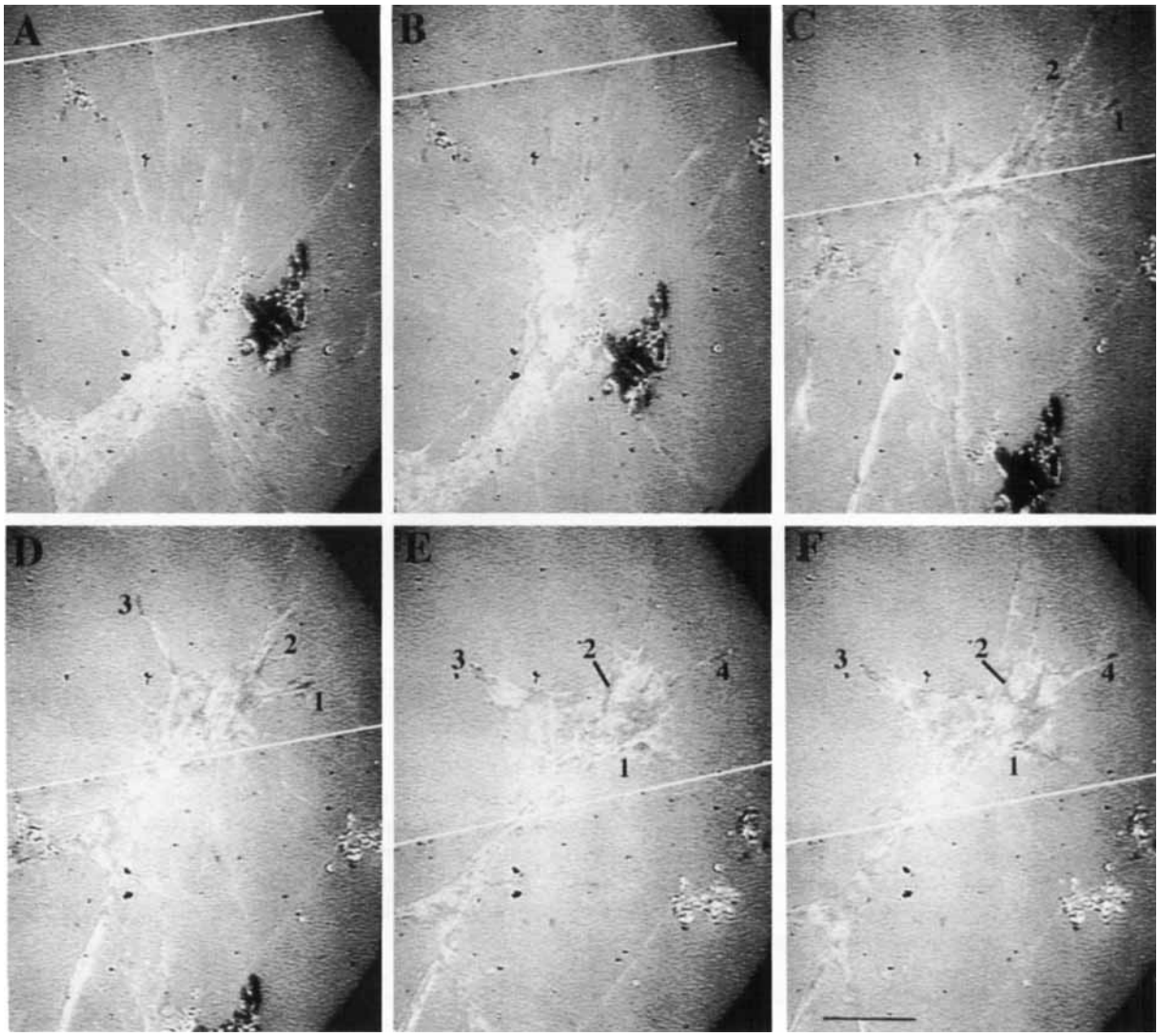

Figure 3 IRM video images from a 45-min sequence of a growth cone crossing from a LM-treated substratum to a stripe of FN-treated substratum. The border is marked with a white line. The first focal contacts marked in frame C were on the FN-treated surface. Time after frame A for each subsequent frame: B, 13 min; C, 27 min; D, 32 min; E, 41 min; F, 45 min. Scale bar = 10 μm.

corded over 16 min and illustrates the same growth cone that is shown in [Figs. 2,4(B)], but before it crossed the border from LM to FN. On LM, this growth cone did not make dark stable contacts, as it did after it crossed onto FN. [Fig. 4(E)] is the average of 30 images recorded over 15 min while the growth cone in Figure 3 was on LM before crossing the border to FN. Only one contact, marked with an arrowhead in [Fig. 4(E)], was seen in the averaged image of the growth cone on LM. Unlike the stable contacts on FN, it was not located at the front of the growth cone.

These dark, stationary contacts made by DRG growth cones migrating on FN resemble fibroblastic focal contacts (Izzard and Lochner, 1980) because they first appear beneath active filopodia, their IRM image is dark, and they are immobile as a growth cone migrates. However, their identification is less sure than fibroblastic focal contacts, because these contacts were smaller and their IRM images were less intense. In addition, it is suggested that some dark areas of IRM images are higher order reflections from areas that are not close to the substratum (Todd et al., 1988). Therefore, to gain additional evidence on the nature of these contacts that DRG growth

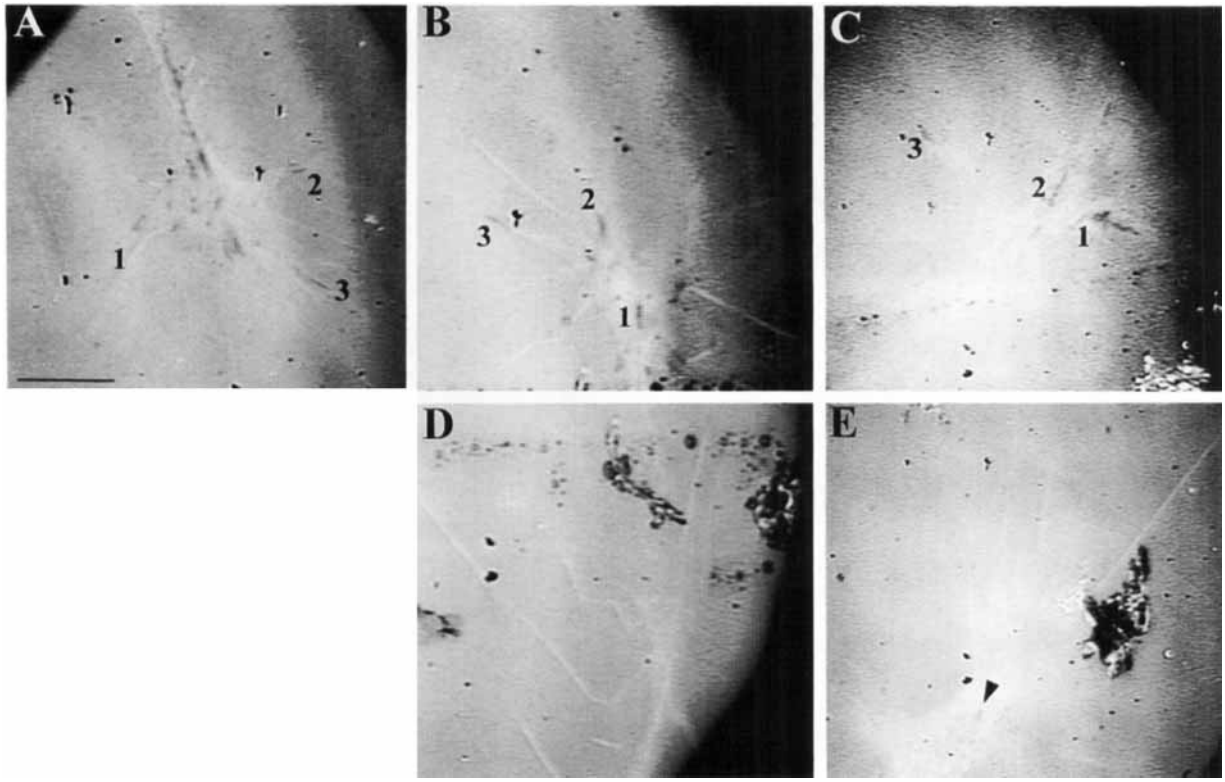

Figure 4 Averaged images from the sequences pictured in Figures 1, 2, and 3. Frame A is the average of 32 images recorded over 16 min between frames B and E of Figure 1. Frame B shows the average of 32 images recorded over 16 min between frames B and F of Figure 2. Frame C shows the average of 26 images recorded over 13 min between frames C and F of Figure 3. Frame D is the average of 32 images recorded over 16 min and illustrates the same growth cone that is shown in Figures 2 and 4(B), before it crossed from LM to FN. Frame E is the average of 30 images recorded over 15 min when the growth cone shown in Figure 3 was on LM, before crossing to FN. The arrowhead in frame E marks one dark contact that appeared in the averaged images of the growth cone on LM. Scale bar = 10 μm.

cones make on FN-treated substrata, we used antibodies against protein components of focal contacts.

Four antibodies against components of focal contacts were used. We localized FN receptors, integrin heterodimers, with a polyclonal antibody against the β1 integrin subunit. We used a monoclonal antibody to localize paxillin, a phosphoprotein that is concentrated in focal contacts, where it may bind vinculin (Turner et al., 1990; Turner, 1991, 1994). A polyclonal antibody was used to localize zyxin, a recently described protein of focal contacts (Crawford and Beckerle, 1991; Crawford et al., 1992). Finally, monoclonal and polyclonal antibodies against phosphotyrosine were used, because focal contacts contain high levels of phosphorylated proteins (Maher et al., 1985; Burridge et al., 1992).

Western Blot Analysis

To demonstrate that the immunocytochemical staining we observed was due to the presence of paxillin, zyxin, and β1 integrin, we did western blot analysis of homogenates of chicken fibroblasts (as a positive control), embryonic DRGs, and embryonic forebrain. Figure 5 is a western blot of gels that were immunolabeled for paxillin, zyxin, and β1 integrin. The blots indicate that the antibodies recognized proteins of the appropriate apparent molecular weights for paxillin (68×10^3 M_r) (Turner, 1994) and zyxin (82×10^3 M_r) (Sadler et al., 1992) in the lysates from fibroblasts, forebrain, and DRGs. The blot stained for β1 integrin indicates that the antibody recognized a polypeptide of the appropriate apparent molecular weight (116×10^3 M_r) (Tomaselli et al., 1993). Al-

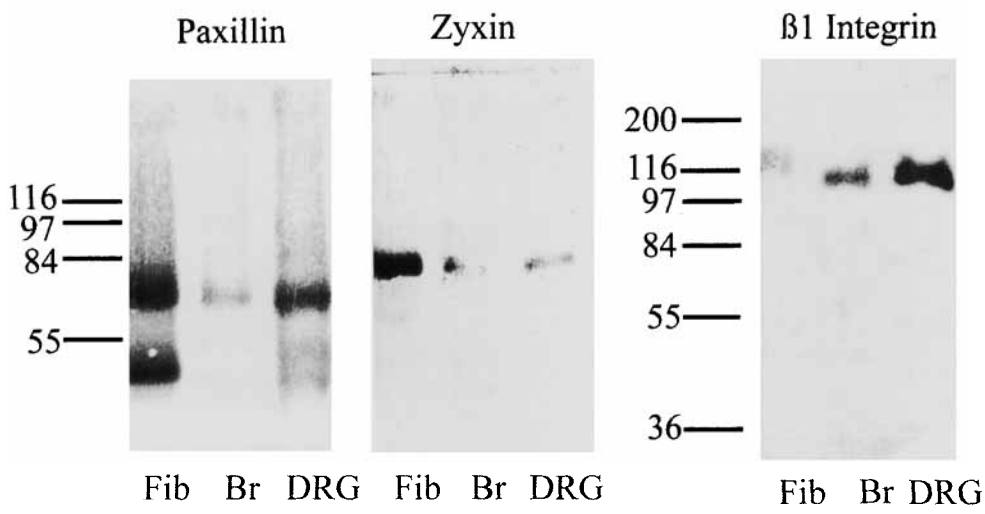

Figure 5 Western blot analysis of homogenates from chick fibroblasts, forebrain, and DRG; paxillin, 68×10^3 M_r; zyxin, 82×10^3 M_r; β1 integrin, 116×10^3 M_r.

though these western blots were not done with proteins from purified chick DRG growth cones, which would be very difficult to isolate, the results indicate that these antibodies respectively recognize proteins of appropriate molecular sizes in chick neural tissues.

Correlated IRM and Immunocytochemistry

IRM images of living DRG growth cones on a FN-treated substratum were recorded, then, the cells were fixed and stained with antibodies against components of focal contacts. These growth cones were relocated and immunofluorescence images were recorded. [Fig. 6(A)] shows a phase-contrast view of a living growth cone, and [Fig. 6(B)] shows the IRM image of this growth cone 15 min later, immediately before fixation. Finally, [Fig. 6(C)] shows immunofluorescence labeling of this growth cone with antiphosphotyrosine. Labeling for phosphotyrosine was concentrated at several sites that were dark contacts in the IRM image of [Fig. 6(B)]. Figure 6 (D, E, F, G) shows images of a growth cone that was recorded alive as phase contrast [Fig. 6(D)] and IRM images [Fig. 6(E)], and then fixed, and stained with antiphosphotyrosine [Fig. 6(F)] and anti-β1 integrin [Fig. 6(G)]. Again, labeling for these focal contact components was concentrated at sites that appeared as dark growth cone-substratum contacts by IRM. This evidence supports our proposal that DRG growth cones make contacts on FN-treated substrata that resemble focal contacts.

Immunocytochemistry

Correlated live IRM and subsequent immunocytochemical staining of growth cones is difficult and often unsuccessful. Thus, we used immunocytochemistry alone to ask whether paxillin and zyxin, two other proteins of fibroblast focal contacts, are also concentrated at sites at the leading growth cone margin that are strongly labeled by antiphosphotyrosine. Cultures of sensory neurons on FN- and LM-treated coverslips were incubated 24 h, fixed, and double labeled with antibodies against either β1 integrin, paxillin, or zyxin, and against phosphorylated tyrosine residues. When the primary antibodies were omitted, binding of the secondary antibodies to growth cones was weak and not different from binding to the noncellular substratum.

[Fig. 7(A–F)] shows three images for each of two growth cones double labeled with a rabbit polyclonal antibody against β1 integrin [Fig. 7(A, D)], PY20, against phosphorylated tyrosine residues [Fig. 7(B, E)], and a merged image of both antibody labels [Fig. 7(C, F)]. Anti-β1 integrin was distributed over the surface of both growth cones, although there was intense labeling of some filopodial tips, as we previously reported [Fig. 7(A, C, D, F, arrowheads)] (Letourneau and Shattuck, 1989). PY20, the antiphosphotyrosine antibody, was distributed as spots and as short streaks beneath the body of the growth cone and the leading margin [Fig. 7(B, E)]. The staining in short streaks at the bases of filopodia, along filopodia, and at the leading margin is typical of sites in which putative focal contacts were seen beneath growth cones

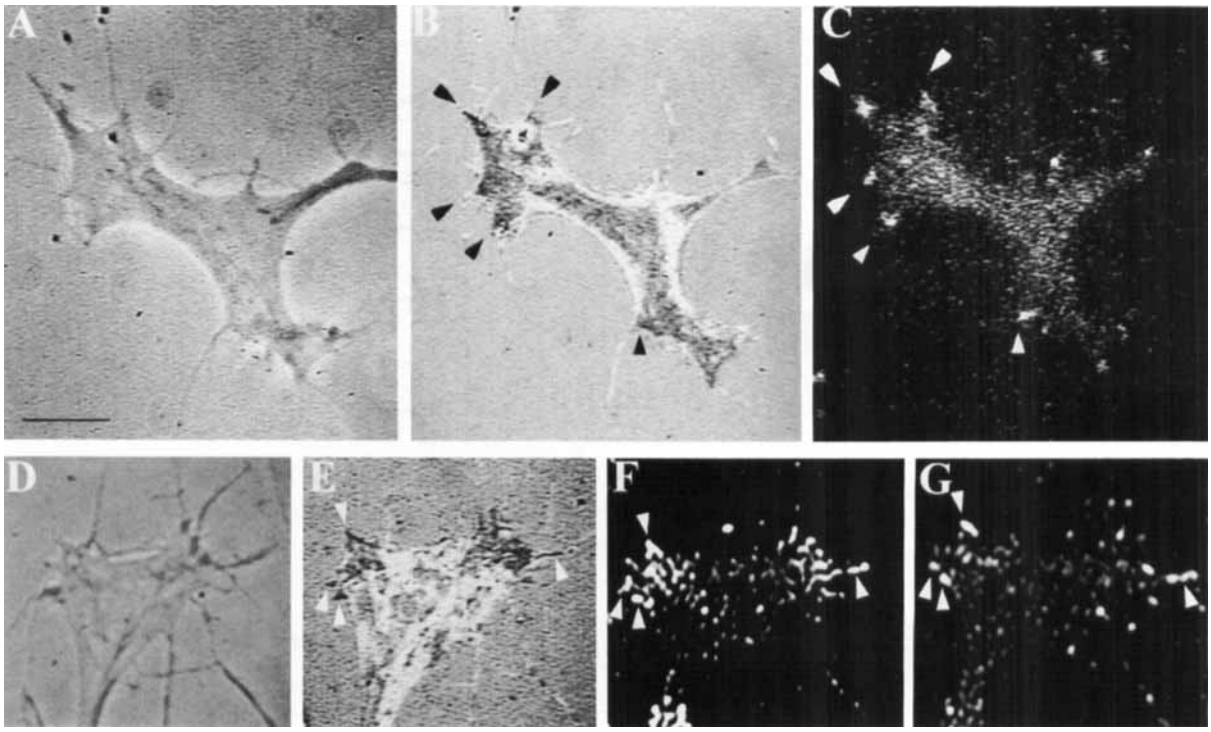

Figure 6 Antibodies that label fibroblast focal contacts also label putative focal contacts of DRG growth cones on FN. Phase contrast (A) and IRM (B) images were recorded just before fixing the cells on the microscope stage. The immunofluorescence staining shows concentrated labeling with antiphosphotyrosine at several sites (C, arrowheads) that also appeared as putative focal contacts (B, arrowheads). Another growth cone was recorded live with phase contrast (D) and IRM (E) and then immediately fixed and subjected to immunofluorescence staining for phosphotyrosine (F) and β1 integrin (G). Dark putative focal contacts (E, arrowheads) were also sites that labeled strongly for both phosphotyrosine and β1 integrin immunoreactivity (F and G, arrowheads, respectively). Scale bar = 8 μm.

[Figs. 1, 2, and 3]. Anti-β1 integrin was also co-concentrated with PY20 at these sites. The frequency of coincident staining with PY20 and anti-β1 integrin at sites that were characteristic of focal contacts was determined for 25 growth cones. Of 122 sites that were labeled with PY20, 88% also showed increased labeling with anti-β1 integrin. However, filopodial tips, which were often strongly labeled by anti-β1 integrin, were not usually also labeled by PY20, the antiphosphotyrosine. Of 72 filopodial tips that showed concentrated staining with anti-β1 integrin, only 17% also had concentrated labeling with PY20.

[Fig. 7(G–I)] presents three images of a growth cone that was double labeled with antibodies against β1 integrin [Fig. 7(G)] and paxillin [Fig. 7(H)]. [Fig. 7(I)] is a merged image of both labels. The distribution of labeling for β1 integrin is similar to the anti-integrin labeling seen in Fig. 7(A, D), and the distribution of labeling for paxillin was similar to the labeling seen for phosphotyrosine in [Fig. 7(B, E)]. Intense antipaxillin immunofluorescence was seen at the front margin of the growth cone and at the bases of filopodia, where stable dark contacts were characteristically located by IRM [arrowheads in Fig. 7(H, I)].

[Fig. 7(J, K, L)] presents images of a growth cone that was double labeled with fluorescein-conjugated phalloidin [Fig. 7(J)] and antizyxin [Fig. 7(K)]. A merged image of these two stains is seen in [Fig. 7(L)]. Streaks of antizyxin staining appeared at the bases of filopodia, similar locations where dark, putative focal contacts were often seen by IRM [Fig. 7(K, L, arrowheads)].

[Fig. 8] shows double labeling of two growth cones on FN with antibodies against phosphotyrosine [Fig. 8(A)] and paxillin [Fig. 8(B)] and phosphotyrosine [Fig. 8(C)] and zyxin [Fig. 8(D)]. In both cases antibody labeling for the proteins and the antiphosphotyrosine labeling showed co-distribution at sites that are characteristic of putative focal contacts [Fig. 8(A, B, C, D arrowheads)]. The

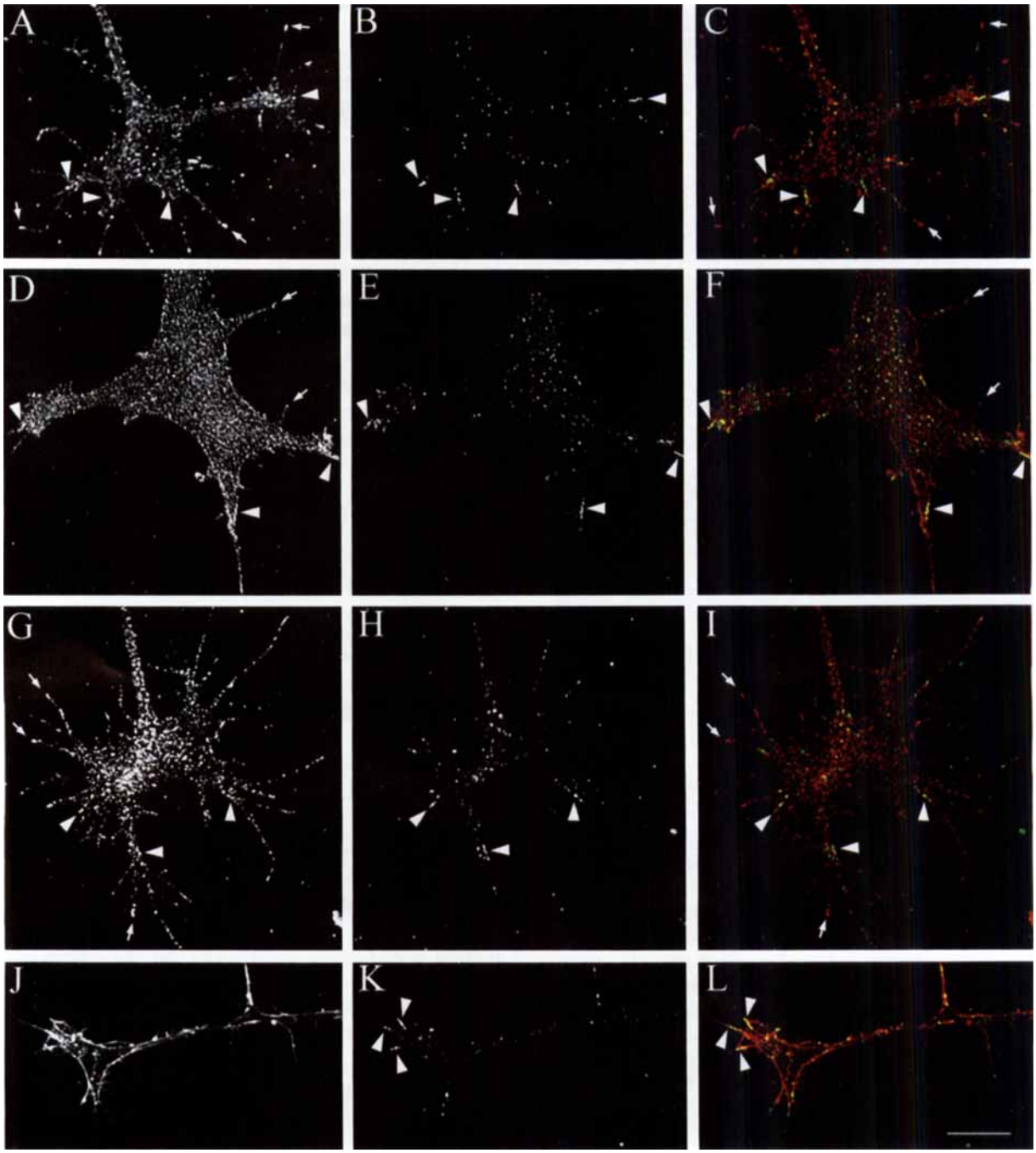

Figure 7 Double labeling and merged images of growth cones, cultured on FN-treated substrata and stained with antibodies against focal contact proteins. Two growth cones were double labeled for β1 integrin (A, D) and phosphotyrosine (B, E). A merged image of the two labels is presented in C and F, respectively. Antiphosphotyrosine and anti-integrin labeling is co-localized at sites where putative focal contacts were typically observed by IRM (A, B, D, and E, arrowheads). Filopodial tips that labeled strongly for β1 integrin in images (A and D, arrows) were not labeled by antiphosphotyrosine (C and F, arrows). (G, H, I) A growth cone double labeled for β1 integrin (G) and paxillin (H) is shown, and a merged image of the two labels is presented in (I). Antipaxillin staining was intense in short streaks where focal contacts were typically observed by IRM (G, H, I, arrowheads). Filopodial tips that were intensely labeled for β1 integrin in image (G, arrows) were not labeled by antipaxillin (I, arrows). (J, K, L) show a growth cone double labeled with fluorescent phalloidin (J) and antizyxin (K). A merged image of the two labels is presented in (L). Intense labeling for zyxin (K and L, arrowheads) was seen beneath the proximal ends of filopodia and the growth cone margin. Scale bar = 10 μm.

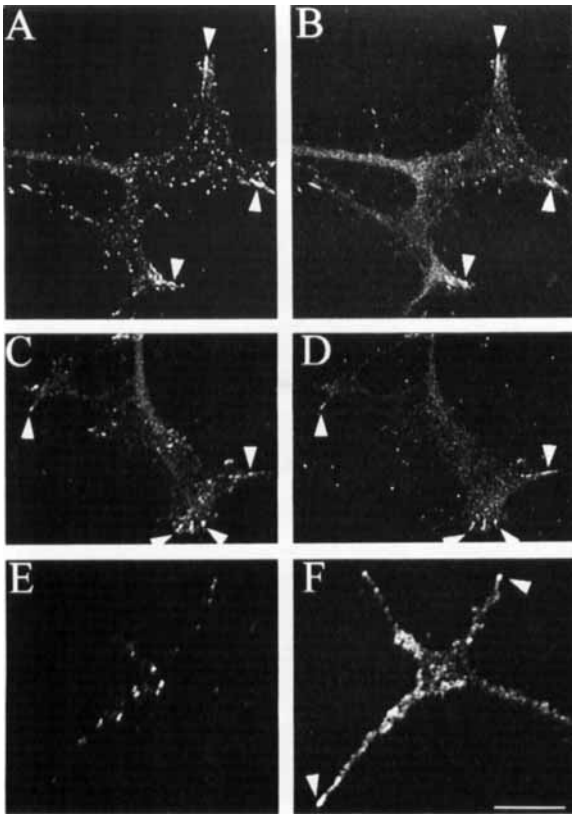

Figure 8 (A and B) A growth cone that was cultured on FN and double labeled for phosphotyrosine (A) and paxillin (B) is shown. Both antibodies were concentrated in streaks located at sites where focal contacts are often seen by IRM (A and B, arrowheads). (C and D) A growth cone on FN that was double labeled for phosphotyrosine (C) and zyxin (D) is shown. Streaks of double labeling (C and D, arrowheads) show the co-localization of zyxin and phosphorylated tyrosine residues at sites where focal contacts were typically seen. (E and F) A growth cone that was cultured on a LM-treated substratum and double labeled for phosphotyrosine (E) and β1 integrin (F) is shown. Antiphosphotyrosine was concentrated in punctate sites, but not in streaks like at focal contacts. Anti-β1 integrin labeling was concentrated at some filopodial tips (arrowheads). Scale bar (A–D) = 10 μm; (E, F) = 8 μm.

frequency of double labeling at sites that were characteristic of focal contacts was determined for 20 growth cones that were double labeled with antiphosphotyrosine and antipaxillin and for 25 growth cones that were double labeled with antiphosphotyrosine and antizyxin. Of 80 sites that were labeled by antiphosphotyrosine, 91% were co-labeled by antipaxillin, and of 107 sites that were labeled by antiphosphotyrosine, 87% were co-labeled by antizyxin.

Sensory neurons were also cultured on LM-treated coverslips and stained with the same antibodies. The labeling of DRG growth cones was diffuse or punctate with all antibodies. We did not see intense staining or co-distribution of antiphosphotyrosine with anti-β1 integrin, antipaxillin, or antizyxin that would indicate that these proteins are concentrated into putative focal contacts on LM. [Fig. 8(E, F)] illustrates a growth cone on a LM-treated substratum that was doubled labeled with antiphosphotyrosine [Fig. 8(E)] and anti-β1 integrin [Fig. 8(F)]. The antiphosphotyrosine labeling showed some punctate staining, but the labeling did not resemble the putative focal contacts of growth cones on FN-treated substrata. Anti-β1 integrin was distributed across the surfaces of growth cones and was sometimes concentrated at filopodial tips, as we saw on FN. These results were consistent with those from our IRM images, that is, growth cones on LM do not form contacts that resemble focal contacts.

Effects of the Tyrosine Kinase Inhibitor Herbimycin A

These immunocytochemical results indicate that growth cones migrating on a FN-treated substratum make contacts that contain several components of focal contacts, including tyrosine-phosphorylated proteins. Burridge et al. (1992) reported that a tyrosine kinase inhibitor, herbimycin A (Fukazawa et al., 1991), inhibited the formation of focal contacts and stress fibers by fibroblasts and reduced the tyrosine phosphorylation of paxillin. To examine whether tyrosine phosphorylation has similar roles in growth cone migration on a FN-treated substratum, we determined the effects of herbimycin A on growth cone migration on FN- and LM-treated substrata.

The results of these experiments, reported in Table I, show that growth cones migrating on FN were inhibited by concentrations of herbimycin A that inhibited focal contact formation by fibroblasts (Burridge et al., 1992). The drug effects were concentration dependent, and after the addition of 0.5 or 1.0 μM herbimycin A, most growth cones either stopped advancing or the neurites began to retract, as if the growth cones were detached from the substratum. In support of these measurements of growth cone migration, IRM observations of three growth cones migrating on FN showed that dark, focal-like contacts were not made after the addition of 0.5 or 1.0 μM herbimycin A. Growth cone migration on LM-treated substrata was also sensitive to 1.0 μM herbimycin A, but the extent of inhibi-

Table 1 Effect of Herbimycin A on Growth Cone Migration

Substratum	Advance before/ Advance after	Advance before/ Stop, Retract after
Fibronectin		
DMSO only	100% (12)	0%
0.05 μM herbimycin A	55% (6)	45% (5)
0.5 μM herbimycin A	36% (22)	64% (39)
1.0 μM herbimycin A	15% (4)	85% (22)
Laminin		
0.05 μM herbimycin A	85% (11)	15% (2)
0.5 μM herbimycin A	88% (21)	12% (3)
1.0 μM herbimycin A	67% (29)	33% (14)

DRG explants were cultured for 24 or 48 h in 35 mm tissue culture dishes coated overnight with 20 μg/ml FN or LM. A dish was placed on a warmed microscope stage, and images of a microscope field containing several growth cones were recorded once per minute for at least 45 min before and 45 min after the addition of herbimycin A, dissolved in DMSO. Those growth cones that were advancing at the time of addition of herbimycin A were scored as either advancing, stopped, or retracting after the addition of herbimycin A. The number of growth cones that were scored for each condition are reported in parentheses.

tion at all concentrations was much less on LM than on FN. Certainly, caution is necessary when using kinase inhibitors. However, we confined our study to measurements of growth cone migration only in a 45-min period after adding the drug. Our results support the conclusion that protein tyrosine phosphorylation is involved in growth cone migration on FN, whereas tyrosine phosphorylation is less important for growth cone migration on LM. This finding is consistent with our IRM and immunocytochemical staining results reported herein.

DISCUSSION

Adhesive interactions have two roles in guiding growth cones to synaptic targets: (1) adhesive bonds stabilize elongating nerve fibers against intrinsic tensions, and (2) receptor-ligand interactions generate transmembrane signals that may regulate growth cone function and, perhaps, gene expression. Previous studies have shown that the filopodia of chick DRG growth cones distinguish FN- from LM-treated surfaces, and the growth cones respond with changes in migratory behaviors (Gomez and Letourneau, 1994; Kuhn et al., 1995). In this study we used IRM to examine the interactions of growth cones with FN- and LM-treated substrata, and we used immunocytochemistry to determine whether components found in fibroblastic focal contacts are concentrated at adhesive sites of growth cones. Our evidence indicates that the filopodia of DRG growth cones initiate contacts on FN, but not on LM, that resemble focal contacts. In addition, the effects of an inhibitor of tyrosine kinases also indicate that tyrosine phosphorylation has a distinct role in the migration of DRG growth cones on FN-treated substrata, compared with LM. Our results are consistent with the hypothesis that differences in transmembrane interactions with the cytoskeleton and cytoplasmic signaling are important in growth cone recognition of different adhesive molecules during growth cone navigation.

The formation of focal contacts by migrating fibroblasts was observed with IRM by Izzard and Lochner (1976, 1980). Key characteristics of focal contact are that: (1) they are formed beneath microspikes or lamellipodia at the leading margin of a migrating cell; (2) they are formed ahead of existing focal contacts; (3) once formed, they remain stationary; and (4) they disappear behind the leading edge or under the body of a moving cell. Although previous studies contained static IRM images of growth cone–substratum interactions (Letourneau, 1979; Gundersen, 1988; Letourneau et al., 1988; Wu and Goldberg, 1993; Gomez and Letourneau, 1994; Zheng et al., 1994), no one has reported prolonged observations. Growth cone–substratum contacts are difficult to visualize, because growth cones and their actin filament bundles and networks are smaller than fibroblasts, and the areas of contact are small and have a less dark IRM appearance. However, with improved imaging offered by the combination of a Zeiss Antiflex objective, a SIT camera and image processing

software, we recorded time lapse IRM sequences of growth cones on FN and LM. Growth cones make contacts with FN that have the dynamic features of Izzard and Lochner's description of focal contacts (Figs. 1, 2, 3, and 4). These contacts first appeared as small dark areas, often beneath the tip of a filopodium, and the contacts remained stationary as the growth cone advanced.

IRM evidence alone does not provide structural or biochemical detail of growth cone–substratum interactions. We acquired additional evidence from immunocytochemistry. Using antibodies against proteins that are concentrated in fibroblastic focal contacts, we found that proteins with appropriate sizes of β1 integrin, paxillin, and zyxin were recognized in homogenates of chick brain and DRGs (Fig. 5). The combination of IRM records of live growth cones with fixation and immunocytochemical labeling showed that these dark contacts were strongly labeled with antibodies against phosphotyrosine and anti-β1 integrin (Fig. 6). Additional double labeling showed that β1 integrin, paxillin, and zyxin were co-concentrated at sites that labeled strongly for proteins with phosphorylated tyrosine residues (Figs. 7 and 8), another characteristic of focal contacts (Maher et al., 1985). We previously found that chick neuronal growth cones contain other proteins that are concentrated at fibroblastic focal contacts, alpha-actinin, talin, and vinculin (Letourneau and Shattuck, 1989; Cypher and Letourneau, 1991). We conclude that growth cones on a FN substratum make contacts with the dynamics and many of the components that are present in fibroblastic focal contacts.

These putative focal contacts of DRG growth cones may have two roles. First, actin filaments are linked to the plasma membrane at focal contacts (Heath and Dunn, 1978; Romer et al., 1992), indicating that these contacts can be anchor points against which traction can pull a growth cone toward the contact site. Zheng et al. (1994) measured the force required to detach growth cones from substrata, and they found that filopodia were frequently sites of such strong contact that the filopodia stretched or broke rather than detach from the substratum. The putative focal contacts of DRG growth cones first appeared beneath filopodia, possibly allowing filopodia to get traction to influence growth cone migration.

Second, the presence of zyxin and elevated tyrosine phosphorylation at the putative focal contacts suggests they are sites of cell regulation. The presence of zyxin has implications for signaling in growth cones, because zyxin contains copies of the LIM motif, which is a polypeptide domain found in several transcription factors and in proteins with interesting expression during development (Sadler et al., 1992). The formation of fibroblastic focal contacts or the clustering of integrins leads rapidly to elevated phosphorylation of focal contact components, including paxillin and pp125^{fak}, a tyrosine kinase localized to focal contacts (Kornberg et al., 1991; Burridge et al., 1992; Lipfert et al., 1992). Protein kinases in focal contacts are implicated in several signaling pathways that regulate cell behaviors (Kornberg et al., 1991; Schaller et al., 1992; Juliano and Haskill, 1993; Schaller and Parsons, 1994). Although we found no evidence by immunocytochemistry and western blot for the expression of pp125^{fak} in DRG neurons, using two commercially available antibodies, several other protein tyrosine kinases are present in nerve growth cones (Maness et al., 1988; Sobue and Kanda, 1988; Hanissian et al., 1992; Meyerson et al., 1992; Bixby and Jhabvala, 1993; Ignelzi et al., 1994; Helmke and Pfenninger, 1995).

A tyrosine kinase inhibitor, herbimycin A, inhibits growth cone migration on FN. These results, similar to previous findings with fibroblasts (Burridge et al., 1992), are consistent with the idea that tyrosine phosphorylation of focal contact proteins is involved in growth cone migration on FN. Clearly, kinase inhibitors should be used with caution, because (1) they are not specific, (2) we do not know which tyrosine kinases exist in DRG growth cones, and (3) we have not examined how the drug treatments affect protein phosphorylation. However, we confined our measurements of the drug's effects to a 45-min period after adding the drug, and the only parameter we measured was growth cone migration on FN versus on LM. The results are consistent with a proposal that adhesive interactions of DRG growth cones with FN involve a different second messenger pathway than interactions with LM.

How do DRG growth cones make these putative focal contacts on FN, but not LM? One explanation is that adhesive interactions with the two molecules differ, because FN and LM each have several cell recognition sites that bind different integrin heterodimers on DRG neurons (Humphries et al., 1988; Tomaselli et al., 1993; Letourneau et al., 1994b). Different integrin heterodimers have different interactions with cytoplasmic components (Sastry and Horwitz, 1993; Pasqualini and Hemler, 1994). In addition, FN and LM are bound by other distinct receptors on neurons, such as pro-

teoglycans and glycosyltransferases (Letourneau et al., 1994b). Finally, LM and FN are multifunctional proteins, and it is proposed that LM has domains that interfere with neuronal-substratum adhesion (Calof and Lander, 1991; Calof et al., 1994), perhaps including focal contacts. We do not propose that proteins of focal contacts, like paxillin or zyxin, have no roles in growth cone interactions with LM. Rather, we suggest that on LM these proteins are not organized into the same functional and structural sites as on FN.

The significance of these dark growth cone-substratum contacts and their cytoskeletal organization and protein tyrosine phosphorylation is that they may be important in DRG growth cones being able to distinguish FN from LM (Gundersen, 1987; Gomez and Letourneau, 1994; Kuhn et al., 1995). When filopodia on FN or LM contact the alternative substratum, growth cones initiate changes in their direction and rate of migration. The differences that we found in cytoskeletal organization and protein tyrosine phosphorylation between contacts with FN versus with LM may lead to changes in either actin filament organization and function in filopodial and lamellipodial protrusion, or in microtubule advance in growth cones. These are the two major cytoskeletal activities that generate growth cone turning or changes in migration rate (Stossel, 1993; Letourneau, 1996).

Our results are consistent with other evidence that neurite outgrowth on different cell adhesion molecules involves distinctive transmembrane signaling (Bixby and Jhabvala, 1992; Hynes and Lander, 1992; Doherty and Walsh, 1994; Doherty et al., 1994; Williams et al., 1994). Much evidence for this hypothesis is based on studies using inhibitors and blockers. Our report is significant, because we have shown a specific pattern of tyrosine phosphorylation and localization of cytoskeletal proteins in growth cones migrating on FN that is different from growth cones on LM. This is direct evidence that interactions with different cell adhesion molecules have different transmembrane effects on growth cone organization and protein phosphorylation.

Several other recent articles report results related to growth cone–substratum contacts and protein tyrosine-phosphorylation. Wu and Goldberg (1993) reported concentrated antiphosphotyrosine staining of *Aplysia* neuronal growth cones at the tips of filopodia that were not attached to a polylysine substratum. It is difficult to compare their findings with ours, because we studied growth cones migrating on purified ECM molecules, rather than polylysine, and it is unknown whether *Aplysia* growth cones express integrins and other components of focal contacts. Wu et al. (1994) also reported tyrosine phosphorylation at filopodial tips of sympathetic neuronal growth cones. We have often seen strong labeling with anti-β1 integrin at filopodial tips of DRG growth cones (Fig. 7) (Letourneau and Shattuck, 1989), but only in 17% of our observations was this accompanied by antiphosphotyrosine labeling. This diversity in the staining of filopodial tips may reflect differences in filopodial motility between the two studies. In addition, these different results may arise from the use of different neuronal types and substratum molecules that would result in dissimilar growth cone–substratum interactions.

PC12 cells and chick DRG neurons on substrata treated with LM or collagen form point contacts, where integrins interact with the cytoskeleton (Arregui et al., 1994). We also observed punctate immunofluorescence staining with the antibodies we used, but we did not assess whether phosphotyrosine was co-localized at these sites with integrin, paxillin, or zyxin. Neither did we determine the relationships of the punctate staining to our IRM images. We agree with Arregui et al. (1994) that these point contacts are not focal contacts and they may have a role in growth cone migration, when focal contacts are not made (Tawil et al., 1993).

In summary, DRG growth cones on a FN-treated substratum make substratum contacts that form in a manner and contain concentrations of specific proteins and tyrosine-phosphorylation that are characteristic of fibroblastic focal contacts. Similar growth cone–substratum contacts were not formed on LM. These specialized contacts are initiated at the distal portions and tips of filopodia, and they remain stationary, as the growth cone migrates forward. These results indicate that DRG growth cones discriminate FN- from LM-treated substrata through distinctive interactions between ECM molecules and filopodial receptors that lead to differences in the organization of cytoskeletal components and the regulation of protein function by phosphorylation.

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REFERENCES

ARREGUI, C. O., CARBONETTO, S., and MCKERRACHER, L. (1994). Characterization of neural cell adhesion sites: point contacts are the sites of interaction between integrins and the cytoskeleton. *J. Neurosci.* **14**:6967–6977.

BIXBY, J. L. and JHABVALA, P. (1992). Inhibition of tyrosine phosphorylation potentiates substrate-induced neurite growth. *J. Neurobiol.* **23**:468–480.

BIXBY, J. L. and JHABVALA, P. (1993). Tyrosine phosphorylation in early embryonic growth cones. *J. Neurosci.* **13**:3421–3432.

BRELJE, T. C., WESSENDORF, M. W., and SORENSON, R. L. (1993). Multicolor laser scanning confocal immunofluorescence microscopy: practical application and limitations. In: *Methods in Cell Biology* B. Matsumoto, Ed, Academic Press, New York, pp. 97–181.

BURRIDGE, K., TURNER, C. E., and ROMER, L. H. (1992). Tyrosine phosphorylation of paxillin and pp125FAK accompanies cell adhesion to extracellular matrix: a role in cytoskeletal assembly. *J. Cell Biol.* **119**:893–903.

CALOF, A. L. and LANDER, A. D. (1991). Relationship between neuronal migration and cell-substratum adhesion: laminin and merosin promote olfactory neuronal migration but are anti-adhesive. *J. Cell Biol.* **115**:779–794.

CALOF, A. L., CAMPANERO, M. R., O'REAR, J. J., YURCHENKO, P. D., and LANDER, A. D. (1994). Domain-specific activation of neuronal migration and neurite outgrowth-promoting activities of laminin. *Neuron* **13**:117–130.

CRAWFORD, A. W. and BECKERLE, M. C. (1991). Purification and characterization of zyxin, an 82,000-dalton component of adherens junctions. *J. Biol. Chem.* **266**:5847–5853.

CRAWFORD, A. W., MICHELSEN, J. W., and BECKERLE, M. C. (1992). An interaction between zyxin and α-actinin. *J. Cell Biol.* **116**:1381–1393.

CYPHER, C. and LETOURNEAU, P. C. (1991). Identification of cytoskeletal, focal adhesion, and cell adhesion proteins in growth cone particles isolated from developing chick brain. *J. Neurosci. Res.* **30**:259–265.

DEPASQUALE, J. A. and IZZARD, C. S. (1987). Evidence for an actin-containing cytoplasmic precursor of the focal contact and the timing of incorporation of vinculin at the focal contact. *J. Cell Biol.* **105**:2803–2809.

DOHERTY, P. and WALSH, F. S. (1994). Signal transduction events underlying neurite outgrowth stimulated by cell adhesion molecules. *Curr. Opin. Neurobiol.* **4**: 49–55.

DOHERTY, P., FURNESS, J., WILLIAMS, E. J., and WALSH, F. S. (1994). Neurite outgrowth stimulated by the tyrosine kinase inhibitor herbimycin A requires activation of tyrosine kinases and protein kinase C. *J. Neurochem.* **62**:2124–2131.

FUKAZAWA, H., LI, P.-M., YAMAMOTO, C., MURAKAMI, Y., MIZUNO, S., and UEHARA, Y. (1991). Specific inhibition of cytoplasmic tyrosine kinases by herbimycin A *in vitro*. *Biochem. Pharmacol.* **42**:1661–1671.

GOMEZ, T. M. and LETOURNEAU, P. C. (1994). Filopodia initiate choices made by sensory neuron growth cones at laminin/fibronectin borders *in vitro*. *J. Neurosci.* **14**:5959–5972.

GUNDERSEN, R. W. (1987). Response of sensory neurites and growth cones to patterned substrata of laminin and fibronectin in vitro. *Dev. Biol.* **121**:423–431.

GUNDERSEN, R. W. (1988). Interference reflection microscopic study of dorsal root growth cones on different substrates: assessment of growth cone-substrate contacts. *J. Neurosci. Res.* **21**:298–306.

HANISSIAN, S. H., CHATILA, T. and SAHYOUN, N. E. (1992). Association of neuronal pp60c-src with growth cone glycoproteins of rat brain. *J. Neurobiol.* **23**:803–813.

HEATH, J. P. and DUNN, G. A. (1978). Cell to substratum contacts of chick fibroblasts and their relation to the microfilament system. A correlated interference-reflexion and high voltage electron-microscope study. *J. Cell Sci.* **29**:197–212.

HEIDEMANN, S. R., LAMOUREUX, P., and BUXBAUM, R. E. (1991). On the cytomechanics and fluid dynamics of growth cone motility. *J. Cell Sci.* **15**(suppl.):35–44.

HELMKE, S. and PFENNINGER, K. H. (1995). Growth cone enrichment and cytoskeletal association of non-receptor tyrosine kinases. *Cell Motil. Cytoskel.* **30**: 194–207.

HUMPHRIES, M. J., AKIYAMA, S. K., KOMIRIYA, A., OLDEN, K., and YAMADA, K. M. (1988). Neurite extension of chicken peripheral nervous system neurons on fibronectin. Relative importance of specific adhesion sites in the central cell-binding domain and the alternatively spliced type III connecting segment. *J. Cell Biol.* **106**:1289–1298.

HYNES, R. O. (1992). Integrins: versatility, modulation, and signaling in cell adhesion. *Cell* **69**:11–25.

HYNES, R. O. and LANDER, A. D. (1992). Contact and adhesive specificities in the associations, migrations, and targeting of cells and axons. *Cell* **68**:303–322.

IGNELZI, M. A., JR., MILLER, D. R., SORIANO, P., and MANESS, P. F. (1994). Impaired neurite outgrowth of src-minus cerebellar neurons on the cell adhesion molecule L1. *Neuron* **12**:873–884.

IZZARD, C. S. and LOCHNER, L. R. (1976). Cell-to-substrate contacts in living fibroblasts: an interference reflexion study with an evaluation of the technique. *J. Cell Sci.* **21**:129–159.

IZZARD, C. S. and LOCHNER, L. R. (1980). Formation

of cell-to-substrate contacts during fibroblast motility: an interference-reflexion study. *J. Cell Sci.* **42**:81–116.

JULIANO, R. L. and HASKILL, S. (1993). Signal transduction from extracellular matrix. *J. Cell Biol.* **120**: 577–585.

KATER, S. B. and MILLS, L. R. (1991). Regulation of growth cone behavior by calcium. *J. Neurosci.* **11**: 891–899.

KORNBERG, L. J., EARP, H. S., TURNER, C. E., PROCKOP, C., and JULIANO, R. L. (1991). Signal transduction by integrins: increased protein tyrosine phosphorylation caused by clustering $\beta 1$ integrins. *Proc. Natl. Acad. Sci. USA* **88**:8392–8396.

KUHN, T. B., SCHMIDT, M. F., and KATER, S. B. (1995). Laminin and fibronectin guideposts signal sustained but opposite effects to passing growth cones. *Neuron* **14**:275–285.

LANKFORD, K. L. and LETOURNEAU, P. C. (1989). Evidence that calcium may control neurite outgrowth by regulating the stability of actin filaments. *J. Cell Biol.* **109**:1229–43.

LANKFORD, K. L. and LETOURNEAU, P. C. (1991). Roles of actin filaments and three second-messenger systems in short-term regulation of chick dorsal root ganglion neurite outgrowth. *Cell Motil. Cytoskel.* **20**: 7–29.

LETOURNEAU, P. C. (1975). Possible roles for cell-to-substratum adhesion in neuronal morphogenesis. *Dev. Biol.* **44**:77–91.

LETOURNEAU, P. C. (1979). Cell-substratum adhesion of neurite growth cones, and its role in neurite elongation. *Exp. Cell Res.* **124**:127–138.

LETOURNEAU, P. C. (1989). Nerve cell shape. In: *Cell Shape, Determinants, Regulation and Regulatory Roles* F. Bronner and W. D. Stein, Eds. Academic Press, New York, pp. 247–289.

LETOURNEAU, P. C. (1996). The cytoskeleton in nerve growth cone motility and axonal pathfinding. *Perspect Dev. Neurobiol.* (in press).

LETOURNEAU, P. C. and CYPHER, C. (1991). Regulation of growth cone motility. *Cell Motil. Cytoskel.* **20**: 267–271.

LETOURNEAU, P. C. and SHATTUCK, T. A. (1989). Distribution and possible interactions of actin-associated proteins and cell adhesion molecules of nerve growth cones. *Development* **105**:505–519.

LETOURNEAU, P. C., PECH, I. V., ROGERS, S. L., PALM, S. L., MCCARTHY, J. B., and FURCHT, L. T. (1988). Growth cone migration across extracellular matrix components depends on integrin, but migration across glioma cells does not. *J. Neurosci. Res.* **21**:286–297.

LETOURNEAU, P. C., SHATTUCK, T. A., ROCHE, F. K., TAKEICHI, M., and LEMMON, V. (1990). Nerve growth cone migration onto Schwann cells involves the calcium-dependent adhesion molecule, N-cadherin. *Dev. Biol.* **138**:430–442.

LETOURNEAU, P. C., GOMEZ, T. M. and SNOW, D. M. (1994a). Growth cone motility: substratum-bound molecules, cytoplasmic [Ca^{++}] and Ca^{++}-regulated proteins. *Prog. Brain Res.* **102**:35–48.

LETOURNEAU, P. C., CONDIC, M. L., and SNOW, D. M. (1994b). Interactions of developing neurons with the extracellular matrix. *J. Neurosci.* **14**:915–928.

LIPFERT, L., HAIMOVICH, B., SCHALLER, M. D., COBB, B. S., PARSONS, J. T. and BRUGGE, J. S. (1992). Integrin-dependent phosphorylation and activation of the protein tyrosine kinase $pp125^{FAK}$ in platelets. *J. Cell Biol.* **119**:905–912.

MAHER, P. A., PASQUALE, E. B., WANG, J. Y. J., and SINGER, S. J. (1985). Phosphotyrosine-containing proteins are concentrated in focal adhesions and intercellular junctions in normal cells. *Proc. Natl. Acad. Sci. USA* **82**:6576–6580.

MANESS, P. F., AUBRY, M., SHORES, C. G., FRAME, L., and PFENNINGER, K. H. (1988). *c-src* gene product in developing rat brain is enriched in nerve growth cone membranes. *Proc. Natl. Acad. Sci. USA* **85**:5001–5005.

MEYERSON, G., PFENNINGER, K. H., and PAHLMAN, N. E. (1992). A complex consisting of pp60c-src/pp60c-srcN and a 38 kDa protein is highly enriched in growth cones from differentiated SH-SY5Y neuroblastoma cells. *J. Cell Sci.* **103**:233–243.

PASQUALINI, R. and HEMLER, M. E. (1994). Contrasting roles for integrin $\beta 1$ and $\beta 5$ cytoplasmic domains in subcellular localization, cell proliferation and cell migration. *J. Cell Biol.* **125**:447–460.

ROGERS, S. L., EDSON, K. J., LETOURNEAU, P. C., and MC LOON, S. C. (1986). Distribution of laminin in the developing nervous system of the chick. *Dev. Biol.* **113**:429–435.

ROGERS, S. L., LETOURNEAU, P. C., and PECH, I. V. (1989). The role of fibronectin in neural development. *Dev. Neurosci.* **11**:248–265.

ROMER, L. H., BURRIDGE, K., and TURNER, C. E. (1992). Signaling between the extracellular matrix and the cytoskeleton: tyrosine phosphorylation and focal adhesion assembly. *Cold Spring Harbor Symp. Quant. Biol.* **57**:193–202.

SADLER, I., CRAWFORD, A. W., MICHELSEN, J. W., and BECKERLE, M. C. (1992). Zyxin and cCRP: two interactive LIM domain proteins associated with the cytoskeleton. *J. Cell Biol.* **119**:1573–1588.

SASTRY, S. K. and HORWITZ, A. F. (1993). Integrin cytoplasmic domains: mediators of cytoskeletal linkages and extra- and intracellular initiated transmembrane signaling. *Curr. Opin. Cell Biol.* **5**:819–831.

SCHALLER, M. D. and PARSONS, J. T. (1994). Focal adhesion kinase and associated proteins. *Curr. Opin. Cell Biol.* **5**:705–709.

SCHALLER, M. D., BORGMAN, C. A., COBB, B. S., VINES, R. R., REYNOLDS, A. B., and PARSONS, J. T. (1992). pp125FAK, a structurally unique protein tyrosine kinase associated with focal adhesions. *Proc. Natl. Acad. Sci. USA* **89**:5192–5196.

SOBUE, K. and KANDA, K. (1988). Localization of

pp60c-src in growth cones of PC12 cells. *Biochem. Biophys. Res. Commun.* **157**:1383–1389.

STOSSEL, T. P. (1993). On the crawling of animal cells. *Science* **260**:1086–1094.

TAWIL, N., WILSON, P., and CARBONETTO, S. (1993). Integrins in point contacts mediated cell spreading: factors that regulate integrin accumulation in point contacts vs. focal contacts. *J. Cell Biol.* **120**:261–271.

TODD, I., MELLOR, J. S., GINGELL, D. (1988). Mapping cell-glass contacts of Dictyostelium amoebae by total internal reflection aqueous fluorescence overcomes a basic ambiguity of interference reflection microscopy. *J. Cell Sci.* **89**:107–114.

TOMASELLI, K. J., DOHERTY, P., EMMETT, C. J., DAMSKY, C. H., WALSH, F. S., and REICHARDT, L. F. (1993). Expression of β1 integrins in sensory neurons of the dorsal root ganglion and their functions in neurite outgrowth on two laminin isoforms. *J. Neurosci.* **13**:4880–4888.

TOWBIN, H., STAEHELIN, T., and GORDON, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**: 4350–4354.

TURNER, C. E. (1991). Paxillin is a major phosphotyrosine-containing protein during embryonic development. *J. Cell Biol.* **115**:201–207.

TURNER, C. E. (1994). Paxillin: a cytoskeletal target for tyrosine kinases. *Bioessays* **16**:47–52.

TURNER, C. E., GLENNEY, J. R., and BURRIDGE, K. (1990). Paxillin: a new vinculin-binding protein in focal adhesions. *J. Cell Biol.* **111**:1059–1068.

WILLIAMS, E. J., WALSH, F. S., and DOHERTY, P. (1994). Tyrosine kinase inhibitors can differentially inhibit integrin dependent and CAM-stimulated neurite outgrowth. *J. Cell Biol.* **124**:1029–1038.

WU, D.-Y. and GOLDBERG, D. J. (1993). Regulated tyrosine phosphorylation at the tips of growth cone filopodia. *J. Cell Biol.* **123**:653–644.

WU, D.-Y., WANG, L.-C., GOLDBERG, D. J., and MASON, C. A. (1994). Tyrosine phosphorylation at filopodial tips of vertebrate growth cones. *Soc. Neurosci. Abstr.* **20**:1081.

ZHENG, J., BUXBAUM, R. E., and HEIDEMANN, S. R. (1994). Measurements of growth cone adhesion to culture surfaces by micromanipulation. *J. Cell Biol.* **127**:2049–2060.