Distribution and possible interactions of actin-associated proteins and cell adhesion molecules of nerve growth cones

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Summary

Actin filaments and their interactions with cell surface molecules have key roles in tissue cell behaviour. Axonal pathfinding during embryogenesis, an especially complex cell behaviour, is based on the migration of nerve growth cones. We have used fluorescence immunocytochemistry to examine the distribution in growth cones, their filopodia and lamellipodia of several actin-associated proteins and nerve cell adhesion molecules. The leading margins of chick dorsal root ganglion nerve growth cones and their protrusions stain strongly for f-actin, filamin, a-actinin, myosin, tropomyosin, talin and vinculin. MAP2 is absent from DRG growth cones, and staining for spectrin/fodrin extends into growth cones, but not along filopodia. Thus, organization of the leading margins of growth cones may strongly resemble the leading lamella of migrating fibroblasts. The adhesion-mediating molecules integrin, L1, N-CAM and A-CAM are all found on DRG neurites and growth cones. However, filopodia stain relatively more strongly for integrin and L1 than for A-CAM or N-CAM. In fact, the $180 \times 10^3 M_r$ form of N-CAM may be absent from most of the length of filopodia. DRG neurones cultured in cytochalasin B display differences in immunofluorescence staining which further emphasize that these adhesion molecules interact differentially with the actin filament system of migrating growth cones. Several models for neuronal morphogenesis emphasize the importance of regulation of the expression of adhesion molecules. Our results support hypotheses that cellular distribution and transmembrane interactions are key elements in the functions of these adhesion molecules during axonal pathfinding.

Key words: actin, adhesion, growth cone, motility, pathfinding, nerve growth, cell adhesion, protein.

Introduction

Actin filament systems and their interactions with molecules that face the extrinsic environment have key roles in tissue cell behaviour. Cell migration, cell adhesion, cell shape and cell communication all involve structural associations of actin filaments with components that face both the internal and external faces of the plasma membrane. A particularly accessible system for study of cytoskeletal–membrane interactions is the focal contacts or adhesion plaques of fibroblastic cells on in vitro surfaces coated with molecules of extracellular matrices. At these sites actin-associated proteins, including a-actinin, vinculin, and talin, participate in the linkage of actin filament bundles to integrins, which are transmembrane receptors for extracellular matrix proteins (Burridge et al. 1987; Buck & Horwitz, 1987; Hynes, 1987). These associations may participate in the generation of mechanical forces and other activities related to cell movement, adhesion or shape. Cell–cell adhesions may involve similar supramolecular complexes, although it is not clear whether the components of adhesion plaques are involved in the same ways (Geiger et al. 1987).

Axonal pathfinding during embryogenesis is based on the migratory activities of nerve growth cones (Bentley & Caudy, 1983; Bentley & Keshishian, 1982; Letourneau, 1985; Raper et al. 1983a,b, 1984; Tosney & Landmesser, 1985). The leading margins of growth cones contain dense networks and bundles of actin filaments. Many regulatory actin-associated proteins have been localized in growth cones, although the published studies are too diverse to provide a clear picture of actin filament organization in growth cones. Several adhesion-mediating molecules have been characterized and localized in developing neural tissues, and models for neuronal morphogenesis propose close regulation of the expression of these and other adhesion molecules (Chuoug et al. 1987; Edelman, 1988; Hatta et al. 1987; Letourneau, 1985). The models predict that there is temporal, spatial and cell-specific regulation of the distribution and activity of adhesion molecules on nerve growth cones. However, few studies have closely examined the distributions of identified adhesion molecules on growth cones and their filopodial and lamellipodial protrusions.
Thanks to the generous donation of antibodies by several colleagues, we have examined the distribution of eight actin-associated proteins and four cell adhesion molecules on neurites and growth cones of chick embryo sensory neurons. We have used immunocytochemistry, because we wanted to examine growth cone morphology and because biochemical purification of growth cones from a single neural type is extremely difficult. Our results suggest that many of the actin-associated proteins of fibroblast adhesion plaques may be present in growth cones and may be concentrated in the leading edge and its protrusions. The surfaces of sensory growth cones stain with antibodies against the cell adhesion molecules, integrin, N-CAM, 8D9 (equivalent to Ng-CAM or L1), and A-CAM (equivalent or closely related to N-cadherin). However, filopodial and lamellipodial staining varies with different antibodies, indicating that growth cone behavior involves more than the mere presence of certain adhesive ligands. In addition, growth cones treated with low concentrations of the drug cytochalasin B show differences in the co-distribution of actin, actin-associated proteins, and adhesion molecules which suggest that adhesive ligands interact differently with the actin filament system. Thus, a firm understanding of growth cone navigation and neuronal morphogenesis will require more information about the basic cell biology of these actin-associated and adhesion-mediated molecules.

Materials and methods

Cell culture

Dorsal root ganglia (DRG) were removed from 10- or 11-day-old chicken embryos and were dissociated with trypsin, as previously described (Letourneau, 1975). The cell suspensions were plated in a 50:50 mixture of F14 nutrient mixture (Gibco, Grand Island, NY) and F14 medium, previously conditioned by dense monolayers of chick heart cells (Ludueña, 1973). The medium also contained 10 ng/ml-1 nerve growth factor (a gift from Dr Bernard Mirkin, University of Minnesota). Cells that were exposed to cytochalasin B (CB; Sigma Chemical Corp., St Louis, MO) were treated with 0-3 µg/ml-1 CB dissolved in DMSO. CB was added at the beginning of the culture period. All cells were plated on polyornithine-treated glass coverslips (Letourneau, 1975) in 35 mm plastic Petri dishes (Falcon, Becton-Dickinson Laboratory, Lincoln Park, NJ), and the dishes were incubated at 37°C in a 5% CO2 humidified incubator.

Antibodies

Antisera and antibodies to the following proteins were generously provided by the indicated individuals: human platelet myosin (polyclonal antiserum, Dr Keigi Fujiwara, National Cardiovascular Center, Japan; Fujiwara & Pollard, 1976); chicken α-actinin, talin, vinculin, brain spectrin (polyclonal antiserum, Dr Keith Burridge, University of North Carolina; Burridge & Farquaher, 1982; Burridge et al. 1982); chicken gizzard tropomyosin (polyclonal antiserum, Dr James Lin, University of Iowa; Matsumura et al. 1983); rat brain fodrin (affinity-purified polyclonal antibody, Dr Mark Willard, Washington University; Levine & Willard, 1981), rat MAP2 (monoclonal, Dr Lester Binder, University of Alabama-Birmingham; Binder et al. 1984), chicken integrin (polyclonal antiserum, Dr Alan Horwitz, University of Illinois; Bozycek & Horwitz, 1986); chicken N-CAM, 8D9 (monoclonals and IgG fraction of polyclonal anti-avian L1, Dr Vance Lemmon, University of Pittsburgh; Lagenauer & Lemmon, 1987; Lemmon & McLoon, 1986; Lemmon et al. 1982); 180 × 10^6 M, form of mouse N-CAM (rat monoclonal clone 481, Dr Melitta Schachner, University of Heidelberg, West Germany; Pollerberg et al. 1985); chicken cardiac A-CAM, gizzard vinculin (monoclonals, Dr Benjamin Geiger, Weizmann Institute, Israel; Geiger et al. 1987), chicken N-cadherin (polyclonal antiserum, Dr M. Takeichi, Kyoto University, Japan) human plasma fibronectin (affinity-purified polyclonal antibody, Dr L. Furcht, University of Minnesota; Palm & Furcht, 1983). Rabbit antibodies to chicken gizzard filamin and erythrocyte spectrin were purchased from Miles Scientific, Naperville, IL. A monoclonal antibody to chick brain β-tubulin was purchased from Amersham Corp., and fluorescent secondary antibodies were purchased from Organon Teknika-Cappel, Malvern, PA. Rhodamine- and fluorescein-derived phalloidin were purchased from Molecular Probes, Eugene, OR.

Immunocytochemistry

The standard fixative was 4% paraformaldehyde in Ca²⁺- and Mg²⁺-free PBS (CMF-PBS). Sometimes 0-01-0-04% glutaraldehyde was also present. Cultures were fixed for 20 min after first rinsing with warm serum-free medium. After fixation, the coverslips were rinsed and incubated 10 min in 1 mg/ml-1 sodium borohydride in CMF-PBS. Next, the cells were soaked for 15 min in CMF-PBS with 5 mg/ml-1 BSA (Miles Scientific). If the cells were labelled with fluorescent phalloidin, it was added at 3 × 10⁻⁷ M to the soak at this time. Next the cells were exposed to the primary antibodies for 60 min, diluted in CMF-PBS with BSA at dilutions recommended by their donors. The coverslips were then rinsed, soaked, incubated with 1/100 dilutions of the secondary antibodies for 60 min, rinsed, soaked again and finally mounted on glass slides in a mounting medium containing polyvinyl alcohol.

This basic procedure was done in three ways, according to when the cells were extracted with Triton X-100. (1) Cultures were fixed, but not extracted with Triton X-100 at any time during the procedure. (2) Cultures were fixed, rinsed and then exposed to 0-1% Triton X-100 in the first soak and every subsequent step. (3) All cultures that were to be labelled with antibodies to actin-associated proteins were fixed and extracted simultaneously with Triton X-100 added to the fixation solution, and then Triton X-100 was present in all subsequent solutions. In procedure (1) the cells are not extracted, but we have good evidence using a monoclonal anti-tubulin that primary and secondary antibody molecules can enter unextracted embryonic cells to produce intracellular labelling in large areas of cytoplasm. Procedure (2) promotes access of antibodies to intracellular antigens and does not seem to remove the cell surface proteins whose distributions we examined. Procedure (3) results in loss of membrane components and many soluble proteins, but retains actin-associated proteins that are bound to the Triton X-100-resistant cytoskeleton.

Photography

Photographs of stained cells were taken on a Zeiss Model IM microscope, using a 35 mm camera with Kodak Tri-X film. A 63× planapochromat lens was used in all cases, and exposure times were 25–50 s.
Results

Distribution of actin-associated proteins

As found previously, the staining with fluorescent phalloidin indicated that actin filaments are highly concentrated in the leading motile margins of DRG growth cones, while actin filaments are less concentrated in neurites and proximal portions of growth cones (Letourneau, 1981, 1983; Letourneau et al. 1986). This agrees with electron microscopic observations of dense networks and bundles of microfilaments at the leading edges of growth cones, while in neurites microfilaments are primarily found in networks of short filaments in the subcortical cytoplasm (Letourneau, 1979, 1983).

Actin-crosslinking proteins

Filamin and α-actinin are two actin-associated proteins that form physical connections between actin filaments, creating three-dimensional networks in isolated preparations (Pollard & Cooper, 1986; Stossel, 1984). Immunoreactivity for both these proteins was concentrated in the leading margins and filopodia of growth cones, suggesting that these proteins participate in the organization of actin networks and bundles in the motile margin (Fig. 1A–C). Two other proteins that can cross-link actin filaments, spectrin (Pollard & Cooper, 1986; Stossel, 1984) and MAP2 (Vallee et al. 1984) may also be present in DRG neurites, but immunoreactivity for these proteins was very different from staining for filamin and α-actinin. As shown previously for sympathetic neurones in vitro (Peng et al. 1986), strong MAP2 staining occurred in the cell soma and proximal portions of neurites, but distal neurites and growth cones were completely unstained by several monoclonal antibodies to MAP2 (not shown). DRG neurites and growth cones were stained with three different polyclonal antibodies against chick erythrocyte spectrin, chicken brain spectrin (fodrin) and rat brain spectrin (fodrin), respectively. The staining pattern of all of these antibodies was very similar. Immunoreactivity for spectrin or fodrin was strong along neurites and into the body of growth cones, but filopodia were unstained or were stained only at their bases (Fig. 1D,E). Thus, the organization of networks and bundles of actin filaments at the growth cone margin and in its dynamic protrusions may involve filamin and α-actinin, but our immunocytochemical evidence suggests that fodrin (spectrin) and MAP2 are not similarly involved in growth cone motility.

Myosin and tropomyosin

Myosin is a very interesting actin-associated protein, because of its role in the production of mechanical forces. An antibody to human platelet myosin, which has previously been used to stain chick fibroblast cells (Tomasek et al. 1982), labelled DRG neurites and growth cones. In cells that were double labelled for tubulin and myosin (Fig. 2A,B), myosin immunoreac-

![Fig. 1. (A) Filamin immunoreactivity in DRG growth cone (G) and neurites (N). Note that filopodia stain particularly brightly (arrows). (B) Immunoreactivity for α-actinin in growth cone leading margin. Note coincidence of α-actinin staining with distribution of F-actin, as revealed by phalloidin staining in C. (D) Immunoreactivity for rat fodrin is characteristic of staining by antibodies to rat fodrin, chick fodrin, or chick erythrocyte spectrin. Neurites and main body of growth cones are stained, but filopodia are not stained or only proximal portions of filopodia stain for fodrin. Compare anti-fodrin staining of filopodia (arrows) to filopodial staining with fluorescent phalloidin as shown in E. x900.](image-url)
tivity extended from neurites into filopodia, beyond the
distal ends of microtubules, which stopped at the bases
of filopodia (Letourneau, 1979, 1983; Letourneau et al.
1986). Preimmune serum from the rabbit that was
immunized with human platelet myosin did not stain
growth cones and neurites above background levels
(Fig. 10), indicating that anti-myosin antibodies are
binding specifically to the DRG growth cones and
filopodia. Filopodia and lamellipodia also stained
strongly with a polyclonal antiserum that recognizes all
forms of chicken tropomyosin (Fig. 2C,D). Tropomyo-
sin binds to the sides of actin filaments and protects
them from spontaneous fragmentation and from frag-
mentation induced by several proteins (Pollard &
Cooper, 1986). Tropomyosin regulates actin-myosin
interaction in striated muscle, but its role in non-muscle
cells is unclear.

Membrane attachment proteins
It has already been noted that staining with three
different antibodies against spectrin/fodrin, which is
involved in attachment of actin filaments to the erythro-
cyte membrane, was diminished or absent in the leading
margins and filopodia of growth cones. Two prominent
components of fibroblast adhesion plaques, talin and
vinculin, seemed to be present in the motile portions of
growth cones, although the staining intensity for these
proteins was lower than in the larger focal contacts of
nearby fibroblasts. Staining for talin and vinculin ex-
tended out along the total length of filopodia, in a
pattern which was distinctly different from immuno-
reactivity for spectrin or fodrin (Fig. 3A–E). Staining
of growth cones and filopodia was not seen when the
anti-talin serum was preadsorbed with purified talin
(Fig. 10B). Thus, talin and vinculin may be involved in
the interactions of actin filaments and the plasma
membrane during growth cone motility and adhesion.

Cell surface adhesion molecules
Immunostaining with antibodies to cell adhesion mol-
ecules was done on cultures that were fixed and not
extracted, and with cultures that were extracted with
TX-100 after fixation. The staining appeared similar in
both cases, and all the pictures here are from cultures
that were extracted after fixation. In any case, one
should not assume that antibodies do not enter fixed,
unextracted chick embryo neurones. We have good
examples in which microtubules were brightly stained
by anti-tubulin throughout large portions of fixed,
unextracted chick neurones. Adhesion of growth cones
to fibronectin, laminin, and other components of extra-

Fig. 2. (A) Neurite and growth cone staining by antibody to
human platelet myosin. (B) Microtubule staining with
monoclonal antibody to β-tubulin. Myosin staining extends
from neurites throughout growth cone and along filopodia
(arrows). Microtubules pass from neurite into growth cone,
but do not extend the length of filopodia. (C) Staining for
tropomyosin in growth cone leading margin is very similar
to colocalization of f-actin with fluorescent phallolidin, as
seen in D. ×840.

Fig. 3. (A) Immunoreactivity for talin is present in leading
margin and extends the lengths of growth cone filopodia
(arrows). Immunoreactivity for vinculin (B and C) is also
relatively concentrated in filopodia (arrows), as seen by
comparison with phallolidin binding (D and E). ×880.
cellular matrices involves the integrin family of matrix receptors (Bozyczko & Horwitz, 1986; Letourneau et al. 1988; Tomaselli et al. 1986). Staining with a polyclonal antiserum to β-integrin indicated that integrin molecules are present on DRG neurites, growth cones and along the complete length of filopodia (Fig. 4A–D). The fluorescent staining of filopodia was often bright, and a unique feature of the anti-integrin staining was that tips of filopodia often stained particularly intensely. The comparison of integrin staining with staining by fluorescent phalloidin clearly showed that the bright terminal staining for integrin was at the ends of filopodia. This is important in considering the role of integrins in growth cone contact with other cells or surfaces that contain components of extracellular matrices.

DRG neurites were also stained by antibodies to three adhesive glycoproteins implicated in neurite elongation, avian L1 (Ng-CAM, NILE), N-CAM and A-CAM (N-cadherin). Immunoreactivity with the monoclonal antibody 8D9 indicated that avian L1 is present on neurites, growth cones and filopodia. The most striking observation was that the immunoreactivity for L1 on filopodia was often quite strong, and the coincident staining with 8D9 and fluorescent phalloidin indicated that this glycoprotein is present on the total length of filopodia (Fig. 5A–D). A polyclonal antibody against avian L1 produced the same strong labelling of filopodia as the 8D9 monoclonal (not shown). The strong staining of filopodia for L1 was quite unlike staining with antibodies to A-CAM, N-cadherin or N-CAM. Immunoreactivity for A-CAM or N-cadherin was present on neurites, growth cones and filopodia, although the staining was continuous and might have been stronger on neurites than on the leading edges of growth cones and their protrusions (Fig. 6A,B). This certainly differed from the labelling with anti-integrin or 8D9. Filopodia were also labelled quite differently by two different antibodies against N-CAM. An antibody (1A6) that recognizes the three polypeptide forms of N-CAM (120, 140 and 180×10^3) stained neurites and growth cones in a continuous manner, but like the

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**Fig. 4.** (A and C) Labelling of neurites and growth cones with anti-integrin. Note bright spot of integrin staining at tips of many filopodia (arrows). This terminal staining is seen only with anti-integrin among the cell adhesion molecules. Corresponding staining with fluorescent phalloidin shows the length of filopodia in B and D. ×945.

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**Fig. 5.** (A and C). Neurite labelling with anti-chicken L1. Staining of many filopodia is particularly strong and extends the length of filopodia (arrows). Filopodial length is shown by corresponding labelling with fluorescent phalloidin in B and D. ×880.
staining with anti-A-CAM, anti-N-CAM staining of filopodia was not stronger than the staining of neurites (Fig. 6C,D). A striking difference in anti-N-CAM staining of filopodia vs growth cones or neurites was seen with a monoclonal antibody (clone 481; Pollerberg et al. 1985), which recognizes only the $180 \times 10^3 M_r$ form of N-CAM. This antibody stained growth cones and the bases of filopodia strongly, but the middle and distal portions of filopodia were unstained by this antibody to the $180 \times 10^3 M_r$ form of N-CAM (Fig. 7). This is interesting, because of the reported selective binding of purified spectrin to the $180 \times 10^3 M_r$ form of N-CAM (Pollerberg et al. 1986, 1987). Remember that immuno-reactivity for spectrin and fodrin was similarly reduced in filopodia.

**Cell surface and cytoskeletal staining of cytochalasin-treated neurones**

On an adhesive substratum, neurite elongation occurs at reduced levels and with abnormal growth cone motility in the presence of low levels of the drug cytochalasin B (CB; Marsh & Letourneau, 1984). In the presence of 0-3 μg ml$^{-1}$ CB, filopodia and lamellipodia are not extended from neurite tips, although blunt protrusions and small blebs are produced. CB disrupts the polymerization and organization of the actin fila-

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**Fig. 6.** Labelling with anti-A-CAM is shown in A. Surfaces of neurite, growth cone and filopodia are all stained, although neurite stains most strongly. (B) Corresponding binding of phalloidin. Similar differences in the intensity of neurite and filopodial staining are seen on neurites labelled with an antibody to all forms of N-CAM (C). Staining is continuous from neurites to growth cones, but fluorescence of filopodia appears less than neurites. Corresponding phalloidin staining is shown in D. ×900.

**Fig. 7.** Selective labelling for N-CAM$_{180}$ on growth cones is shown in A with corresponding phalloidin labelling shown in B. The main growth cone bodies stain strongly for N-CAM$_{180}$ (arrows in A), but filopodia are not labelled by anti-N-CAM$_{180}$ (arrows in B). The large flat cell in B is a fibroblast. ×900.
ment system in growth cones, and we used CB to examine the relationships between actin filaments, actin-associated proteins, and cell adhesion molecules under these altered conditions.

**Actin-associated proteins**

In DRG neurones cultured 24 h in 0-3 μg ml⁻¹ CB, the ends of many neurites contained dense spots or foci of staining with fluorescent phalloidin. This staining corresponded to previous observation in whole-mounted CB-treated DRG neurites of dense masses from which many short actin filaments projected (Marsh & Letourneau, 1984). Several of the actin-associated proteins showed colocalization with the concentrated staining by fluorescent phalloidin in CB-treated neurites. Strong immunoreactivity for filamin, tropomyosin, myosin, talin and vinculin coincided with bright regions of staining with fluorescent phalloidin (Fig. 8A–J; α-actinin was not examined). On the other hand, staining for fodrin or spectrin was not concentrated at the distal areas of bright phalloidin staining (Fig. 8K,L). Immunoreactivity for fodrin/spectrin was strong in neuronal perikarya and along the length of neurites, but staining of distal neurites and neurite tips for fodrin or spectrin was much reduced. Thus, the different distribution of spots of dense phalloidin staining, similar to what was induced by CB.

**Cell surface adhesion molecules**

Immunoreactivity of CB-treated neurones with antibodies to integrin showed a strong localization with spots of dense phalloidin staining, similar to what was seen with several actin-associated proteins. The immunoreactivity for integrin was much more intense at neurite tips, where phalloidin bound, than anti-integrin staining elsewhere along CB-treated neurites (Fig. 9A,B). This differed from staining with antibodies against N-CAM, A-CAM or LI. These antibodies tended to stain neuronal perikarya and the proximal portions of neurites more strongly and uniformly than anti-integrin (Fig. 9C–H). Sometimes, immunoreactivity for L1 and A-CAM was enhanced at distal sites of bright phalloidin staining, but this was relative to adjacent regions, and not as strong as along the proximal portions of the same neurites. In summary, immunostaining of CB-treated neurites for N-CAM, A-CAM, and L1 tended to be similar, while integrin staining was different, in that it was strongest at distal sites that corresponded to concentrated phalloidin staining.

**Controls for immunocytochemistry and immunoreaction with transferred proteins**

Most of the antibodies used in this study were directed against chicken proteins, so problems of cross species reactivity did not exist. Antibodies acquired from other researchers have also been well characterized in papers cited in this text. Most of the actin-associated proteins were purified from non-nervous tissues, and it is possible that the antibodies may react with different antigens in cells from the nervous system. In addition, fixation and extraction of growth cones might produce altered protein conformations that are abnormally recognized by specific antibodies. For most of the antibodies that we used, we did not have access to preimmune serum or antiserum that were preadsorbed with the antigen. Thus, we are cautious, and claim only that this immunocytochemical staining is one type of evidence that these antigens or molecules like these antigens are present in growth cones and their filopodia. Some of the staining that was done with control antisera is shown in Fig. 10. Our experience with these controls and many other antibodies indicates that growth cones and filopodia do not bind antibodies generally or in a non-specific manner. Over many experiments with antibodies against extracellular and intracellular antigens such as laminin, desmin, S100 protein or fibronectin, growth cones were unstained, while nearby non-neuronal cells were strongly stained. Fig. 10F shows an example of strong staining of fibroblasts for fibronectin, while adjacent growth cones were unstained. In addition, the fact that filopodia remained unstained, while nearby growth cone regions stained with antibodies against tubulin (Fig. 2B) or neurofilament proteins (Shaw et al. 1981) is another indication that filopodia remain intact and are not stained non-specifically or artifactually with our procedures.

To characterize further some of the antigens that were localized in DRG growth cones, Western blot analysis was done by reaction of several antibodies against proteins from chick embryo brain. 12-day chick embryo brains were dissected, and a procedure for ‘growth cone’ isolation was carried out (Dr Christopher Cypher, personal communication), according to Pfenninger et al. (1983). The ‘growth cone’ preparations were extracted, subjected to electrophoresis and transferred to nitrocellulose paper (Sheir-Neiss et al. 1978; Towbin et al. 1979). The Western blot analysis involved three of the antibodies that were used for immunostaining; anti-human platelet myosin, anti-chicken gizzard talin, anti-chicken gizzard talin preadsorbed with purified talin, anti-chicken gizzard tropomyosin, anti-chicken N-CAM, and anti-chicken L1. The anti-myosin reacted with a single band at a size appropriate for the high molecular weight chain of myosin, anti-talin reacted with a single band at the proper size for talin, preadsorbed anti-talin showed no reactivity with the transferred proteins, anti-tropomyosin reacted with several bands that were appropriate for different tropomyosin isoforms, and anti-N-CAM and anti-L1 reacted with several bands at expected molecular weight ranges of these glycoproteins. From this we can conclude only that these antibodies are binding to proteins with the appropriate size of their specific antigens. Much work beyond the scope of this study will be required to prove that these particular proteins are contained in growth cones and that our immunostaining has labelled only these proteins in DRG growth cones.
Fig. 8. Immunoreactivity for several actin-associated proteins and corresponding phalloidin staining in neurones cultured in 0.3 μg ml⁻¹ CB for 24 h. (A) Filamin staining is seen along neurite (n) and is strong at neurite tip, which is strongly stained by phalloidin, as seen in B. (C) Immunoreactivity for tropomyosin is strong at the tips of a branched neurite. These sites coincide with strong labelling by phalloidin (D). (E) Immunoreactivity for myosin is concentrated at neurite tips (arrows) and several other points (arrows) around a neurone and an adjacent non-neuronal cell, which are also stained by fluorescent phalloidin in F. Immunolabelling for the membrane-associated proteins talin (G) and vinculin (I) also shows concentrations of staining at neurite tips and other points (arrows in G and I), which are strongly labelled by phalloidin in H and J. On the other hand, staining for fodrin (K) is strong in the neuronal soma and proximal neurites, but the neurite tips, which contain concentrated binding of phalloidin (L), show reduced staining for fodrin. ×900.

Discussion

We have labelled the neurites and growth cones of chick DRG neurones with a battery of antibodies against actin-associated proteins and cell adhesion molecules. Although previous studies have looked for most, if not all, of these molecules on elongating neurites, no other study has analysed the distribution of so many actin-associated and adhesion molecules in the growth cones of a single neuronal type. In addition, most of the published reports have not depicted growth cones with sufficient resolution to see the distribution of antigens on growth cones, filopodia and lamellipodia. Thus, our results provide new information about the potential roles of these molecules in the navigational behaviour of nerve growth cones. Because we did not have access to preimmune sera and preadsorbed antisera for many of the antibodies, we are cautious and state that our staining is one type of evidence for the presence of these antigens or related molecules in growth cones.

Actin-associated proteins

Our immunostaining indicated that the leading margins of DRG growth cones, filopodia and lamellipodia, contain myosin, tropomyosin, filamin, α-actinin, talin and vinculin. In previous work, myosin, tropomyosin and α-actinin have been demonstrated in growth cones, but immunoreactivity for filamin was negative (Jockusch & Jockusch, 1981; Kuczmarski & Rosenbaum, 1979; Letourneau, 1981; Roisen et al. 1978; Shaw et al.)
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1981). Anti-vinculin stained neurite tips of NGF-treated PC12 cells (Halegoua, 1987), although filopodial staining was not shown. Our demonstration of anti-talin staining is the first indication of talin in growth cones and their filopodia. Staining for MAP2 was absent from DRG growth cones and staining for spectrin/fodrin was present in growth cones, but absent from filopodia. Growth cone immunoreactivity for spectrin/fodrin has been reported, although it is unclear whether filopodia were stained (Koenig et al. 1985).

The activities of these actin-associated molecules must be intricately coordinated during growth cone motility. Although the specific roles of these molecules are unknown, possible functions can be listed. Filamin and α-actinin, which cross-link actin filaments, may participate in organizing actin filament networks and bundles at the leading edge (Pollard & Cooper, 1986; Stossel, 1984). Filamin immunoreactivity of filopodia was especially strong, indicating that this protein may be critical in the formation of actin bundles. Tropomyosin binds along actin filaments and may regulate actin–myosin interactions (Pollard & Cooper, 1986). Tropomyosin may also stabilize actin filaments against attack by fragmenting molecules such as actin depolymerizing factor, which is present in DRG growth cones (Bamburg & Bray, 1987; Bernstein & Bamburg, 1982). Myosin is potentially important in generating the tensions that filopodia and lamellipodia exert on adherent sites. Immunoreactivity for myosin in growth cones has been demonstrated several times, but labelling of distinct structural components is not reported (Kuczynski & Rosenbaum, 1979; Letourneau, 1981; Roisen et al. 1978). Understanding actomyosin-related tensions in growth cones is further complicated, because actin–myosin interactions are regulated by other actin-associated proteins (Pollard & Cooper, 1986; Stossel, 1984).

The evidence with three different antibodies that spectrin/fodrin is much diminished in filopodia and lamellipodia is interesting. This may be related to the molecular interactions of spectrin with cytoplasmic
Fig. 9. Immunoreactivity for several cell adhesion glycoproteins and corresponding phalloidin staining in neurones cultured in 0-3 μg ml⁻¹ CB for 24 h. Immunoreactivity for integrin in A is strongly concentrated at neurite tips and at a point of neurite-neurite adhesion (arrow), in a distribution that is very similar to the phalloidin binding (B). Immunostaining for L1 (C) is very strong along proximal neurite, and L1 staining is sometimes enhanced at neurite tips (arrows), where phalloidin labelling is high (D). Staining for A-CAM is continuous along neurites (E) and sometimes is relatively concentrated at neurite tips where phalloidin staining is strong (F). N-CAM staining is stronger on neuronal soma and along neurite (G), and a faint appearance of N-CAM staining is seen at the neurite tip (arrow), which is strongly stained for phalloidin (H). ×900.
Fig. 10. Controls for non-specific staining. (A) Neurones are very weakly stained when the primary antibody is preimmune serum from the rabbit used to produce anti-human platelet myosin. The thicker soma (s) is visible, but the neurite and growth cone (arrow) are not labelled. (B) Some fluorescent staining remains along neurites (n) when anti-talin antiserum is preadsorbed with purified talin, but labelling of growth cones and filopodia is abolished. (C) Staining with fluorescein–phalloidin shows a growth cone (arrow) and a fibroblast (f) but, when the primary antibody is normal rabbit serum, the cells are not labelled by a rhodamine–goat anti-rabbit antibody (D). (E) Two fibroblasts (f) and two growth cones (arrows) are labelled by fluorescein–phalloidin, while in F the fibroblasts are strongly labelled by anti-fibronectin, but the growth cones are unlabelled.

surfaces of membranous organelles and the plasma membrane (Burridge et al. 1982; Koenig & Repasky, 1985; Levine & Willard, 1981). If most of the spectrin/fodrin is bound to membranous structures, then spectrin/fodrin may not be free to diffuse forward with the expanding cytoplasmic matrix of protruding filopodia and lamellipodia. Other actin-associated proteins, filamin, tropomyosin, α-actinin and myosin, may not be bound to membranes and, thus, be more free to diffuse forward and be incorporated into expanding actin filament networks and bundles of extending filopodia and lamellipodia. Other factors, such as accessibility of specific binding sites on actin filaments, may also influence which actin-associated proteins are pres-
ent within filopodia and lamellipodia. However, a major determinant may be whether a molecule is soluble and can rapidly enter an extending protrusion.

Cell surface adhesion molecules

Our data indicate that the filopodial surfaces of DRG growth cones contain the four adhesion molecules, integrin, N-CAM, A-CAM (N-cadherin) and L1. Yet, there are differences in staining that may be related to associations with the cytoskeleton and perhaps also related to their mode of insertion into the plasma membrane. Immunoreactivity on cultured neurones has been shown for all these molecules (Bozyczko & Horwitz, 1986; Grumet et al. 1984; Hatta et al. 1987; Lemmon & McLoon, 1986; Pollerberg et al. 1985, 1987; Rathjen & Schachner, 1984; van den Pol et al. 1986). However, staining for all four has not been done on the same neuronal type with the same procedures, and the morphological resolution of staining on growth cones and filopodia was not clear in most cases (except van den Pol et al. 1986). We have not examined the effects of substratum on the distribution of surface adhesion molecules. It is possible that molecular components of the substratum may interact with and influence the distribution of particular adhesion molecules. This will be considered in future work, utilizing substrata treated with purified components of extracellular matrices or cell adhesion molecules (Lagenaur & Lemmon, 1987).

Caution is appropriate when comparing the distributions of these adhesion molecules. Neurites, growth cones and filopodia have different contours and thicknesses, which influence the fluorescence intensity of a given area. Thus, different fluorescence intensities may not accurately reflect actual differences in density of a surface antigen on filopodia vs the body of a growth cone vs a neurite. However, a fair estimate of relative differences in the distribution of different adhesive ligands may be gained from comparing the relative staining intensities of filopodia, growth cones and neurites. For example, filopodial staining for integrin and L1 (8D9) was equal to or greater than the strongest neurite staining for integrin or L1 but, with the anti-N-CAM antibody which recognizes all forms of N-CAM and with anti-A-CAM, the fluorescence of neurites appeared stronger than that of filopodia. One interpretation of this is that integrin and L1 are relatively more concentrated on filopodia compared to neurites than is N-CAM and A-CAM. Without direct evidence we cannot determine the actual difference in filopodial vs neurite staining for integrin and L1, and we cannot say that integrin and L1 are actually more concentrated on filopodia than N-CAM and A-CAM. These differences do indicate, however, that addition or stabilization of integrin and L1 on the surfaces of growth cones and filopodia is different from what occurs for N-CAM and A-CAM. A further interpretation is that the functions of integrin and L1 may be related to their concentration on filopodia and their availability to mediate growth cone contacts with cells or extracellular surfaces.

An antibody that recognizes only the 180×10^3 M_r form of N-CAM (N-CAM_{180}) labelled neurites and the bodies of growth cones, but filopodia were unstained. N-CAM_{180} is less mobile on the surfaces of neuroblastoma cells than the 140×10^3 M_r form of N-CAM, and related to this, the membrane–cytoskeletal protein spectrin binds only to N-CAM_{180} (Pollerberg et al. 1985, 1986, 1987). Immunofluorescence for spectrin/fodrin was also much reduced in filopodia, and a transmembrane association between these molecules may control the distribution of N-CAM_{180}. The more widespread N-CAM labelling of growth cones by the antibody 1A6 indicates that these DRG growth cones contain more than one form of N-CAM. Perhaps anchorage of N-CAM_{180} to the membrane cytoskeleton restricts the movement of N-CAM_{180} onto the surface of extending filopodia. With its linkage to the membrane cytoskeleton, N-CAM_{180} may be involved in stabilizing the cohesion of neurite fascicles, while the more mobile forms of N-CAM, which extend onto filopodia, may mediate adhesive contacts of growth cones with other N-CAM-containing surfaces. In this way the different forms of N-CAM may serve different functions, while they are present simultaneously on growth cones.

Our immunostaining indicated that A-CAM (N-cadherin) is present on DRG neurites, growth cones and filopodia. Filopodia did not appear to be more strongly stained by anti-A-CAM than neurites. Multiple developmental roles have been proposed for A-CAM or N-cadherin (Hatta et al. 1987; Takeichi, 1988). In vitro effects of anti-N-cadherin indicate that N-cadherin is involved in neurite elongation on the surfaces of astrocytes and Schwann cells (Tomaselli et al. 1988), but the role of N-cadherin in fasciculation or growth cone migration on the surfaces of other neurones or neuronal processes is unclear.

The staining of CB-treated neurites reinforces the idea that these adhesion molecules interact differently with the actin filament system. Integrin appeared to be strongly concentrated at the tips of CB-treated neurites, where talin and other actin-associated proteins may be linked to actin filament densities. This is consistent with the reported binding of talin to the cytoplasmic domain of integrin (Buck & Horwitz, 1987; Horwitz et al. 1996). In a less dramatic fashion, neurite tips, which stained brightly with fluorescent phalloidin, sometimes showed local concentrations of staining for L1, N-CAM and A-CAM. Interactions of these molecules with talin have not been demonstrated, although there is evidence that members of the cadherin family are linked to actin filaments (Hirano et al. 1987). Vinculin, but not talin, has been suggested to be involved in the association of cadherins with actin filaments (Geiger et al. 1987).

**Hypothesis**

It is uncertain how these adhesive components are added to the surface of elongating neurites or how they interact with the cytoskeleton during growth cone motility (Pfenninger & Maylie-Pfenninger, 1981; Small et al. 1984). From our staining of normal and CB-treated neurites, we propose a mechanism for regulation of the distribution of these surface ligands. Protein and phospholipid components may be added to
the growth cone surface near where they are delivered by transport along microtubules, which extend into the growth cone. These membrane components are then available to move onto elongating filopodia. Transmembrane associations with cytoskeletal structures may influence this process, and integrin may be carried forward and/or stabilized on filopodia by linkage to talin (Horwitz et al. 1986), while, conversely, movement of N-CAM₈₀ onto filopodia is inhibited by linkage to spectrin. The concentration of L1 on filopodia may also be mediated by transmembrane associations. A-CAM and the lower molecular weight forms of N-CAM both showed no greater fluorescence on filopodia compared to the adjacent growth cone and neurite. Perhaps these membrane components are not strongly linked to the actin filament system, and their distribution may reflect bulk flow of membrane components onto extending filopodia.

In conclusion, our immunocytochemical evidence is consistent with the proposition that the leading margins of growth cones contain actin-associated proteins that are involved at the leading lamellae of fibroblasts. Protrusion, adhesion and the exertion of mechanical tensions are common behaviours at the front margins of growth cones and migrating fibroblasts (Burridge et al. 1987; Buck & Horwitz, 1987; Letourneau, 1985). Differences in the relative concentrations and regulation of actin and actin-associated proteins may determine distinctive growth cone size and shape compared to fibroblasts, as well as features, such as filopodial vs lamellipodial protrusion. The immunocytochemical evidence for simultaneous expression of several adhesion molecules suggests a redundant adhesive system on growth cones. In vitro experiments with blocking agents indicate that neurite elongation on surfaces of astrocytes, muscle cells, other neurites, or extracellular matrices involves multiple adhesive ligands, but in each situation a particular ligand(s) may dominate (Bixby et al. 1987; Chang et al. 1987; Keilhauer et al. 1985; Letourneau et al. 1988; Rathjen et al. 1987; Rogers et al. 1983; Stallcup & Beasley, 1985; Tomaselli et al. 1986, 1988). In addition, these adhesion molecules display differences in filopodial distributions and relations with actin filaments which indicate that they really do not have redundant roles in neurite elongation. For example, integrin and L1 may be important in adhesive contacts that are initiated by filopodia, while A-CAM and N-CAM may be important in the stabilization of adhesive contacts of neurites with other surfaces. The separate distributions and associations of different forms of N-CAM further indicates the potential diversity in adhesive interactions.

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