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Association of Ezrin Isoforms With the Neuronal Cytoskeleton

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We are studying the changes in the organization of the cytoskeleton which accompany expression of differentiated neuronal morphology. Of particular interest is the elaboration of growth cones, the motile domains of the neuronal plasma membrane, and the cytoskeletal structures that underlie them. A candidate for a component of the growth cone cytoskeleton of cultured hippocampal neurons is the antigen recognized by the monoclonal antibody, 13H9 (Birgbauer and Solomon, *J Cell Biol* 109:1609–1620, 1989; Goslin et al., *J Cell Biol* 109:1621–1631, 1989). That antibody binds strongly to growth cones, but barely stains neurites. The characterization of the antigen, both biochemical and microscopic, suggests that it may interact with microfilaments and microtubules. We have established that 13H9 recognizes a subset of the isoforms of ezrin (unpublished results). Here, we describe the properties and localization of ezrin isoforms in differentiating neuronal cells, using two *in vitro* systems and developing spinal cord. In embryonal carcinoma cells, both the abundance of ezrin and the proportion of ezrin associated with the cytoskeletal fraction increase upon induction of neuronal differentiation with retinoic acid. In the neuronal cells within such cultures, the 13H9-positive forms of ezrin are enriched in the growth cone, while the bulk of ezrin identified by a polyclonal antibody shows no specific localization. In mouse DRG neurons, 13H9 staining is asymmetrically distributed along the edges of the complex growth cones of these cells. Staining of developing spinal cord in rat embryos also demonstrates that the 13H9-positive forms of ezrin do not colocalize with the majority of ezrin. These results support the conclusion that ezrin is a component of the neuronal cytoskeleton, and that the localization of covalently distinct isoforms of the protein may be significant for growth cone function.

Key words: growth cone, actin-associated protein, microtubule-associated protein, neuronal differentiation

INTRODUCTION

Cell motility, to produce new cell shapes or to move the cell across a substratum, is ultimately a function of the leading edge of the cell. Membrane specializations such as lamellipodia and filopodia, and membrane domains such as ruffling edges or growth cones, are the sites at which cells undergo the deformations that are fundamental to movement. The expression and function of these edges clearly depend upon appropriate organization of the cytoskeleton; that fact is established by a long list of drug interference experiments and morphological correlations. Among the issues that remain to be resolved is how cytoskeletal elements interact with one another and with other cellular structures to produce the organizations required for cell motility. These interacting proteins need not be distributed uniformly along the length of cytoskeletal fibers, but instead may be restricted to domains of cells where crucial events in motility occur.

One possible candidate for such a protein was identified by analyses of the marginal band of avian erythrocytes. Although the marginal band probably is not involved in typical motility, it does represent a domain where microtubules, microfilaments, and the cell surface are in close proximity. A monoclonal antibody, called 13H9, binds to the position of the marginal band even in the absence of assembled microtubules, as assayed by immunofluorescence (Birgbauer and Solomon, 1989). That same antibody applied to hippocampal neurons in culture recognizes an epitope in growth cones but is barely detectable in the shafts of neurites. The staining pattern of 13H9 on growth cones is similar to that of phalloidin; however, unlike F-actin, the 13H9 localiza-

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tion is dependent upon the maintenance of intact, assembled microtubules (Goslin et al., 1989). The antigen is also greatly enriched in ruffling edges of migrating fibroblastic cells (Birgbauer, 1991). These results, and other experiments, suggest that the 13H9 antigen may interact with both microtubules and microfilaments (Birgbauer and Solomon, 1989). We now know that 13H9 antigen recognizes a subset of the isoforms of the protein ezrin (Bretscher, 1983). The epitope is specified by less than 13 amino acids of the ezrin sequence, and the isoforms are generated by either covalent modifications or alternative splicing (unpublished results). Here, we have used 13H9 and another antibody, 904, which recognizes most if not all isoforms of ezrin to characterize the behavior of this protein in neurons, both in vivo and in vitro. The results show that the 13H9-positive isoforms of ezrin are localized differently than is the majority of cellular ezrin, supporting the conclusion that these forms of ezrin are a component of the neuronal cytoskeleton in domains specialized for motility.

MATERIALS AND METHODS

Culture of Embryonal Carcinoma Cells

The embryonal carcinoma (EC) cell line P19 was obtained from M. McBurney, University of Ottawa. Cells were cultured as previously described (Rudnicki and McBurney, 1987), except that 0.5 μ M retinoic acid (RA) was used to induce neuronal cells (Dinsmore and Solomon, 1991). Uninduced EC cells were plated onto coverslips coated with 0.1% gelatin (Sigma Chemical Co., St. Louis, MO), allowed to grow for 1 day, and then fixed with 4% paraformaldehyde in PBS. RA-induced cells were trypsinized from dishes, dispersed by pipetting, and plated onto coverslips coated either with 50 μ g/ml laminin (GIBCO Laboratories, Grand Island, NY) or with 0.1 mg/ml polylysine and then 20 μ g/ml laminin.

Antibodies

Anti-ezrin serum 904 is a rabbit polyclonal serum raised against ezrin purified from intestinal epithelia, kindly provided by P. Matsudaira (Whitehead Institute and M.I.T., Cambridge, MA). Rabbits were injected subcutaneously with 50 μ g of ezrin in Freund's complete adjuvant, followed by two boosts, at 3-week intervals, with 50 μ g of ezrin in Freund's incomplete adjuvant. The specificity of 904 for ezrin is based on several criteria. Briefly, 904 binds to several spots of about 80 kd resolved by two-dimensional gel electrophoresis of material from chicken erythrocytes; it binds to bacterially expressed ezrin fusion proteins specified by at least two distinct domains of the human ezrin cDNA; and antibodies affinity purified from those fusion proteins give blot-

ting patterns of animal cell ezrin identical to that obtained with the whole serum.

Monoclonal antibody 13H9, of the IgM class, has been previously described (Birgbauer and Solomon, 1989); its characterization has been extended. Briefly, a 13 amino acid domain of the ezrin sequence is both necessary and sufficient for 13H9 binding. In two-dimensional separations (iso-electric focusing followed by SDS-PAGE) of chicken erythrocyte ezrin, only a minor subset of 904-positive spots is stained by 13H9.

A control monoclonal IgM, AD4, which was made against mouse CD8 antigen, was kindly provided by D. Raulet (Berkeley). Culture supernatants of AD4 were used at the same concentration as 13H9. The anti-tubulin antibody (6-1D) is a mouse monoclonal IgM described previously (Magendantz and Solomon, 1985). The anti-neurofilament antibody, RT97, is a mouse monoclonal antibody previously described (Wood and Anderton, 1981). Rhodamine-, Texas Red-, and FITC-conjugated second antibodies were obtained from Organon Teknika-Cappel, Malvern, PA.

Immunofluorescence of Embryonal Carcinoma Cells

Cells were fixed by underlaying the medium with 4% paraformaldehyde in PBS with 10% sucrose, then permeabilizing with 0.5% Triton X-100 in PBS for 5 min. Cells were stained with 13H9 or 904, followed by FITC-conjugated goat anti-mouse Ig and Texas Red-conjugated goat anti-rabbit IgG secondary antibodies. Prior to the primary antibody incubations, coverslips were blocked for 20 min at 37°C with 10% normal goat serum and 1% BSA in PBS. The antibody incubations were carried out in the presence of 1% BSA (Sigma Chemical Co., St. Louis, MO) in PBS. Coverslips were mounted onto slides using gelvatol mounting media containing 15 mg/ml 1,4-diazabicyclo-[2.2.2]-octane, an anti-fade agent (Aldrich Chemical Co., Milwaukee, WI). They were examined on an Axioplan microscope (Carl Zeiss, Inc., Thornwood, NY) using a 100 $\times$, 1.3 NA, objective. Photography was done using Kodak Tri-X 400 film.

Fractionation and Protein Analysis of EC Cells

To prepare lysates, EC cells were resuspended in PBS with protease inhibitors (leupeptin, pepstatin, PMSF, TAME, soybean trypsin inhibitor, and aprotinin), then diluted into gel-loading buffer to a final concentration of 2% SDS and 5% β -mercaptoethanol and boiled for 2 min. Subcellular fractionation of EC cells was performed as follows (with the above protease inhibitors present in all buffers): Cells on tissue culture dishes were washed with PBS, then washed with PM2G (0.05M PIPES, 2 M glycerol, 5×10^{-3} M EGTA, 10^{-3} M MgSO_4 , pH 6.9), and then extracted for 10 min at

37°C with 0.1% NP-40 in PM2G. The dishes were scraped and the suspension pelleted; supernatants were saved as the "NP-40 SOLUBLE" fraction. The pellet was washed once with PM2G, and then extracted for 20 min on ice with 5 mM CaCl₂ in 0.05 M Pipes pH 6.9 and 5 mM MgSO₄. After pelleting again at 15,000g for 10 min, the supernatant was saved as the "CALCIUM EXTRACTABLE" fraction. The "SDS RESIDUE" fraction was then obtained by dissolving the pellet in gel loading buffer (containing 2% SDS and 5% β -mercaptoethanol).

For immunoblotting, samples separated on a 7.5% polyacrylamide gel with a 5% polyacrylamide stacker, according to the method of Laemmli (1970), were transferred to nitrocellulose electrophoretically and probed by slight modifications of standard procedures (Tobin et al., 1979). The nitrocellulose filters were stained for total protein with 0.2% Ponceau S (Sigma Chemical Co., St. Louis, MO) in 3% trichloroacetic acid, photographed, and then the Ponceau S was washed away. The nitrocellulose filters were blocked with 2% hemoglobin in PBS with 0.05% sodium azide. Antibody incubations were done in the same buffer. Following the primary and secondary antibody incubations, blots were washed in blocking buffer that contained 0.1% SDS and 0.05% NP-40. ¹²⁵I-labeled goat anti-mouse Ig and protein A were obtained from New England Nuclear (Boston, MA).

Culture and Staining of DRG Neurons

Spinal ganglia were dissected from adult female CD-1 mice. Dorsal root ganglion neurons were isolated as described by Lindsay (1988). The culture medium was 45% Hams F12, 45% Dulbecco's modified Eagle's medium, and 10% heat-inactivated horse serum, plus penicillin (25 IU/ml), streptomycin (25 μ g/ml), glutamine (1 mM) and 2.5 S nerve growth factor (1 ng/ml). Cells were cultured on glass coverslips pretreated with laminin (20 μ g/ml). After about 6 hr in culture, cells were fixed in 10% formalin and 5% sucrose in PBS, and permeabilized with 1% Triton X-100. Cells were stained with either 13H9 or with phalloidin coupled to rhodamine (Molecular Probes, Inc., Junction City, OR) as described above.

Immunostaining of Rat Embryo Sections

Rat embryos (Sprague-Dawley) were staged by embryonic day, with date of sperm positivity defined as E0. Embryos were dissected from pregnant rats (Taconic Farms) and fixed overnight by immersion in ice cold 4% paraformaldehyde in 0.1 M sodium phosphate, pH 7.4. The fixed embryos were washed thoroughly with PBS, then washed with 5% sucrose in PBS, and then equilibrated with 15% sucrose in PBS. The embryos were

embedded in 7.5% gelatin in PBS plus 15% sucrose. Prior to sectioning, the embryos were embedded in OCT compound and frozen on dry ice. 14- μ m cryostat sections were mounted onto subbed microscope slides and stored frozen until stained. Subbing of slides was achieved by treating Premium microscope slides (Fisher) with 0.25% gelatin plus 0.25 mg/ml Chrom-Alum. Sections were blocked for 1–3 hr at room temperature with 10% goat serum plus 1% BSA in PBS prior to antibody incubations. The primary antibodies were diluted in the above buffer and incubated on the sections overnight at 4°C. The sections were washed four times, and then incubated at room temperature for 1–5 hr with rhodamine- or Texas Red-labelled secondary antibodies in the above buffer. After washing four times, the sections were dehydrated by washing sequentially in 70% ethanol, twice in 100% ethanol, and twice in xylene. Coverslips were then mounted with Pro-Texx, and the slides examined on a Zeiss Axiophot microscope with a 10 $\times$, 0.30 NA lens or a 25 $\times$, 0.80 NA lens.

RESULTS

An Embryonal Carcinoma Model System to Study Ezrin During Neuronal Differentiation

To study expression and localization of ezrin during neuronal differentiation, we have taken advantage of an embryonal carcinoma cell line, P19, characterized by McBurney and colleagues (McBurney et al., 1982). In normal culture medium, these cells grow and divide as undifferentiated cells. They can be induced to differentiate into either neuronal cells or muscle cells by exposing aggregates in suspension to either retinoic acid (RA) or dimethyl sulfoxide (DMSO), respectively. In each case, the assays for differentiation are expression of characteristic morphologies and molecular markers (Jones-Villeneuve et al., 1982; Sharma and Notter, 1988). In particular, 50 to 70% of the RA-induced cells display neuronal morphology: they develop well-defined growth cones and send out long thin processes that frequently branch. The rest of the cells in the culture are large and flattened, but do not extend processes and do not express neuronal markers (Dinsmore and Solomon, 1991).

Quantitative and Qualitative Changes in Ezrin as a Result of P19 Cell Differentiation

We analyzed the levels of ezrin during EC cell differentiation on immunoblots with the polyclonal anti-ezrin serum 904 (see Materials and Methods). The level of ezrin in RA-induced EC cells was compared to that present in uninduced and DMSO-induced EC cells. Equal amounts of protein from whole cell lysates were loaded onto a gel, and immunoblots were probed with

Fig. 1. Immunoblot of EC cell extracts probed with anti-ezrin serum 904. **a:** Whole cell lysates of uninduced (**lane 1**), RA-induced (**lane 2**), and DMSO-induced (**lane 3**) EC cell were loaded for equal protein, and immunoblots probed with antiserum 904. The position of molecular weight markers, given in kd, is shown at the left. **b:** Subcellular fractionation of uninduced (**lane 1**), RA-induced (**lane 2**), and DMSO-induced

(**lane 3**) EC cells, assayed with anti-ezrin serum 904. Sequential extractions were performed to produce a "NP-40 SOLUBLE" fraction, a "CALCIUM EXTRACTABLE" fraction, and an "SDS RESIDUE" fraction. Each lane was loaded for equal protein. Because only one band from EC cell extracts is immunoreactive with 904 (see panel a), only this region is shown in panel b.

904 (Fig. 1a, lanes 1–3). By comparing exposures of different lengths, we estimate that there is about a two-fold increase in the level of ezrin in the RA-treated cultures. On the other hand, incubation with DMSO to induce muscle differentiation resulted in a twofold to fourfold decrease in ezrin protein.

The association of ezrin with the cytoskeleton, as assayed by extraction with nonionic detergent, also is altered upon RA treatment. The undifferentiated, neuronal, and muscle cultures were extracted sequentially, using a scheme previously devised to analyze microtubules (Solomon et al., 1979; Materials and Methods). About 75% of the total cell protein is released by initial extraction with 0.1% NP-40 in PM2G ("NP-40 SOLUBLE," Fig. 1b), a buffer that preserves cellular microtubules. The proteins that remain behind in such detergent-extracted cytoskeletons can be further fractionated by a subsequent extraction with the same buffer plus 5×10^{-3} M CaCl_2 ("CALCIUM EXTRACTABLE," Fig. 1b), which depolymerizes microtubules and solubilizes associated components. The proteins remaining ("SDS RESIDUE," Fig. 1b), about 15% of the total cell pro-

tein, are solubilized by dissolving the remaining cytoskeletal fraction in SDS.

In both uninduced and treated P19 cells, ezrin is detected in each of the three fractions (Fig. 1b). However, the fractionation of ezrin is altered by RA-induced neuronal differentiation. A higher proportion of the protein remains associated with the cytoskeleton after NP-40 extraction in neuronal cultures than in undifferentiated cultures (Fig. 1b, compare lanes 1 and 2). That material is partially released by a subsequent extraction with 5×10^{-3} M calcium, while the rest is solubilized only by dissolving the entire cytoskeleton in SDS. Components of microtubules in EC cells, including both assembled tubulin and minor microtubule associated proteins (MAPs), are released upon extraction with Ca^{++} in this procedure (Dinsmore and Solomon, 1991). However, it is clear that ezrin does not behave as do other known MAPs. In particular, pretreatment of cells with nocodazole before extraction completely depolymerizes the microtubules, but does not diminish the amount of ezrin released in the Ca^{2+} extractable fraction (data not shown). And unlike known MAPs, the association of

much of the ezrin with the detergent-extracted cytoskeleton is refractory to Ca^{2+} ions, and remains behind in the residue fraction. These results rule out the possibility that cytoskeletal ezrin is bound exclusively to the microtubules, but do not preclude ezrin from interacting with microtubules and with some other component of the cytoskeleton (Birgbauer and Solomon, 1989).

Localization of Ezrin Isoforms in Undifferentiated and Neuronal EC Cells

We have used immunofluorescence to localize the distribution of ezrin in both undifferentiated and neuronal P19 cells, using the polyclonal anti-ezrin serum 904 and the monoclonal antibody 13H9. As described in Materials and Methods, 904 binds to many if not all forms of ezrin, while 13H9 binds only to some isoforms of the molecule.

The immunofluorescence staining experiments reveal that the 13H9-positive isoforms of ezrin are localized differently than the majority of cellular ezrin. In undifferentiated cells (Fig. 2a,b), antiserum 904 gives bright, diffuse cellular staining throughout the cytoplasm, as well as staining of tufts and filopodia at the edges of cells. 13H9 stains tufts and filopodia quite brightly, but stains the cytoplasm only faintly (Fig. 2c,d). Staining of neuronal cells with 904 (Fig. 2e,f) gives intense staining of cell bodies, while the thin neurites and growth cones stain only very faintly, visible only when high concentrations of antiserum are used and the photograph is overexposed (as in Fig. 2f). At higher magnification, there is no apparent specific staining of growth cone components (data not shown). The same results are obtained when 904 is affinity purified by elution from polypeptides specified by the ezrin cDNA (data not shown). Therefore, the difference between 13H9 and 904 staining is a consequence of the differential localization of the 13H9-positive isoforms of ezrin, rather than some other activity of the 904 serum. In contrast, 13H9 stains growth cones very brightly (Fig. 2g,h). Viewed at higher magnification, the 13H9 staining clearly aligns with the prominent phase dense striations of the growth cone lamellipodia (Fig. 2i,j), a pattern very similar to that described for 13H9 staining of hippocampal neurons in culture (Goslin et al., 1989). In neuronal P19 cells, the 13H9 staining is confined largely to the growth cones; there is some diffuse cell body staining (Fig. 2g,h), and even fainter staining of neurites not visible in prints optimized to show growth cone staining. The fractionation of the 13H9-positive isoforms of ezrin, as a correlate of the immunofluorescence, is not available because the blotting signal is as yet unsatisfactory; perhaps because so little of the antigen is present (unpublished results).

Asymmetric Localization of Ezrin in Growth Cones of Dorsal Root Ganglia Neurons

Neuronal cells with different growth cone morphologies show strikingly different patterns of 13H9 localization. In the neuronal cells derived from the P19 cultures (Fig. 2e-j), and in the hippocampal neurons studied previously (Goslin et al., 1989), the growth cones are symmetrical fans with multiple filopodia. The 13H9 isoforms of ezrin in these cells are localized in striations symmetrically arrayed across the breadth of the growth cone and extending into filopodial projections. This pattern suggests that 13H9-positive isoforms of ezrin are localized along the longitudinally oriented bundles of actin filaments observed in the distal portions and the filopodia of many types of growth cones (Forscher and Smith, 1988). The morphology of growth cones expressed by adult mouse dorsal root ganglia (DRG) neurons is more complex. These neurons rapidly extend large flattened growth cones that, during the first 12 hr in culture, break up into smaller, irregularly shaped growth cones. The growth cones bear a small number of filopodia (typically less than ten), which emerge from points all along the growth cone perimeter. In these growth cones, the most intense staining of F-actin is separate from that of 13H9. As shown in Figure 3b, these filopodia, and the growth cone edges that lie between them, are strongly and relatively uniformly stained with phalloidin, to visualize F-actin. In contrast, 13H9 (Fig. 3a) binds strongly to some growth cone edges and filopodia, but only weakly to others. This result suggests that, at least in DRG neurons, there are molecular specializations within the growth cone represented by the 13H9-positive isoforms of ezrin that are not apparent from morphology or actin-staining alone. Similarly, staining of DRG growth cones with the polyclonal anti-ezrin serum 904 gives diffuse and faint staining (data not shown).

Ezrin Localization in the Developing Nervous System

The results above suggest that, in neuronal cells growing in vitro, the 13H9-positive forms of ezrin are greatly enriched in growth cones, distinct from the distribution of the bulk of ezrin forms. To determine whether or not the antigens also are segregated in neuronal tissue in vivo, we applied the 904 and 13H9 reagents to a developing system undergoing neuronal differentiation and axonal extension: the rat spinal cord from embryonic days 11 to 15. The spinal cord has been used to characterize a variety of neuronal antigens and their expression during development (Dodd et al., 1988; Rathjen et al., 1987; Yamamoto et al., 1986). Cells of the spinal cord begin to differentiate at about days E10–E11 in an anterior-to-posterior gradient in the rat (Altman and Bayer, 1984). From E10 to E15, the dif-

Fig. 2. Immunofluorescence of EC cells with 13H9 and 904. **a-d**: Uninduced EC cells were visualized by phase (a,c) or immunofluorescence (b,d) microscopy, after staining with (b) 904 or (d) 13H9. **e-j**: RA-induced EC cells were visualized by phase (e,g,i) or immunofluorescence (f,h,j) microscopy after

staining with 904 (f) or 13H9 (h,j). The 904 immunofluorescence (f) is overexposed to show faint staining in neurites and growth cones. The cell shown in panels i and j is enlarged to show the growth cone. Bars = 20 μ m (a,e); 10 μ m (i).

Fig. 3. Double immunofluorescence of a DRG neuron growth cone, stained with (a) 13H9 and (b) phalloidin. The neurite leading to this growth cone is marked by the arrow. Bar = 10 μ m.

ferent spinal cord neurons differentiate and send out axons (Altman and Bayer, 1984): motor neurons between days E10 and E12; the intermediate gray neurons between days E11 and E14; and finally, the substantia gelatinosa neurons between days E13 to E15. During the same period of time, from E11 to E14, the sensory neurons of the dorsal root ganglia (DRG) are also differentiating. This system is well suited for examining neurons and their axonal growth cones in vivo.

Cryostat sections of E11, E12, E13, and E15 rats were stained; the basic staining pattern was similar in all stages examined, and the data from E12 and E15 rat sections are presented here.

E12 Stage rat spinal cord. At E12, neuronal differentiation has recently begun, and some neurons have sent out axons. These differentiated neurons are predominantly motor neurons in the ventral horn. However, the majority of the cells in the spinal cord at this stage are still undifferentiated. 13H9 stains most of the cells, and there are no cell types or embryonic regions that are conspicuously enriched in the 13H9 antigen (data not shown). However, the 13H9 staining is not uniform throughout the section but shows a markedly cellular distribution. The 13H9 and anti-tubulin staining patterns are distinct. In particular, there is bright anti-tubulin staining of axon tracts that is not seen with 13H9. The anti-tubulin antibody also stains the notochord and the region around it more brightly than does 13H9; the basis of this bright notochord staining is not known. Antiserum 904 stains most cells throughout the section, more uniformly than does 13H9, especially at the level of individual cells. The most striking distinction is the strong staining by 904 of the floor plate, a structure stained only very weakly by 13H9.

E15 Stage rat spinal cord. At stage E15, many neurons of different classes have differentiated and sent out axons, and the region of undifferentiated cells has

shrunk to less than half the spinal cord. 13H9 stains most cell types (Fig. 4a), similar to the pattern at E12, and there are no cell types that are dramatically brighter with 13H9. However, cartilage is almost devoid of 13H9 staining, and the floor plate stains only weakly. A control IgM antibody does not show any staining (Fig. 4f). Again, the 13H9 staining pattern (Fig. 4a) can be compared with the anti-tubulin pattern (Fig. 4b). There is a significant difference between the 13H9 and anti-tubulin staining of the axon tracts, which are demarcated by anti-neurofilament antibody (Fig. 4e). The axon tracts along the ventrolateral and dorsal margins of the spinal cord stain brightly with anti-tubulin but only faintly with 13H9. In addition, the neuronal cells of the DRG stain slightly more with anti-tubulin than do the cells surrounding them, while with 13H9, the DRG staining is equal to or less than that of the surrounding cells. We also note the bright staining with anti-tubulin antibody of the vertebral cartilage, both that located ventral and lateral to the DRG, and that surrounding the notochord.

By stage E15, differences between the total anti-ezrin staining with 904 (Fig. 4c) and the 13H9 staining pattern (Fig. 4a) have become more marked than at stage E12. Again, the staining with 904 is more uniform across the section and less cellular than the 13H9 staining or the anti-tubulin staining. Several regions stain more brightly with 904. First, the floor plate at day E15 stains very strongly with 904; this floor plate staining is even more pronounced than the staining at E12. Second, 904 also stains strongly the neuroepithelial cell layer surrounding the central canal of the developing spinal cord. Third, 904 staining is brighter than 13H9 staining in the DRG. Fourth, there is some increased staining of axon tracts along the periphery of the spinal cord, though not nearly as bright as the anti-tubulin staining of these axon tracts. Finally, there is bright staining of the notochord with the 904 antiserum; its significance is not known.

Fig. 4. Adjacent 14- μ m cryostat sections of E15 rat spinal cord stained with (a) 13H9; (b) anti-tubulin; (c) anti-ezrin serum 904; (e) anti-neurofilament; (f) control IgM. Panel d is a dark-field image of panel a. The regions labelled in panel d are

the roof plate (rp), ventral horn (VH), the axon tracts (AT), the floor plate (fp), the dorsal root ganglia (DRG), the notochord (NC), and forming cartilage (cl). Bar = 100 μ m.

The weak staining of axon tracts with 13H9 can be seen clearly on sections that cut through a ventral root. Figure 5 shows 13H9 staining compared with anti-tubulin on sections containing a ventral root. The anti-tubulin antibody stains the axon bundles of the ventral root very intensely, as well as the axons in the ventral funiculus of the spinal cord (Fig. 5c). 13H9, however,

stains the axon tracts very faintly, and indeed the ventral root appears almost devoid of 13H9 staining (Fig. 5b). The higher magnification of Figure 5 shows more clearly the cellular staining with 13H9 and anti-tubulin. Neither 13H9 antigen nor tubulin fill the entire cytoplasm uniformly, although they do not appear to be localized to a particular region of the cells.

Fig. 5. Higher magnification image of a ventral root seen in two adjacent sections stained with (b) 13H9 and (c) anti-tubulin. Panel a is a phase image of the section in panel B. The spinal cord (sc) and the ventral root (vr) are labelled in panel a. Bar = 100 μ m.

DISCUSSION

The experiments described above were based on the observation that the growth cones of cultured hippocampal neurons bind an anti-ezrin antibody with considerable specificity. The present results generalize the original observation by showing that ezrin is present in the growth cones of other cultured neurons, specifically those of differentiated embryonal carcinoma cells and adult mouse dorsal root ganglia. In all three cases, the antibody binds significantly more strongly to the growth cone than to the neuritic shafts. However, the patterns within the growth cones differ. In both hippocampal and EC cell neurons, the 13H9-positive isoforms of ezrin are primarily in parallel striations within the symmetrical fan-shaped growth cones. In the more complex growth cones of the DRG cells, those isoforms are largely at the edges and filopodia, and the pattern is not symmetrical. Instead, some domains of the growth cone appear to be stained significantly more than others. This distribution of ezrin isoforms may reflect the activity of the growth cone. For example, the isoforms may be enriched in domains of the growth cone that are motile. It should be possible to resolve the relationship between function and distribution of ezrin by following—or even entraining—live growth cones, and then fixing them for immunofluorescence.

The application of two anti-ezrin reagents with different specificities makes clear that the localization of ezrin to growth cones is a property of only some isoforms—those recognized by the monoclonal antibody,

13H9. In contrast, a polyclonal antiserum that is likely to recognize all isoforms of ezrin (904) stains predominantly cell bodies, and stains neuritic shafts and growth cones of neuronal EC cells only very faintly. The same segregation may also occur in vivo. Sections through the developing rat nervous system demonstrate that ezrin is present in virtually all cells, but that regions that are rich in axons are significantly depleted of 13H9 staining. This difference in staining intensity is consistent with relative depletion of the 13H9-positive isoforms from neurites and their enrichment in growth cones, but a direct test of that possibility will require analysis at the level of immunoelectron microscopy. Such experiments are planned.

Our results on developing rat spinal cord can be compared with those reported on chicken embryos (Everett and Nichol, 1990) with both a monoclonal antibody, 3D11, and an anti-ezrin polyclonal on developing chicken spinal cord. The results are similar to those reported here for 904: staining at the cellular level throughout the cytoplasm, and some enrichment of staining in the DRG. However, 3D11 stains almost exclusively the dorsal root ganglia in 7.5-day-old chick embryos. This result differs from that reported here—staining of most cell types—and is surprising given the wide range of cell types in which ezrin has been found. At least some of these differences may arise from the fact that day 7.5 chick embryos represent a later developmental stage than the rat embryos used in the present study (Wentworth, 1984).

Finally, these experiments reveal two characteristics of the association of ezrin with the cytoskeleton. First, upon induction of neuronal differentiation in EC cells, the level of ezrin increases and, more important, the proportion of ezrin apparently associated with the cytoskeleton also increases markedly. Second, the association with growth cones is particularly striking for a subset of the ezrin isoforms—those that are 13H9-positive. As the molecular basis for 13H9 binding is elucidated, it should be possible to determine if it is directly related to the interaction with other structures of the growth cone, such as the F-actin filaments.

The role of ezrin in organizing the cytoskeleton is unlikely to be restricted to neuronal cells. The protein itself is apparently ubiquitous. More to the point, the isoforms that align with F-actin in the growth cone—the isoforms that bind 13H9—are present in specialized domains of other cells as well: the ruffling edge of fibroblasts and the marginal band of nucleated erythrocytes (Birgbauer and Solomon, 1989; Birgbauer, 1991). These localizations and other results suggest that ezrin may be capable of multiple associations with the cytoskeleton and perhaps with the plasma membrane as well. In addition, the homology between ezrin and band 4.1 in a region of the latter suspected to mediate binding to glyophorin (Gould et al., 1989; Rees et al., 1990) suggests that this protein may also interact with integral membrane proteins. These lines of evidence suggest that isoforms of ezrin may mediate functional connections between the cytoskeleton and the cell cortex at sites where motility events occur.

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REFERENCES

- Altman J, Bayer SA (1984): "The Development of the Rat Spinal Cord." Berlin: Springer-Verlag, pp 1–166.
- Birgbauer E, Solomon F (1989): A marginal band-associated protein has properties of both microtubule- and microfilament-associated proteins. *J Cell Biol* 109:1609–1620.
- Birgbauer E. (1991) "Cytoskeletal Interactions of Ezrin in Differentiated cells." Cambridge: MIT Press.
- Bretscher A (1983): Purification of an 80,000-dalton protein that is a component of the isolated microvillus cytoskeleton, and its localization in nonmuscle cells. *J Cell Biol* 97:425–432.
- Dinsmore JH, Solomon F (1991): Inhibition of MAP2 expression affects both morphological and cell division phenotypes of neuronal differentiation. *Cell* 64:817–826.
- Dodd J, Morton SB, Karagogeos D, Yamamoto M, Jessell TM (1988): Spatial regulation of axonal glycoprotein expression on subsets of embryonic spinal neurons. *Neuron* 1:105–116.
- Everett AW, Nichol KA (1990): Ezrin immunoreactivity in neuron subpopulations: Cellular distribution in relation to cytoskeletal proteins in sensory neurons. *J Histochem Cytochem* 38:1137–1144.
- Forscher P, Smith SJ (1988): Actions of cytochalasins on the organization of actin filaments and microtubules in a neuronal growth cone. *J Cell Biol* 107:1505–1516.
- Goslin K, Birgbauer E, Banker G, Solomon F (1989): The role of cytoskeleton in organizing growth cones: A microfilament-associated growth cone component depends upon microtubules for its localization. *J Cell Biol* 109:1621–1631.
- Gould KL, Bretscher A, Esch FS, Hunter T (1989): cDNA cloning and sequencing of the protein-tyrosine kinase substrate, ezrin, reveals homology to band 4.1. *EMBO J* 8:4133–4142.
- Jones-Villeneuve EMV, McBurney MW, Rogers KA, Kalnins VI (1982): Retinoic acid induces embryonal carcinoma cells to differentiate into neurons and glial cells. *J Cell Biol* 94:253–262.
- Laemmli UK (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685.
- Lindsay RM (1988): Nerve growth factors (NGF, BDNF) enhance axonal regeneration but are not required for survival of adult sensory neurons. *J Neurosci* 8:2394–2405.
- Magendantz M, Solomon F (1985): Analyzing the components of microtubules: Antibodies against chertins, associated proteins from cultured cells. *Proc Natl Acad Sci USA* 82:6581–6585.
- McBurney MW, Jones-Villeneuve EMV, Edwards MKS, Anderson PJ (1982): Control of muscle and neuronal differentiation in a cultured embryonal carcinoma cell line. *Nature* 299:165–167.
- Rathjen FG, Wolff JM, Chang S, Bonhoeffer F, Raper JA (1987): neurofascin: A novel chick cell-surface glycoprotein involved in neurite-neurite interactions. *Cell* 51:841–849.
- Rees DJG, Ades SE, Singer SJ, Hynes RO (1990): Sequence and domain structure of talin. *Nature* 347:685–689.
- Rudnicki MA, McBurney MW (1987) Cell culture methods and induction of differentiation of embryonal carcinoma cell lines. In Robertson EJ (ed): "Teratocarcinomas and Embryonic Stem Cells: A Practical Approach." Oxford: IRL Press Limited, pp 19–49.
- Sharma S, Notter MFD (1988): Characterization of neurotransmitter phenotype during neuronal differentiation of embryonal carcinoma cells. *Dev Biol* 125:246–254.
- Solomon F, Magendantz M, Salzman A (1979): Identification with cellular microtubules of one of the co-assembling microtubule-associated proteins. *Cell* 18:431–438.
- Tobin H, Staehelin T, 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.
- Wentworth LE (1984): The development of cervical spinal cord of the mouse embryo. II. A golgi analysis of sensory, commissural, and association cell differentiation. *J Comp Neurol* 222:96–115.
- Wood JN, Anderton BH (1981): Monoclonal antibodies to mammalian neurofilaments. *Biosci Rep* 1:263–268.
- Yamamoto M, Boyer AM, Crandall JE, Edwards M, Tanaka H (1986): Distribution of stage-specific neurite-associated proteins in the developing murine nervous system recognized by a monoclonal antibody. *J Neurosci* 6:3576–3594.