Surface and Cytoskeletal Markers of Rostrocaudal Position in the Mammalian Nervous System

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To identify cell surface molecules that define position in the mammalian nervous system, we previously characterized the binding of two monoclonal antibodies, ROCA1 and ROCA2, to adult rat sympathetic ganglia and intercostal nerves. The binding of ROCA1 is highest in rostral ganglia and nerves and declines in a graded manner in the caudal segments. ROCA2 labels the same cells in ganglia and nerves as ROCA1, but not in a position-selective manner. We now show by immunoblot analysis that ROCA1 recognizes two antigens in membrane/cytoskeletal fractions of peripheral nerves and ganglia: (1) a Triton X-100-insoluble, 60 kDa protein and (2) a Triton X-100-soluble, 26 kDa protein. The 60 kDa protein is expressed at higher levels in rostral than in caudal intercostal nerves, and is identified as the intermediate filament protein peripherin. In contrast, it is the ROCA1 epitope on the 26 kDa protein, and not the protein itself, that is preferentially visualized immunohistochemically in rostral nerves and ganglia. We suggest that the ROCA1 epitope on the 26 kDa protein is masked in sections of caudal nerves and ganglia. Amino acid sequence data obtained from the affinity-purified 26 kDa protein indicate significant homology with human CD9, a cell surface protein implicated in intercellular signaling in hematopoietic cells. These results suggest that intermediate filament gene expression and epitope masking on the cell surface may be involved in functions related to position in the nervous system.

[Key words: rostrocaudal position, gradients, epitope masking, intermediate filaments, peripherin, CD9]

It is thought that positional information at the cellular level plays a key role in pattern formation (Wolfert, 1989). The molecular mechanisms underlying the specification of positional information have been studied in Drosophila and mice. Homeotic selector gene expression, for instance, specifies segmental differences in Drosophila embryos (Lewis, 1963, 1978; Nusslein-Volhard and Wieschaus, 1980), and is restricted to particular domains along the anterior–posterior axis of the mouse embryo in the brain and spinal cord, and in many mesoderm-derived tissues (Holland and Hogan, 1988; Kessel and Gruss, 1990; McGinnis and Krumlauf, 1992). The anterior–posterior expression boundaries of these genes correlate with their relative 3′-5′ positions on the chromosomes of both Drosophila and mice (Akam, 1989; Duboule and Dolle, 1989; Graham et al., 1989). Molecular analyses have shown that the transcription factors encoded by many of these genes regulate the specification of positional information by an array of hierarchical and combinatorial interactions (Ingham, 1988). Mutations in the homeotic genes affect regional specification in both Drosophila and mice (Nusslein-Volhard and Wieschaus, 1980; Chisaka and Capecchi, 1991; Mouëllic et al., 1992), and can lead to alterations in the pathways of axonal growth (Doe et al., 1988; Luškin et al., 1991; Chisaka et al., 1992). The latter results suggest that the specification of neuronal connections may be based, in part, on the downstream products of the homeotic genes.

While these genes regulate segmental identity, they encode DNA-binding proteins localized to the cell nucleus, and thus are not able to convey positional information directly to neighboring cells. It seems likely that these positional regulators activate target genes in a cascade, leading to the expression of particular cell surface and secreted proteins that influence intercellular interactions. The identification of downstream targets of homeotic gene control has only recently begun (Gould et al., 1990; Immergluck et al., 1990; Reuter et al., 1990; St. Johnston et al., 1990). The neural cell adhesion molecule (NCAM) has a homeodomain binding site in its promoter region (Hirsch et al., 1991), and HOX 2.5 and 2.4 can modulate transcription from this promoter (Jones et al., 1992).

Additional candidates for surface molecules that may supply positional information in the vertebrate nervous system are those whose distribution reflects particular patterns of synaptic connections. Considerable attention has focused on the retinotectal system, where Sperry's chemoaffinity hypothesis postulates that the specificity of connections is established through matching biochemical differences among cells in the retina with corresponding differences among cells in the tectum (Sperry, 1963). A number of cell surface proteins display intriguing patterns of expression in this regard. TRAP (temporal retinal axon protein) is asymmetrically distributed in the nasal–temporal axis of the retina (McLoon, 1991), the TOP, and TOPα proteins are distributed in dorsoventral and anteroposterior topographic gradients in both retina and tectum (Trisler, 1990), and a 33 kDa membrane glycoprotein is enriched in the posterior tectum (Stahl et al., 1990). Moreover, an antiserum raised against pos-
terior membranes that recognizes the 33 kDa protein abolishes both the ability of these membranes to collapse retinal growth cones and to specifically inhibit temporal retinal axon growth (Stahl et al., 1990). Thus, this protein may be responsible for the preferential growth of temporal retinal axons on anterior membranes, through its ability to specifically inhibit or repulse temporal growth cones (Walter et al., 1987, 1990; Muller et al., 1990). In addition, an isomorph of aldehyde dehydrogenase (AHD-2) is present at much higher levels in the dorsal as compared to the ventral retina (McCaffery et al., 1991). Since AHD-2 oxidizes retinaldehyde to retinoic acid (Lee et al., 1991), and recent results have demonstrated asymmetrical retinoic acid synthesis along the dorsalventral axis of the retina (McCaffery et al., 1992), it seems possible that retinoic acid plays a role in the specification of positional information in this system.

Cell surface epitopes are asymmetrically distributed within the retinotectal system, as well. For example, the JONES carbohydrate epitope present on several gangliosides (Schlosshauer et al., 1988) is distributed in a strong dorsoventral gradient in the developing retina (Constantine-Paton et al., 1986; Reinhardt-Maelicke et al., 1990). Multiple monoclonal antibodies (mAbs) specific for a 43 kDa protein involved in protein translation identify a dorsovertical asymmetry in the embryonic retina (McCaffery et al., 1990; Rabacchi et al., 1990). Despite the pronounced immunohistochemical gradient displayed by the binding of these mAbs, however, the 43 kDa protein itself is not asymmetrically distributed. Rather, the differential binding appears to be due to a difference in conformation or accessibility; the 43 kDa protein is more labile to proteolysis in the dorsal retina, and the epitopes in the ventral retina can be exposed by an appropriate fixation protocol (McCaffery et al., 1990; Rabacchi et al., 1990).

The connections between preganglionic neurons in the spinal cord and postganglionic sympathetic neurons also form a highly stereotyped pattern, with a rostrocaudal gradient of specificity (Langley, 1892, 1895; Purves and Lichtman, 1978). Moreover, transplantation experiments in this system suggest that neurons and muscles may share cell surface molecules that define rostrocaudal position (Purves et al., 1981; Wigston and Sanes, 1982, 1985). Two experimental paradigms have identified molecules that are of interest in this regard. A > 100-fold rostrocaudal gradient of CAT levels is detected in muscles of adult mice determined by the method of Lowry et al. (1951). This material, which will be referred to as the membrane/cytoskeletal fraction, was either used immediately or frozen at −80°C until further use. All procedures were carried out at 4°C. Protein concentration was determined by the method of Lowry et al. (1951).

**Triton X-100-soluble and -insoluble fractions.** Tissues were immediately dissected from rats killed by asphyxiation with carbon dioxide, and immediately frozen on dry ice. Upon thawing, tissues were homogenized either by hand or by a Polytron (Brinkman Instruments Co.) in homogenization buffer (50 mM Tris-HCl (pH 7.4), 50 mM NaCl, 5 mM EDTA, 2 mM EGTA) containing five protease inhibitors: 1 mM phenylmethylsulfonyl fluoride, 20 µg/ml aprotinin, 20 µg/ml turkey egg white trypsin inhibitor, 2 mM benzamidine, and 5 mM N-ethylmaleimide. The homogenates were centrifuged at 100,000 × g for 1 hr in a Beckman 70 Ti rotor. The pellets were resuspended in homogenization buffer and washed twice by centrifugation at 100,000 × g for 1 hr and subsequent resuspension. The final pellets were resuspended in homogenization buffer. This Triton X-100-soluble fraction, was either used immediately or frozen at −80°C until further use. All procedures were carried out at 4°C. Protein concentration was determined by the method of Lowry et al. (1951).

**Fusion protein.** The peripherin fusion protein obtained from L. Parveek (Cincinnati, OH) was generated from a nearly full length peripherin cDNA insert containing coding sequence for all but the N-terminal 21 amino acids (56 kDa; Leonard et al., 1988), plus a portion of the Esch 

**Immunoprecipitation**

Triton X-100-solubilized fractions of adult rat sciatic nerves were mixed (1:1) with ROCA1 hybridoma supernatant and rotated end-over-end for 12–16 hr at 4°C. After this incubation, 50 µl of Protein G-Sepharose beads (Sigma) were added to the mixture and the incubation was continued for another 3 hr at room temperature. The beads were then
centrifuged in a microfuge, the supernatant was removed, and the beads were washed three times in Tris-buffered saline (TBS: 0.9% NaCl, 20 mM Tris-HCl, pH 7.4) containing 0.5% Tween-20 by a series of resuspensions and centrifugations. After a final wash in TBS without detergent, the beads were placed in 1× nonreducing sample buffer (see below) for 5 min to elute bound antigens.

Affinity purification

Adult rat peripheral nerve (including sciatic nerve and brachial plexus) membrane/cytoskeletal fractions derived from about 5–10 gm of nerves were solubilized with 1% NP-40. These extracts were then frozen, thawed, clarified by centrifugation at 100,000 × g for 1 hr in a Beckman 70 Ti rotor, and diluted to 0.5% NP-40. This preparation was then incubated end-over-end for 12–16 hr at 4°C with Protein G-Sepharose beads, to which 10 μg of the ROCA1 affinity-purified peripheral nerve proteins were added, and then coupled to a solid, Sigma) and 1% glacial acetic acid for 5 min, to visualize the 26 kDa band. The reaction was terminated with multiple washes in dH2O. After air drying, the nitrocellulose filters were subjected to densitometric scanning using an Ultrascan XL (LKB).

In Figure 7a, the plots generated from densitometric scanning include data from two further immunoblots than the ones in Figure 7a. In these two immunoblots, the NF-M:MBP ratio was significantly less than one. Since in 10 other blots the NF-M:MBP ratio was quite close to one, we did not consider these two results to be representative, and hence eliminated this data from the analysis.

N-terminal amino acid sequence determination

About 2–5 μg of ROCA1-affinity-purified peripheral nerve proteins were subjected to SDS-PAGE and transferred to an Immobilon-P membrane (PVDF, 0.45 μm, Millipore), using the conditions described for transfer to nitrocellulose. The filter was then rinsed in dH2O for 5 min, stained with 0.1% Coomassie Blue R-250 in 20% methanol, and then destained in 50% methanol; 10% glacial acetic acid (aldehyde free, Baker) for 5 min, and then destained in 50% methanol (HPLC grade, Burdick and Jackson), 10% glacial acetic acid (aldehyde free, Baker) for 5–10 min at room temperature. After a final rinse in dH2O for 5–10 min, a small piece of membrane containing only the 26 kDa band was excised, air dried, and stored at –80°C. The N-terminus of the 26 kDa protein was directly sequenced using a gas-phase microsequencer at the Protein Sequencing Core Facility, Beckman Research Institute of the City of Hope (Duarte, CA).

Internal amino acid sequence determination

For the 60 kDa protein, the isolated protein spot was excised from 52 Ponceau-S-stained nitrocellulose transfers of Triton X-100-insoluble adult rat peripheral nerve proteins that had been separated in two dimensions. The pooled nitrocellulose pieces (containing approximately 5 μg of protein) were incubated with 1 μg of trypsin (sequencing grade; Boehringer Mannheim) in 100 mM NH4HCO3, for 16 hr at 37°C. The resulting peptide fragments were separated by high pressure liquid chromatography, and non-tryptic peaks were subjected to microsequencing. The details of this procedure were exactly as described by Tempst et al. (1990). The peptide sequencing was performed by the Caltech Laboratory of Macromolecular Structure Analysis. For the 26 kDa protein, about 10 μg of the ROCA1-affinity-purified peripheral nerve proteins were subjected to SDS-PAGE and transferred to nitrocellulose. After staining with Ponceau-S, to visualize the 26 kDa band, the nitrocellulose was briefly destained with dH2O. The 26 kDa band was then excised and digested with 1 μg of trypsin (sequencing grade, Boehringer Mannheim) in 100 mM NH4HCO3, for 16 hr at 37°C. The remaining steps were identical to those outlined above and described in detail in Tempst et al. (1990). In this case, the microsequencing was performed by the Protein Sequencing Core Facility, Beckman Research Institute of the City of Hope (Duarte, CA).

Immunohistochemistry

1 tissues dissected from freshly killed adult or postnatal rats were embedded in Tissue-Tek O.C.T. compound (Miles), and frozen on dry ice. Ten micrometer sections were cut using a Bright cryostat (Hacker Instruments, Inc.), placed on gelatin-coated slides, and allowed to air dry for 2 hr. For ROCA1 labeling, the sections were washed in phosphate-buffered saline (PBS: 0.8% NaCl, 0.1% Na2HPO4, pH 7.4) three times over 15 min, incubated in blocking buffer (PBS containing 3% rat and 3% goat serum) for 20 min, incubated in ROCA1 hybridoma supernatant for 45 min, washed in blocking buffer three times over 15 min, incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG secondary antibody (Hi-F, Antibodies, Inc.) diluted 1:200 in blocking buffer, for 45 min, and washed in blocking buffer three times over 15 min. For peripherin labeling, sections were washed in PBS three
We previously identified the ROCAl antigen as a 65 kDa protein in membrane/cytoskeletal fractions derived from adult sympathetic ganglia and intercostal nerves (Suzue et al., 1990). A more extensive analysis of the mobility of this protein on SDS-PAGE, through the use of marker proteins with similar molecular weights, indicates that the more accurate size is 60 kDa (data not shown). To characterize the potential membrane association of this protein, we extracted membrane/cytoskeletal preparations of adult rat sciatic nerves with 1% Triton X-100, and performed immunoblots on the detergent-soluble and -insoluble fractions. The 60 kDa protein is only detectable in the Triton X-100-insoluble pellet, suggesting that it is not an integral membrane protein, but instead may be a cytoskeletal or extracellular matrix component (Fig. 1a). In addition, ROCAl also specifically binds a 26 kDa protein that is present exclusively in the detergent-soluble fraction (Fig. 1a). The 60 kDa and 26 kDa proteins are not labeled when ROCAl is omitted from the incubation, nor are they labeled by a variety of other mAbs from the ROCA fusion protocol (data not shown). Thus, it is very likely that the 60 and 26 kDa proteins, which reside in distinct subcellular fractions, share the ROCAl epitope.

We also performed similar immunoblots with ROCAl, a mAb generated in the same fusion, which binds to ganglia and intercostal nerves from all rostral and caudal levels equally well. While ROCAl does not specifically recognize any proteins in the detergent-insoluble fraction, it does strongly bind a 26 kDa protein in the detergent-soluble fraction (Fig. 1b). This finding raised the possibility that ROCAl and ROCAl recognize the same 26 kDa protein. To test this hypothesis we used ROCAl to immuno precipitate the 26 kDa protein from 1% Triton X-100 extracts of sciatic nerve membrane/cytoskeletal preparations. Proteins specifically precipitated by ROCAl were separated by SDS-PAGE and transferred to nitrocellulose, and an immunoblot analysis was then performed using ROCAl. ROCAl clearly recognizes the major 26 kDa protein that was immunoprecipitated by ROCAl (Fig. 2). As described below, the higher-molecular-weight bands observed on this blot are probable glycosylation variants of the 26 kDa protein.

Identification of the 60 kDa ROCAl antigen

The Triton X-100 insolubility of the 60 kDa ROCAl antigen hampered immunoaffinity chromatography purification of this
Figure 3. Two-dimensional gel and immunoblot analysis of Triton X-100-insoluble adult rat sciatic nerve proteins. A 1% Triton X-100-soluble sciatic nerve fraction, prepared as described in Materials and Methods, was subjected to isoelectric focusing in the horizontal dimension (0.2% ampholines, pH 4.5-5.4; Pharmalyte) (O'Farrell, 1975), followed by SDS-PAGE in the vertical dimension (10% acrylamide, 1.5 mm). Proteins were then transferred to nitrocellulose membranes. a, Coomassie blue stains several proteins in the transfer. b, ROCAl hybridoma supernatant binds a single protein spot (small arrow) at 60 kDa and pl 5.0, in a similar transfer. The ROCAl-positive spot is also visualized by Coomassie blue staining (a, small arrow). The pl was estimated from duplicate two-dimensional gels containing these protein standards: rat serum albumin (pl 5.4) and trypsin inhibitor (pl 4.6). The corresponding positions of these marker proteins are designated at the bottom, in the horizontal dimension. Approximately 60 µg of total protein was loaded in each of the gels. Primary antibody binding was visualized with a peroxidase-conjugated goat anti-mouse IgG secondary antibody.

The protein spot was excised from 52 Ponceau-S-stained nitrocellulose transfers of Triton X-100-insoluble adult rat sciatic nerve proteins. The nitrocellulose pieces were then digested with trypsin. The resulting peptide fragments were separated by high-pressure liquid chromatography, and non-trypsin peaks were subjected to microsequencing by Edman degradation (Tempst et al., 1990). Four distinct sequences were obtained, each with striking similarity to sequences present in rat peripherin (Leonard et al., 1988). The sequences of these peptides and the corresponding peripherin residues are aligned in Table 1.

The identification of the 60 kDa ROCAl antigen as peripherin was further demonstrated in two ways. First, we compared the binding of ROCAl and an anti-peripherin antiserum to nitrocellulose transfers of Triton-insoluble adult rat sciatic nerve proteins that had been separated by two-dimensional electrophoresis. The anti-peripherin antiserum binds a spot in the same position as the ROCAl-immunoreactive 60 kDa protein (Fig. 4). Second, we determined whether ROCAl binds recombinant peripherin on immunoblots. The peripherin fusion protein (obtained from L. Parysek) was generated from a nearly full-length peripherin cDNA insert containing coding sequence for all but the N-terminal 21 amino acids (56 kDa) (Leonard et al., 1988), plus a portion of the E. coli trpE open reading frame (37 kDa). ROCAl binds the peripherin fusion protein that migrates with the expected size of 93 kDa (Fig. 5). This result also indicates that the ROCAl epitope is composed of amino acids, not carbohydrate.

Expression pattern of the ROCAl epitope on peripherin

Consistent with the biochemical identification of the 60 kDa ROCAl antigen as peripherin, ROCAl labels the peripherin-positive subpopulation of neurons in sections of early postnatal DRG (Fig. 6a,b). The small-diameter neurons that strongly express peripherin are identified using the anti-peripherin antiserum on serial sections (Fig. 6b). As previously reported for adult sympathetic ganglia (Suzue et al., 1990), however, ROCAl does not label peripherin-positive neurons in sections of adult DRG (Fig. 6c,d). Instead, as in the adult sympathetic ganglia sections, ROCAl labeling appears to be localized outside and
adjacent to the neurons, probably in the satellite (glial) cells of the DRG (Fig. 6c). Immunoblot analysis of membrane/cytoskeletal fractions derived from early postnatal and adult DRG with the anti-peripherin antiserum and ROCA1 demonstrates the presence of peripherin, as well as the ROCA1 epitope on peripherin in both young and mature DRG (data not shown). Thus, the ROCA1 epitope on peripherin is, in fact, present in mature DRG and sympathetic neurons but is not immunohistochemically accessible.

Previous immunoblot analysis demonstrated that the ROCA1 epitope on the 60 kDa protein is expressed in a rostrocaudal gradient in intercostal nerves (Suzue et al., 1990). Having identified the 60 kDa ROCA1 antigen as peripherin, we next asked whether the peripherin protein itself is also distributed in a rostrocaudal gradient using the anti-peripherin antiserum. Membrane/cytoskeletal fractions of adult rat intercostal nerves taken from segments T2–T12 were prepared as previously described (Suzue et al., 1990). Equal amounts of protein from each of the nerve preparations were separated by SDS-PAGE and transferred to nitrocellulose. To document the rostrocaudal distribution of peripherin, an immunoblot analysis was then performed using ROCA1 and the anti-peripherin antiserum. mAbs specific for NF-M and MBP were used as measures of neuronal cytoskeletal proteins and myelin, respectively. As previously reported (Suzue et al., 1990), the binding of ROCA1 is highest in the rostral segments and declines in a graded manner in the more caudal segments relative to NF-M and MBP (Fig. 7). The rostrocaudal difference between levels T2+3 and T12 for ROCA1 binding, normalized to MBP or NF-M, is about fourfold (Fig. 7). The binding of the anti-peripherin antiserum is also highest in the rostral segments. The rostrocaudal difference for the antiserum binding is not as pronounced as with ROCA1, however; anti-peripherin antiserum binding, normalized to MBP or NF-M, is only about twofold greater for T2+3 than for T12 (Fig. 7). The levels of MBP and NF-M show no obvious rostrocaudal differences (Fig. 7). These results indicate that the peripherin protein is expressed at higher levels in extreme rostral versus extreme caudal intercostal nerves. Moreover, the rostrocaudal difference in ROCA1 binding to a rostral intercostal nerve, T4, and the sciatic nerve, normalized to MBP and NF-M, is about sixfold, while it is about threefold for the anti-peripherin antiserum (data not shown).

Expression of the ROCA1 and ROCA2 epitopes on the 26 kDa protein

The ROCA1 antigen, distributed in a dramatic rostrocaudal gradient as visualized by immunohistochemical labeling of adult peripheral nerves and ganglia, is likely to be a surface membrane protein, not a neuronal cytoskeletal component (Suzue et al., 1990). Thus, although peripherin displays rostrocaudal differences, it cannot be the antigen visualized in sections of peripheral nerves and ganglia. Moreover, the complete solubilization with 1% Triton X-100 of the 26 kDa protein antigen from membrane/cytoskeletal fractions of adult rat peripheral nerves is consistent with the 26 kDa protein being the antigen visualized in immunohistochemistry of adult nerves and ganglia. In addition, we compared the tissue distribution of the 26 kDa protein determined by immunoblotting with the immunohistochemical distribution, and find an excellent correlation (Table 2). A key point is that ROCA1 stains sections of adrenal chromaffin cells, epithelial cells in the esophagus, and myelin tracts in the cerebellum, where the 26 kDa, but not the 60 kDa antigen, is found. Thus, the 26 kDa protein is very likely the membrane antigen visualized in sections of adult sympathetic ganglia and intercostal nerves.

We also analyzed the rostrocaudal distribution of the 26 kDa protein. Equal amounts of the membrane/cytoskeletal preparations (derived from intercostal nerves T2+3 through T12) used to generate the immunoblot presented in Figure 7a were separated into Triton X-100-soluble and -insoluble components. Equivalent amounts of detergent-soluble protein from each of the nerve preparations were subjected to SDS-PAGE and transferred to nitrocellulose. Immunoblot analysis with
Peripherin

Figure 6. ROCA1 labels the cytoplasm of peripherin-positive neurons in postnatal, but not adult DRG. a and b, Serial frozen sections of postnatal day 8 (P8) DRG were labeled with ROCA1 and the anti-peripherin antiserum. Both antibodies strongly label the cytoplasm of small-diameter neurons (small arrows). ROCA1 but not the anti-peripherin antiserum also labels glial cells in the center of the ganglion. c and d, A single frozen section of an adult DRG was double labeled with ROCA1 and the anti-peripherin antiserum as described in Materials and Methods. ROCA1 displays little or no labeling of neuronal cytoplasm, but strongly labels satellite cells enwrapping the neurons (c). The anti-peripherin antiserum, on the other hand, labels only neurons (d). Scale bars, 50 μm.

ROCA1 and ROCA2 fails to show significant rostrocaudal differences in the levels of the 26 kDa protein (Fig. 8). The result with ROCA2 is consistent with the lack of an immunohistochemical gradient of this epitope (Suzue et al., 1990). The absence of a detectable rostrocaudal gradient of the ROCA1 epitope on the 26 kDa protein is not, however, consistent with the immunohistochemical distribution of this epitope (Suzue et al., 1990). Thus, it seems likely that the rostrocaudal gradient observed with ROCA1 on tissue sections is not due to enhanced expression of the 26 kDa antigen in rostral nerves. As in the case for the immunohistochemical gradient of the 43 kDa protein in the retina, the ROCA1 gradient in intercostal nerves may be due to a preferential masking of the ROCA1 epitope on this antigen in sections of caudal nerves.

Identification of the 26 kDa protein

ROCA1 immunoaffinity chromatography was used to purify the 26 kDa protein from extracts of adult rat sciatic nerve. Silver-stained gels of a small amount of the column eluate indicate the purity of the preparation (Fig. 9). In addition to the major 26 kDa protein, minor bands migrating at 30 kDa and 24 kDa are also present. It is very likely that the latter two bands are related to the 26 kDa protein, as they are both detected by ROCA2 in immunoblots, and the size of the 30 kDa protein is reduced to 26 kDa after ENDO-F digestion (data not shown). Large amounts of two independently purified preparations were separated by SDS-PAGE and transferred to Immobilon membranes. The Coomassie-stained 26 kDa bands were excised and subjected to microsequencing by Edman degradation. The following consensus N-terminal sequence was obtained:

XVKGGIDEVFYLLF

This sequence is very similar to that of the protein termed CD9 isolated from human platelets (Higashihara et al., 1990; Bouchet et al., 1991; Lanza et al., 1991):

PVKGGTKCIKYLLF

The identity at each end of this sequence (underlined) suggests that the ROCA 26 kDa antigen is either the rat homolog of human CD9, or that it is a nervous system variant of this protein.

We also obtained internal sequence information from this purified ROCA1 antigen. Approximately 10 μg of the ROCA1-affinity-purified 26 kDa protein was subjected to SDS-PAGE, transferred to nitrocellulose, and stained with Ponceau-S. The 26 kDa band was then excised and exhaustively digested with trypsin. The resulting peptide fragments were separated by HPLC,
Figure 7. Peripherin is expressed in a rostrocaudal gradient in adult rat intercostal nerves. Membrane/cytoskeletal fractions of adult rat intercostal nerves taken from various segmental levels were subjected to SDS-PAGE and transferred to nitrocellulose. The nitrocellulose transfers were then cut into three pieces representing different molecular weight ranges, and an immunoblot analysis was performed as described in Materials and
and non-trypsin peaks were subjected to microsequencing by Edman degradation. The following internal amino acid sequence was obtained:

EVQEFYK

Except for the second residue, this sequence is identical to one present immediately adjacent to a trypsin cleavage site in human CD9 (Bouchie et al., 1991; Lanza et al., 1991):

ELQEFYK

This sequence further supports the probable identification of the ROCA 26 kDa protein as a CD9 homolog.

Discussion

In an effort to identify surface molecules that define position in the mammalian nervous system, we generated a mAb, ROCA1, that defines two rostrocaudal gradients: (1) a 60 kDa ROCA1 antigen that is expressed at higher levels in rostral than in caudal intercostal nerves, as detected by immunoblot analysis, and (2) a 26 kDa ROCA1 antigen that is labeled more strongly in rostral than in caudal glial cells, as detected immunohistochemically (Suzue et al., 1990). We show here that two structurally distinct antigens, the cytoskeletal protein peripherin and a probable rat homolog of the leukocyte/platelet surface protein CD9, very likely share the ROCA1 epitope. While peripherin is expressed in a shallow rostrocaudal gradient, it is the ROCA1 epitope on CD9, and not the protein itself, that is preferentially localized in rostral nerves and ganglia. We generated another antibody in the same fusion, ROCA2, that labels the same cells in ganglia and nerves as ROCA1, but not in a position-selective manner (Suzue et al., 1990). Surprisingly, the rat CD9 homolog identified here also bears the ROCA2 epitope.

Peripherin

Peripherin is a 57 kDa, type III, intermediate filament protein (Leonard et al., 1988; Parysek et al., 1988; Landon et al., 1989). The size and Triton X-100 insolubility of the 60 kDa ROCA1 antigen are consistent with it being the cytoskeletal component peripherin. Moreover, ROCA1 stains peripheral but not central neurons in sections of neonatal rat tissues. Three additional lines of evidence identify the 60 kDa ROCA1 antigen as rat peripherin. First, four internal amino acid sequences derived from the purified protein (see Table 1) exhibit high homology with rat peripherin sequences (Leonard et al., 1988). Second, an antiserum specific for peripherin binds a single major protein that migrates at the same position as the ROCA1-immunoreactive spot in two-dimensional immunoblots of membrane/cytoskeletal fractions derived from adult rat sciatic nerves. This further suggests that ROCA1 binds the most abundant peripherin isoform. Two alternatively spliced forms of peripherin are known to be expressed at much lower levels and migrate differently in two-dimensional SDS-PAGE than the major form (Landon et al., 1989). Third, ROCA1 binds a bacterial fusion protein generated from a peripherin cDNA. This further demonstrates that the ROCA1 epitope is composed of amino acids and not carbohydrate.

The initial characterization of ROCA1 binding by immunoblot analysis suggested that the 60 kDa protein was preferentially expressed in rostral versus caudal intercostal nerves (Suzue et al., 1990). The enhanced binding of an antiserum specific for peripherin to rostral nerves, on similar immunoblots, supports this result. Although the difference in antisera binding between extreme rostral and caudal levels is not as pronounced as with ROCA1, both reagents can be used to define rostrocaudal position. This is further supported by the demonstration that peripherin levels, as determined by immunoblot analysis with ROCA1 and the anti-peripherin antisera, are higher in rostral intercostal nerves than in sciatic nerve. The present results extend those of Suzue et al. (1990) by showing that there is a rostrocaudal gradient of peripherin with respect to NF-M, as well as to MBP. This indicates that the binding of ROCA1 and the anti-peripherin antisera does not simply re-

Table 2. The tissue distribution of the 26 and 60 kDa proteins compared with ROCA1 immunohistochemistry

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Triton-soluble and -insoluble fractions derived from various adult and neonatal rat tissues were prepared as described in Materials and Methods. Immunoblot analysis of Triton-soluble and -insoluble fractions with ROCA1 was used to screen for binding to the 26 kDa protein, and the 60 kDa protein, respectively. Cryosections were also generated from the same tissues and processed with ROCA1 immunohistochemistry as described in Materials and Methods. The relative level of labeling of sections and the relative level of binding to the 26 kDa and 60 kDa proteins are indicated qualitatively by −, no labeling of sections or bands; +, light labeling; or ++, bright labeling. Without exception, the level of labeling of sections is reflected in the amount of the 26 kDa protein detected on immunoblots. SCG, superior cervical ganglion.

Methods. a. The top panel shows the binding of anti-NF-M (1:2000 dilution) to the 160 kDa neurofilament (along with degradation products that migrate at slightly lower molecular weights), the middle panel shows the binding of ROCA1 hybridoma supernatant to the 60 kDa peripherin (Per), and the bottom panel shows the binding of anti-MBP (1:500) to the 18.5 kDa and 14 kDa species of MBP present in these nerves. Equal amounts of protein (40 μg) were loaded in each lane. This immunoblot and five similar ones were scanned by laser densitometry. The plots show the mean ROCA1:MBP, ROCA1:NF-M, and NF-M:MBP ratios (set to 1.0 for pooled T2 and T3 segments) for each segment, with the corresponding SEM values.
The 26 kDa protein is not expressed in a rostrocaudal gradient in adult rat intercostal nerves. Triton X-100-soluble fractions derived from equal amounts of the membrane/cytoskeletal preparations used in Figure 7a were subjected to SDS-PAGE and immunoblot analysis. As shown in Figure 7a, the original membrane/cytoskeletal preparations contain essentially equal amounts of MBP along the rostrocaudal axis. The top left panel shows the binding of ROCA1 hybridoma supernatant to the 26 kDa protein, while the top right panel shows the binding of ROCA2 hybridoma supernatant to the 26 kDa and minor, related proteins. Primary antibody binding was visualized with a peroxidase-conjugated goat anti-mouse secondary antibody. Approximately 40 μg of protein was loaded in each lane. The bottom left and right panels illustrate the results of densitometric scans of ROCA1 and ROCA2 binding (normalized to the binding at level T2+3, which was set to 1.0) to the 26 kDa protein, respectively. Neither ROCA1 nor ROCA2 binding reveals significant rostrocaudal differences in the expression of the 26 kDa protein. No rostrocaudal difference in binding to the 26 kDa protein was obvious even when significantly less protein was loaded in each lane, and an immunoblot performed with ROCA2 (data not shown). ROCA2 binding to the minor proteins also fails to show a positional difference. The results in the left panel are in dramatic contrast to the ROCA1 immunohistochemical results showing a dramatic rostrocaudal gradient of labeling in sections of these same nerves (Suzue et al., 1990).

The finding that peripherin expression is enhanced in rostral nerves extends results from other systems demonstrating position-specific expression of peripherin and particular epitopes on NF-M. For example, transcripts of the Xenopus peripherin homolog XIF3 are enriched in the anterior over the posterior early embryonic nervous system (Sharpe et al., 1989). In the retinotectal system, a phosphorylated epitope on NF-M undergoes a complex temporal and spatial regulation during ganglion cell axon outgrowth and synapse formation (Go et al., 1989). Another recently characterized phosphorylated epitope on NF-M is expressed preferentially in the most distal portion of motor axons in the chick embryo. This epitope is labeled by a mAb at a particular time in development when each group of motor axons is known to respond to guidance cues that lead them to their appropriate muscles (Landmesser and Swain, 1992). These findings suggest that intermediate filament gene expression may be position specific as well as cell type specific.

Unlike other type III family members (glial fibrillary acidic protein, desmin, and vimentin), peripherin is exclusively expressed in neurons, and predominantly in the PNS (Portier et al., 1984; Leonard et al., 1988; Parysek and Goldman, 1988; Parysek et al., 1988; Escurat et al., 1990). In contrast to the type IV neurofilament triplet proteins, however, peripherin is not expressed in all peripheral neurons. Immunofluorescence labeling of adult rat sciatic nerve sections with peripherin-specific antisera indicates that peripherin is enriched in small-caliber axons (Parysek et al., 1991). Peripherin is also enriched in small-diameter DRG neurons (Parysek and Goldman, 1988; Escurat et al., 1990; Ferri et al., 1990; Goldstein et al., 1991). ROCA1 also strongly labels the cytoplasm of small-diameter, peripherin-positive neurons in sections of DRG taken from early postnatal rats (Fig. 7a), but the intensity of this staining declines with development, so that ROCA1 fails to label peripherin-positive neurons in sections taken from adult animals. We suggest that the ROCA1 epitope on peripherin is progressively masked in situ during development, apparently by a postcovalent interaction with another molecule. It seems unlikely that a posttranslational modification of the protein directly masks this ep-
ite since it is composed of amino acids, and ROCA1 continues to bind the 60 kDa band on immunoblots of adult DRG proteins.

Developmentally and spatially regulated epitope masking, potentially through protein–protein interactions, has been observed in other systems. For example, an immunohistochemical study has shown that antibody staining of the NILE protein is downregulated in maturing nerves, while Northern and immunoblots demonstrate continued, high-level NILE expression into adulthood (Prince et al., 1991). More relevant to the present study, a mAb (22/18) that likely recognizes an intermediate filament protein specifically labels blastemal cells in sections of regenerating newt limbs, but reacts with a much wider range of tissues on immunoblots (Ferretti and Brockes, 1990). It is believed that 22/18 detects a specific protein conformation that occurs transiently during regeneration and possibly during development (Ferretti and Brockes, 1990). In support of this, treatment of sections with the fixative ethylene glycol bisuccinimide (EGS), but not with paraformaldehyde, reveals the antigen in sections of normal and regenerating limbs in a distribution consistent with the one detected on the immunoblots (Ferretti and Brockes, 1990). Both fixatives cross-link proteins, but the reactive groups in EGS are spaced farther apart than those of paraformaldehyde. Interestingly, treatment of embryonic retina sections with EGS removes the pronounced dorsoventral asymmetry observed immunohistochemically on unfixed or paraformaldehyde-fixed sections with a number of mAbs (see below; Rabacchi et al., 1990).

26 kDa/CD9

Despite the compelling evidence that peripherin is a ROCA1 antigen, immunohistochemical characterization of ROCA1 binding in sections of adult peripheral nerves and ganglia is inconsistent with the known distribution of peripherin. Most conspicuously, ROCA1 stains glia—Schwann cells in nerves and satellite cells as well as Schwann cells in ganglia (Suzue et al., 1990). In addition, ROCA1 strongly labels myelin tracts in the cerebellum, chromaffin cells in the adrenal medulla, and a variety of epithelial cells (present results; S. Tole and P. H. Patterson, unpublished observations). The cell surface localization of the ROCA1 epitope may also be inconsistent with peripherin being the antigen detected immunohistochemically (Suzue et al., 1990; Tole and Patterson, unpublished observations). On the other hand, the Triton X-100 solubility of the 26 kDa ROCA1 antigen, and its tissue distribution as detected by immunoblot analysis strongly suggest that it is the protein detected immunohistochemically in adult tissues. This ROCA1 antigen was not present at sufficiently high concentrations in the membrane cytoskeletal fractions to be detected along with peripherin in our previous analysis (Suzue et al., 1990). The fact that ROCA2 labels the same tissues and cell types as ROCA1, and that it binds the ROCA1-affinity-purified 26 kDa protein, further supports the identification of this protein as the adult ROCA-glia surface antigen detected immunohistochemically.

Based on N-terminal and internal amino acid sequence data from the affinity-purified 26 kDa protein, we have tentatively identified this ROCA1 antigen as the rat homolog of human CD9. CD9 is a major transmembrane protein that has been recently purified from human platelets (Higashihara et al., 1990; Boucheix et al., 1991; Lanza et al., 1991), and whose primary structure has been elucidated by cDNA cloning (Boucheix et al., 1991; Lanza et al., 1991). The human CD9 protein exists in three forms ranging in molecular weight between 22 and 27 kDa (Kersey et al., 1981; Newman et al., 1982; Boucheix et al., 1983; LeBien et al., 1985; Sechafer et al., 1988). This is consistent with ROCA1 affinity column eluates containing three proteins with a similar molecular weight distribution. As in humans, the middle-molecular-weight form is the most abundant in rat, and the three forms are related, since ROCA1 binds each on immunoblots (LeBien et al., 1985; data not shown). In addition, ROCA1 and ROCA2 each bind a major 26 kDa Triton X-100-soluble protein in extracts of rat platelets (data not shown). These results support the probable identification of the 26 kDa ROCA antigen as a CD9 homolog. Interestingly, three N-terminal sequencing runs have yielded a consensus sequence with a string of five amino acids that does not match the corresponding human CD9 residues. This may represent a rat-specific or a tissue-specific sequence. cDNA cloning of rat mRNAs should answer this question.

Human CD9 is abundantly expressed on the surface of developing B-lymphocytes, platelets, eosinophils, basophils, and stimulated T-lymphocytes, and has also been detected in a variety of nonhematopoietic tissues (Kersey et al., 1981; Jones et al., 1982; Boucheix et al., 1987; Boucheix and Benoit, 1988; Von dem Borne et al., 1989). Two studies have used immunological methods to detect CD9 expression in certain human neuroblastoma cell lines (Kemshead et al., 1982; Komada et al., 1983), but in vivo expression of CD9 in the nervous system has not been previously described. A number of perturbation studies have suggested a function for this molecule. mAbs against human CD9 specifically induce homotypic adhesion of pre-B lymphocytes (Maselli-Smith et al., 1990). In platelets, such antibodies induce activation and aggregation, leading to what is termed an adherent phenotype (Jennings et al., 1990; Griffith et al., 1991). This activation also induces an association between CD9 and the GPl1b-IIIa integrin (Slupsky et al., 1989). That this CD9–integrin complex is involved in platelet aggregation is supported by the finding that platelets from patients with Glanzman’s thrombasthenia, which lack this integrin but not CD9, are not activated by anti-CD9 antibodies (Boucheix et al., 1991).
In addition, anti-CD9 antibodies augment neutrophil adherence to endothelial cells, probably due to an adhesion-promoting activation event within the endothelial cell (Forsyth, 1991).

Taken together, these studies suggest a role for CD9 in the signaling involved in the adhesion and activation of blood cells mediated by intercellular cues. CD9 may participate in this function in association with molecules that directly mediate adhesion (e.g., integrins) (Slupsky et al., 1989; Maselis-Smith et al., 1990; Forsyth, 1991). It is logical to ask whether CD9 is involved in adhesion and signaling in the nervous system as well. In addition to Schwann cell surfaces, CD9 is expressed on neuronal surfaces (Tole and Patterson, unpublished observations). Therefore, this protein could play a role in triggering or mediating homo- or heterotypic adhesion among Schwann cells and neurons. Since most mAbs specific for human CD9 are directed against functional epitopes (Boucheix and Benoit, 1988; Griffith et al., 1991), we are currently using ROCA1 and ROCA2 in in vitro perturbation studies.

**Gradients by differential antigen expression and epitope masking**

ROCA1 staining is highest in adult, rostral intercostal nerves and declines in a graded manner in the caudal segments (Suzue et al., 1990). Nonetheless, ROCA1 labels the 26 kDa (CD9 homolog) protein uniformly along the rostrocaudal axis on immunoblots. We suggest, therefore, that the ROCA1 epitope on CD9 is preferentially masked in situ in caudal nerves, probably as a result of a noncovalent interaction with another protein. The masking protein, which by analogy with the hematopoietic system could be an integrin, would thus be preferentially expressed in the caudal nerves. In contrast to the results with ROCA1, the binding of ROCA2 to intercostal nerves detected immunohistochemically is consistent with the binding detected by immunoblot analysis. By either technique, ROCA2 binding does not reveal a rostrocaudal gradient of CD9. The results with ROCA2 independently confirm that the CD9 protein itself is not preferentially expressed in rostral versus caudal nerves. This situation is analogous to that of the expression of a 43 kDa protein in the developing rat retina (McCaffery et al., 1990; Rabacchi et al., 1990). Multiple mAbs immunohistochemically detect a pronounced dorsoventral gradient, while the protein and corresponding mRNA are expressed at equivalent levels in dorsal and ventral retina halves. Thus, it is possible that epitope masking can define position in the adult PNS, as well as in the torso and vertebral column. Since this region-specific pattern of expression persists in newborn mice, HOX 3.1 is a candidate for regulating the expression of proteins like the ones under study here.

**Note added in proof**

We have recently isolated several full-length cDNA clones using PCR with primers representing protein sequences derived from the purified 26 kDa protein and cDNA templates generated from adult rat sciatic nerve and superior cervical ganglion RNA. These clones each display 90% identity (at the amino acid level) with human CD9, and each contain the same string of seven amino acids corresponding to one of the tryptic peptides generated from the purified 26 kDa protein, as well as these N-terminal 14 amino acids: PVKGGSKCKYLLF. The latter sequence is essentially identical to that found within human CD9, and quite similar to the N-terminal sequence derived from the purified 26 kDa protein. The difference between the N-terminal sequence translated from the nucleotide sequence present in the cDNAs and that derived from the protein suggests that another, alternatively spliced, form of CD9 may exist. These cloning data confirm that CD9 is present in the rat nervous system, and support the identification of the 26 kDa antigen as CD9.

**References**


