

An Isoform of Ankyrin Is Localized at Nodes of Ranvier in Myelinated Axons of Central and Peripheral Nerves

Ekaterini Kordeli, Jonathan Davis, Bruce Trapp,[‡] and Vann Bennett

Howard Hughes Medical Institute, Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710; and [‡]Department of Neurology, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Abstract. Two variants of ankyrin have been distinguished in rat brain tissue using antibodies: a broadly distributed isoform (ankyrin_B) that represents the major form of ankyrin in brain and another isoform with a restricted distribution (ankyrin_R) that shares epitopes with erythrocyte ankyrin. The ankyrin_R isoform was localized by immunofluorescence in cryosections of rat spinal cord gray matter and myelinated central and peripheral nerves to: (a) perikarya and initial axonal segments of neuron cells, (b) nodes of Ranvier of myelinated nerve with no detectable labeling in other areas of the myelinated axons, and (c) the axolemma of unmyelinated axons. Immunogold EM on ultrathin cryosections of myelinated nerve showed that ankyrin_R was localized on the cytoplasmic face of the axolemma and was restricted to the nodal and, in some cases, paranodal area. The major isoform of ankyrin in brain

(ankyrin_B) displayed a broad distribution on glial and neuronal cells of the gray matter and a mainly glial distribution in central myelinated axons with no significant labeling on the axolemma. These results show that (a) ankyrin isoforms display a differential distribution on glial and neuronal cells of the nervous tissue; (b) an isoform of ankyrin codistributes with the voltage-dependent sodium channel in both myelinated and unmyelinated nerve fibers. Ankyrin interacts in vitro with the voltage-dependent sodium channel (Srinivasan, Y., L. Elmer, J. Davis, V. Bennett, and K. Angelides. 1988. *Nature (Lond.)*. 333:177-180). A specific interaction of an isoform of ankyrin with the sodium channel thus may play an important role in the morphogenesis and/or maintenance of the node of Ranvier.

THE node of Ranvier in the myelinated nerve fiber is a highly differentiated structure of the axonal plasma membrane (axolemma), where the myelin sheath is interrupted and saltatory conduction of the action potential takes place. Integral membrane proteins involved in saltatory conduction, such as voltage-dependent sodium channels (reviewed in references 11 and 44) and certain isoforms of the Na⁺/K⁺ATPase (2), accumulate in high concentrations at the nodes and are present in very much lower density in the internodal regions of the axolemma underlying the myelin sheath. Because axonal proteins are synthesized in the neuron cell body and subsequently transported in the axon, the node of Ranvier represents an interesting model for the study of the cellular mechanisms involved in the sorting, targeting, and local stabilization of integral proteins participating in specialized membrane domains.

Interactions between integral membrane proteins and cytoskeletal components have been proposed to account for the formation and maintenance of the nodal membrane domain (45). A characteristic dense cytoplasmic coating underlines the nodal axolemma (33), indicating that a differentiated membrane skeleton is associated with the nodal integral proteins. Ankyrin is a peripheral membrane protein of potential relevance to the node of Ranvier, since it interacts in vitro

with both integral membrane proteins known to be concentrated at the nodes: the voltage-dependent sodium channel (38) and the kidney isoform(s) of the Na⁺/K⁺ATPase (23, 28, 31). Ankyrin in erythrocytes links the anion channel (band 3) to a membrane skeleton composed of spectrin and actin (5, 26). Ankyrin is associated with the plasma membrane in many tissues where it is likely to also interconnect integral proteins with the spectrin-based membrane skeleton (16, 29). A major form of ankyrin in brain tissue has been isolated and characterized (12, 13). Brain ankyrin shares antigenic sites and a similar domain structure and binding properties with human erythrocyte ankyrin. Brain and erythrocyte ankyrin differ in relative affinities for membrane sites (14, 15) and for spectrin (9, 19), and are products of distinct genes (13; Otto E., T. McLaughlin, and V. Bennett, manuscript in preparation).

Ankyrin is a diverse family of polypeptides and therefore is likely to be involved in local segregation of integral proteins through selective interactions between different ankyrin isoforms and families of integral proteins. Two isoforms of ankyrin have been reported in brain (30) together with two isoforms of spectrin (24, 34), and can be divided into two groups: those with a restricted localization and those with a broader localization in cells. Isoforms of an-

kyrin and spectrin with a restricted distribution are localized in dendrites and neuronal cell bodies, and are expressed late in development. Isoforms of ankyrin and spectrin (fodrin) with a broader distribution occur along axons as well as cell bodies, and assemble on the plasma membrane early in development. Two isoforms of ankyrin have been also localized recently in kidney tissue: a restricted isoform immunologically related to erythrocyte ankyrin that is present in basolateral domains of only two cell types, and an isoform with a broad distribution on the membranes of different cell types that cross-reacts preferentially with antibody against the major form of ankyrin isolated from brain (15).

We report here that brain tissue contains a restricted isoform of ankyrin (ankyrin_R) that is localized at specific, mainly neuronal sites including perikarya of neuron cells, initial segments of axons and the nodes of Ranvier of myelinated nerves, whereas the major isoform of brain ankyrin (ankyrin_B) displays a broader distribution in both glial and neuronal cells of the central nervous system. A role of the restricted isoform of ankyrin in the formation and maintenance of the node of Ranvier is proposed.

Materials and Methods

Antibodies

Affinity-purified polyclonal antibodies against human erythrocyte ankyrin (8) and human erythrocyte anion channel (reference 7, band 3) were described previously. Antisera against ankyrin_B, the major isoform of ankyrin in brain tissue, were prepared using as an immunogen bovine brain ankyrin that was isolated in a final step of gel filtration in the presence of 1% SDS and combined with glutaraldehyde-activated rabbit serum albumin before immunization. Affinity-purified antibody against brain ankyrin_B was isolated from these antisera as described (13). Antibodies specific for ankyrin_R, the erythroid isoform of ankyrin in brain tissue, were isolated by adsorption of the affinity-purified antibody against human erythrocyte ankyrin with bovine brain ankyrin (ankyrin_B) coupled to agarose, resulting in elimination of Ig cross-reacting with both forms of ankyrin. In some experiments, an affinity-purified antibody against the $M_r = 90,000$ domain of human erythrocyte ankyrin (6) was used, because of its high reactivity in immunocytochemistry.

Procedures

Brain tissue was obtained from rats perfused with 150 mM NaCl, 10 mM sodium phosphate pH 7.5, 5 mM NaEDTA and 2.5 mM diisopropylfluorophosphate (DFP, Sigma) at 4°C for 15 min, to minimize proteolysis and to remove erythrocytes. The tissue was homogenized with a Polytron (Brinkmann Instruments, Westbury, NY) and the postnuclear membrane fraction (30,000 g pellet) isolated as described (12). SDS-PAGE and immunoblot analysis was performed as described (12). Erythrocyte ghost membranes (4) and bovine brain ankyrin (13) were prepared as described. Rat brain ankyrin was partially purified by chromatographic steps of gel filtration, Mono Q ion-exchange chromatography and hydroxylapatite chromatography.

Light Microscopic Immunocytochemistry

Adult male Sprague-Dawley rats were perfused with either 1% NaNO₃, pH 7.5 or PBS containing 5 mM NaEDTA, and then with 4% paraformaldehyde and 0.08 M phosphate buffer, pH 7.5. The spinal cord, ventral and dorsal roots, and sciatic nerve were removed and placed in fixative overnight. For 4- μ m-thick cryostat sections, the tissue was cryoprotected with 0.7 M sucrose, frozen in liquid nitrogen-cooled isopentane, and cut at -20°C. Sections were mounted on egg white-coated glass slides, air dried, and stored at -80°C. For 1- μ m-thick cryosections, the tissue was cryoprotected with 2.3 M sucrose and 30% polyvinyl pyrrolidone. The tissue pieces were mounted on specimen stubs, frozen in liquid nitrogen, and cut at -80°C using an ultracycromicrotome. 1- μ m-thick cryosections were mounted on polylysine-coated glass slides and rinsed in PBS. Sections were immunostained using antibodies at 1-5 μ g/ml by either indirect immunofluorescence

or by the peroxidase-antiperoxidase procedure as described previously (42). After the immunostaining procedure, the sections prepared for immunofluorescence were mounted in 60% glycerol-PBS. Sections stained by the PAP technique were dehydrated and overlaid with coverslips. As a control, a 20-fold molar excess of heat-denatured (60°C, 25 min) antigenic proteins was added to the specific antibodies and allowed to interact for 1-3 h at 0°C, before incubation with sections.

Electron Microscopic Immunocytochemistry

7-d-old Sprague-Dawley rats were perfused with a solution containing 4% paraformaldehyde, 2.5% glutaraldehyde, and 0.08 M phosphate buffer. The L4 and L5 ventral root and dorsal root ganglion were dissected from the animal and placed in fixative overnight. Segments of this tissue (2-4 mm) were cryoprotected with 2.3 M sucrose and 30% polyvinyl pyrrolidone, placed on specimen stubs, and frozen in liquid nitrogen. Ultrathin cryosections (100-120 nm thick) were cut on an ultracycromicrotome maintained at -110°C. The sections were transferred to Formvar- and carbon-coated grids and maintained on 2% gelatin in PBS for 2 h. Sections were immunostained by colloidal gold procedures, postfixed, and embedded in methylcellulose using previously described modifications (42) of standard procedures (37, 41).

Results

Two Isoforms of Ankyrin Are Immunologically Distinguished in Rat Brain

Several findings during isolation of brain ankyrin (13) suggested the possibility that brain tissue contains two forms of ankyrin: (a) a major, extractable form that is the principal component of purified brain ankyrin ($M_r = 220,000$ and 210,000 in bovine brain or 220,000 in rat brain); (b) a second population of ankyrin polypeptides that remain associated with brain membranes even after extensive extraction with a combination of 0.8 M KI and a detergent (13). The retained protein contains an additional polypeptide of $M_r = 186,000$ that is not present in the extracted fraction of ankyrin. The $M_r = 186,000$ polypeptide cross-reacts with antibody against erythrocyte ankyrin, but does not cross-react with antibody against bovine brain ankyrin (Fig. 1, lanes 2 in A and D, respectively). It is of interest that human erythrocyte ankyrin also includes a minor polypeptide of $M_r = 186,000$ (reference 19; band 2.2) in addition to the major polypeptide of $M_r = 215,000$ (band 2.1). These observations suggested the possibility that the $M_r = 186,000$ polypeptide associated with brain membranes represents an isoform of ankyrin closely related to erythrocyte ankyrin and that this polypeptide may be analogous to band 2.2 of erythrocyte membranes.

In this study, the presence of an erythroid isoform of ankyrin in brain has been demonstrated using an erythroid-specific antibody that does not react with the major form of brain ankyrin (Fig. 1). The erythroid-specific antibody was prepared by passage of affinity-purified antibody against erythrocyte ankyrin through an affinity column containing the major form of brain ankyrin. Erythrocyte ankyrin antibody before immunoabsorption with brain ankyrin (Fig. 1 A) cross-reacted with isolated rat brain ankyrin, which migrates as a single polypeptide of $M_r = 220,000$ (lane 3) and with two polypeptides in rat brain homogenates of $M_r = 215,000$ - $220,000$ and 186,000 (lane 2). The antibody depleted by immunoabsorption against brain ankyrin (Fig. 1 B) no longer cross-reacted with isolated rat brain ankyrin (lanes 3, 3'), but still reacted with the two forms of ankyrin of $M_r = 215,000$ (band 2.1) and 186,000 (band 2.2) in human

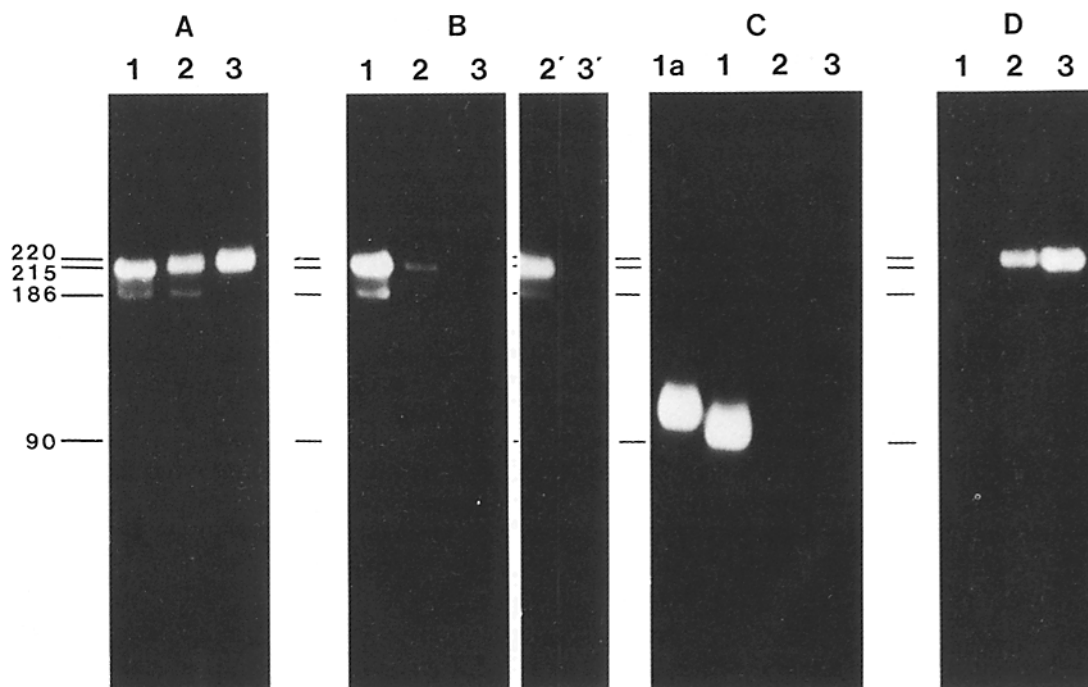


Figure 1. Immunodetection of ankyrin isoforms in rat brain membranes. Human erythrocyte ghost membranes (0.3 μg protein) (lanes 1), rat brain membranes (10 μg protein) (lanes 2), partially purified rat brain ankyrin (2 μg protein) (lanes 3) and rat erythrocyte ghost membranes (0.5 μg protein) (lane 1a in C) were prepared as described in experimental procedures, electrophoresed on SDS-PAGE, transferred to nitrocellulose and immunoblotted with the following affinity-purified antibodies: (A) human erythrocyte ankyrin antibody; (B) human erythrocyte ankyrin antibody followed by depletion of Ig reacting with bovine brain ankyrin epitopes (antibody specific for erythroid ankyrin polypeptides); (C) antibody against human erythrocyte anion channel (band 3); (D) bovine brain ankyrin antibody. Lanes 2' and 3' in B show a 10-times longer exposure of the autoradiogram of lanes 2 and 3. Molecular weight markers are the two human erythrocyte ankyrin polypeptides (ankyrin_(s)_R, $M_r = 215,000$ and $186,000$), the major brain ankyrin (ankyrin_B, $M_r = 220,000$), and human band 3 protein ($M_r = 90,000$).

erythrocyte ghosts (lane 1) and with two polypeptides of $M_r = 215,000$ and $186,000$ in rat brain membranes (lanes 2, 2'). The possibility that the reaction with the erythroid-specific antibody was due to contaminating rat erythrocyte membranes in the brain membrane preparation was ruled out in C, when antibody against the human erythrocyte anion channel (band 3) cross-reacted intensely with both human and rat erythrocyte ghosts (lanes 1 and 1a respectively), but not with brain membranes (lane 2). Thus, an antibody against erythrocyte ankyrin that does not react with the major brain ankyrin isoform still was able to detect a form of ankyrin in brain tissue.

The domain structure of the brain tissue polypeptides cross-reacting with erythrocyte ankyrin antibody was evaluated by limited proteolysis of brain membranes followed by immunoblotting with this antibody (Fig. 2). Antibody specific for erythrocyte ankyrin cross-reacts with two major chymotryptic fragments in brain membranes of $M_r = 72,000$ and $95,000$ (Fig. 2 A) that coincide with the characteristic domains observed with other ankyrins from brain and erythrocytes (6, 13).

Adsorption of erythrocyte ankyrin antibody with purified brain ankyrin (Fig. 1) removed much of the reactivity against brain membrane polypeptides (compare lanes 2 in A and B). In contrast, the adsorbed and unadsorbed antibodies cross-reacted to a similar extent with ankyrin in human erythrocyte ghost membranes (lanes 1 in A and B). Two possible expla-

nations for the weak cross-reaction of the erythroid-specific antibody with ankyrin polypeptides in brain membranes are: (a) the erythroid ankyrin polypeptides are present in very small amounts in brain tissue. However, the initial, unadsorbed erythrocyte ankyrin antibody cross-reacted much more intensely than the adsorbed, erythroid-specific antibody with the $M_r = 186,000$ polypeptide in brain membranes that is related only to the erythroid isoform (lanes 2 in A and B). This comparison indicates that the erythroid-specific antibody has been depleted of much of the cross-reactivity against the erythroid form of ankyrin as well as all of the cross-reactivity towards the major form of ankyrin in brain. Thus the weak reactivity with the erythroid ankyrin antibody probably does not result from a small amount of erythroid ankyrin in brain. (b) The most likely possibility is that the erythroid isoform(s) of ankyrin in brain tissue share antigenic sites with the major form of brain ankyrin. The immunoadsorption with brain ankyrin in this case would have removed those antibodies that cross-reacted with both isoforms.

The antibody raised against purified bovine brain ankyrin (Fig. 1 D) was specific for the major ankyrin isoform in rat brain membranes ($M_r = 220,000$; lane 2) and did not cross-react with human erythrocyte ghost membranes (lane 1). This antibody displayed a cross-reaction with limited epitopes of the antigen, since it did not recognize, in immunoblots, the two major proteolytic fragments of $M_r = 90,000$ -

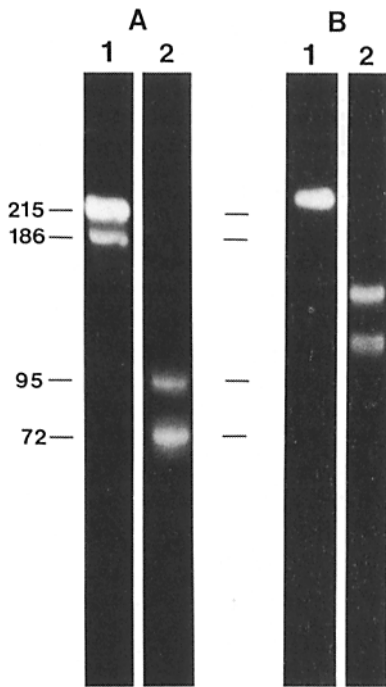


Figure 2. Immunodetection by immunoblot of the cross-reactivity of erythrocyte and brain ankyrin antibodies with proteolytic fragments of the brain ankyrin isoforms. Brain membranes were washed in a solution containing 10 mM sodium phosphate, 1 mM EGTA, 1 mM NaN_3 , and 0.5 mM DTT, pH 7.5, digested with 10 $\mu\text{g}/\text{ml}$ of chymotrypsin for 10 min at 4°C, electrophoresed and immunoblotted as described in the Materials and Methods with antibodies against human erythrocyte ankyrin (A) and bovine brain ankyrin (B). Brain membranes are presented before (lanes 1) and after (lanes 2) proteolysis. Molecular weight markers are the two erythroid ankyrin polypeptides ($M_r = 215,000$ and $186,000$) and the two major proteolytic fragments of them ($M_r = 95,000$ and $72,000$).

95,000 and 72,000 that are common in both erythrocyte and brain ankyrins (Fig. 2 B). However, the selective brain ankyrin antibody exhibits excellent specificity and staining intensity in immunocytochemistry and was used in this study.

The multiple forms of ankyrin in brain as well as kidney (15) pose a problem in nomenclature. We will use the following somewhat arbitrary system in this paper, which is based on the differences between these isoforms in terms of cellular expression and localization in nervous tissue (see immunocytochemistry results) and kidney (15). The erythroid isoform of brain ankyrin will be referred to as a member of the family of restricted isoforms of ankyrin or ankyrin_r. This family of ankyrins can be distinguished based on preferential cross-reaction with antibodies against erythrocyte ankyrin as well as expression and restricted distribution within a limited number of cell types in kidney and nervous tissue. The major form of brain ankyrin will be considered as a member of the family of broadly distributed ankyrins or ankyrin_b, distinguished by a preferential cross-reaction with antibodies against the major ankyrin polypeptide isolated from brain tissue and expression in many types of cells in kidney and nervous tissue.

Ankyrin_b and Ankyrin_r Display a Different Cellular Distribution in the Central Nervous System

The two isoforms of brain ankyrin have been localized by in-

direct immunofluorescence in cryosections of gray and white matter of spinal cord (Figs. 3 and 4, respectively). Erythrocyte ankyrin antibody before and after immunoadsorption with brain ankyrin_b (Fig. 3, a and g, respectively) displayed a punctate labeling at the level of the plasma membrane of the neuron cell bodies within the gray matter. An interesting and consistent observation was that different neuron cells were labeled to a different extent. Labeling on axonal initial segments and starting parts of dendrites was also observed. The specificity of the cross-reaction was tested by incubating the antibodies with either purified erythrocyte ankyrin (Fig. 3, b and h) or purified bovine brain ankyrin_b (Fig. 3, c and i) before incubation with sections. Labeling was entirely displaced by preincubation with erythrocyte ankyrin. A very faint labeling remained around the neuron cells after preincubation of the unadsorbed erythrocyte ankyrin antibody with purified brain ankyrin_b (Fig. 3 c). Thus the two isoforms of ankyrin in nerve tissue share antigenic sites, as suggested by immunoblot results (Fig. 1).

Brain ankyrin antibody (Fig. 3 d) labeled all cell types in the gray matter. Labeling extended up to nerve cell bodies and is likely to include the neuronal plasma membrane. Brain ankyrin antibody also labeled nerve cell bodies and dendrites in sections of cerebellum (data not shown). The nuclei of the neuron cells were also labeled. Labeling of nuclei was specific, since it was displaced by excess of purified brain ankyrin (Fig. 3 f). A common epitope between brain ankyrin_b and a nuclear protein might be responsible for this cross-reaction.

Cross-sections of the white matter (Fig. 4) revealed that the brain ankyrin antibody stains mainly the glial cells of central nerve fibers. A prominent staining occurred on the astrocytic processes that group the nerve fibers into bundles, and on the outer cytoplasmic layers of the oligodendrocytes that surround the central myelinated axons (Fig. 4 b), although compact myelin was not labeled. Identification of the labeled structures as glial processes rather than axons was based on the observation that rings of stain were of large diameter and were touching each other. Axonal staining, in contrast, would result in smaller diameter rings, separated by the thickness of the myelin sheath (compare immunofluorescence staining with similar Differential Interference Contrast micrograph, Fig. 4). The unadsorbed erythrocyte ankyrin antibody reacted only weakly with the oligodendrocytes (Fig. 4 a). Immunoadsorption of the above antibody with brain ankyrin_b considerably decreased cross-reaction with white matter (Fig. 4 c). Absence of labeling on the cross-sectioned internodal axolemma was consistently observed by either ankyrin antibody (Fig. 4, a-c).

Erythrocyte ankyrin antibodies before and after immunoadsorption with brain ankyrin_b display a similar pattern of labeling on spinal cord cryosections (Figs. 3 and 4). This was unexpected since the unadsorbed antibody cross-reacts with both ankyrin_r and ankyrin_b in immunoblots (Fig. 1). In evaluating the relative reactivity of erythrocyte and brain ankyrin_b with this antibody, it should be emphasized that erythrocyte ankyrin (Fig. 1 A, lane 1) was present at a 100-fold lower concentration than the isolated brain ankyrin_b (Fig. 1 A, lane 3). This antibody thus cross-reacts much better with the erythrocyte ankyrin and, therefore, the ankyrin_r isoform than with the major form of ankyrin in brain. In fact, comparison of the labeling pattern of the above cross-sections of

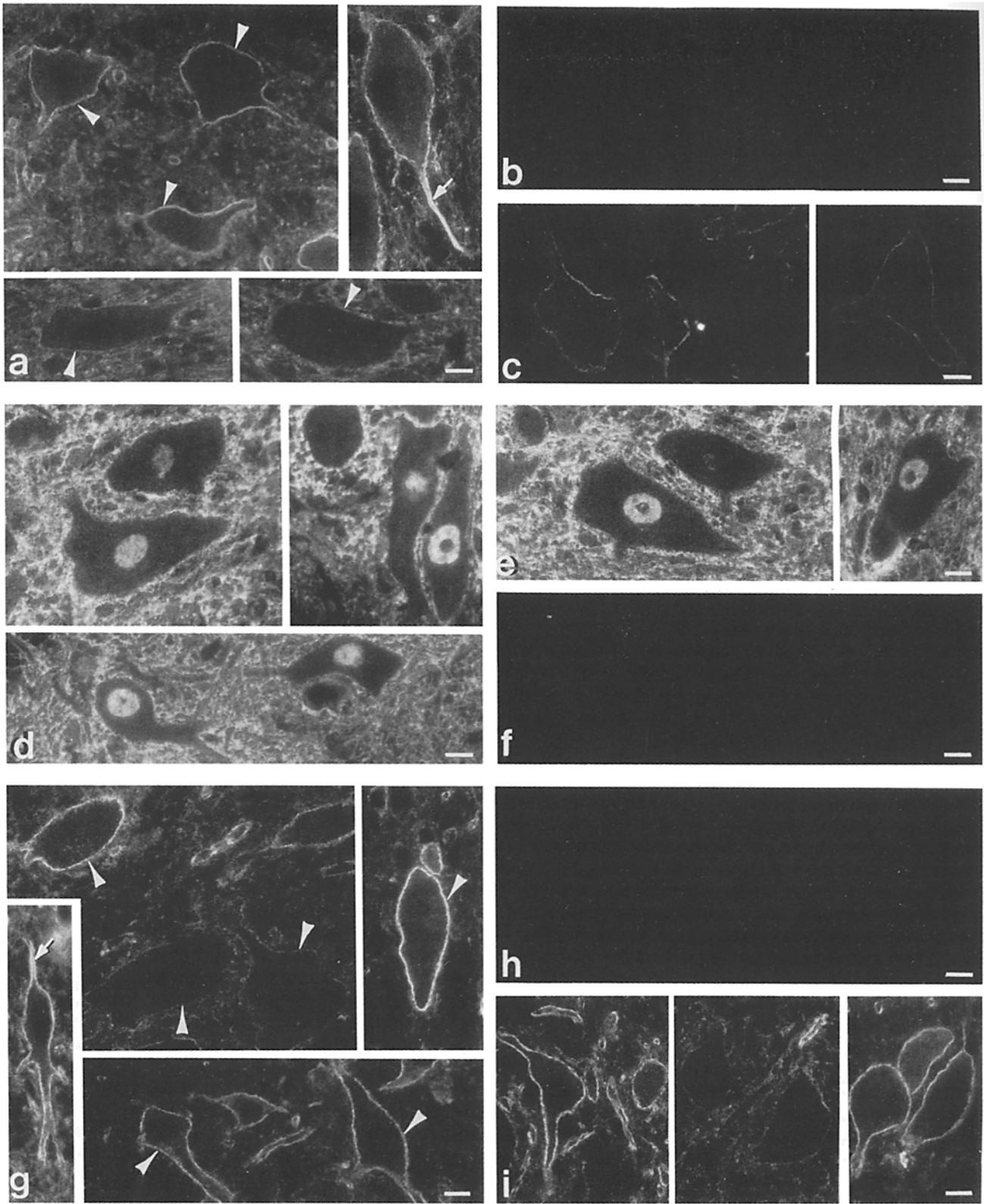


Figure 3. Cellular distribution of brain ankyrin isoforms (ankyrin_B and ankyrin_R) in the grey matter of rat spinal cord. Cross-cryosections of spinal cord grey matter were labeled by indirect immunofluorescence with bovine brain ankyrin antibody (Fig. 3, *d-f*) and human erythrocyte ankyrin antibody before and after depletion of brain ankyrin immunoreactivity (Fig. 3, *a-c* and *g-i*, respectively). *Arrowheads* point at the level of the plasma membrane of neuron cells. *Arrows* (Fig. 3, *a* and *g*) indicate the initial axonal segments of neuron cells. In control experiments, the antibodies have been incubated with a 20-fold molar excess of purified human erythrocyte ankyrin (Fig. 3, *b, e, and h*) and bovine brain ankyrin (Fig. 3, *c, f, and i*) before incubation with the tissue. Bars, 10 μ m.

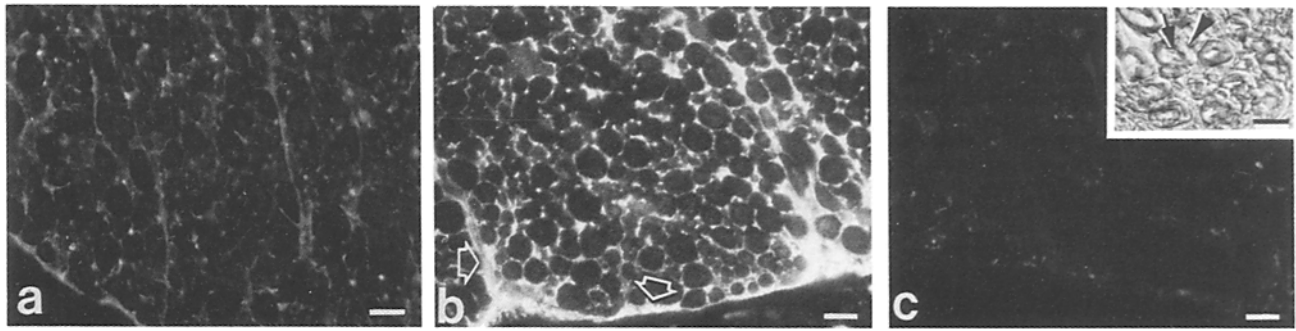


Figure 4. Cellular distribution of brain ankyrin isoforms (ankyrin_B and ankyrin_R in rat central nerve. Cross-cryosections of rat central nerve fibers (white matter of spinal cord) were labeled with bovine brain ankyrin antibody (Fig. 4 *b*) and human erythrocyte ankyrin antibody before and after depletion of brain ankyrin immunoreactivity (Fig. 4, *a* and *c*, respectively). *Inset* in Fig. 4 *c* is a DIC light micrograph of a homologous area of cross-sectioned spinal cord (white matter). *Small arrow* points to a myelinated central axon and *arrowhead* to the myelin sheath. Brain ankyrin antibody labels oligodendrocytes, but not compact myelin, around the central myelinated axons. Subpial astrocytes and their processes are also labeled (Fig. 4 *b*, *open arrows*). Note the absence of labeling on the axolemma with all three antibodies. Bars, 10 μ m.

spinal cord (Figs. 3 and 4) with the three different antibodies used in this study shows that erythrocyte ankyrin antibody before immunoadsorption exhibits, in addition to the specific labeling, a low background labeling comparable to the brain ankyrin antibody labeling pattern.

Ankyrin_R Is Enriched at the Node of Ranvier in Myelinated Axons

Brain ankyrin_R and brain ankyrin_B have been localized by indirect immunofluorescence in longitudinal cryosections of rat myelinated nerve. Erythrocyte ankyrin antibody (Fig. 5) selectively labeled the nodes of Ranvier of myelinated axons on longitudinal sections of the peripheral nervous system (Fig. 5, *a* and *d*) and the central nervous system (Fig. 5 *c*). The internodal axolemma of myelinated axons was essentially unlabeled (Fig. 5, *a*, *c*, and *d*). On thinner sections (1 μ m), labeling occurred mainly near the nodal plasma membrane (Fig. 5, *a*, *c*, and *e*). On thicker (4 μ m) sections, a weaker and punctate labeling sometimes extended to the paranodal area (Fig. 5, *f-h*). Staining also was present within the cytoplasm underlying nodes of Ranvier (Fig. 5, *d* and *h*, *asterisk*). In between the myelinated axons, continuous labeling occurred along elongated structures (Fig. 5 *a*). Comparison with EM images (see below, Fig. 8) strongly suggests that these structures correspond to unmyelinated axons, that are generally of smaller diameter and usually organized in bundles among the myelinated axons. However, cross-reaction with other cell types that exist between myelinated axons, such as fibroblasts, can not be excluded. Nodes of Ranvier were still stained with the erythrocyte ankyrin antibody after depletion of immunoreactivity against brain ankyrin_B (Fig. 5 *i*). Labeling was entirely displaced when the adsorbed antibody was preincubated with erythrocyte ankyrin (Fig. 5 *j*) but not with brain ankyrin_B (Fig. 5 *k*).

Ankyrin_B, which reacts with antibody against the major form of brain ankyrin (Fig. 6), displayed a different localization from ankyrin_R in longitudinal sections of central nerve fibers. Labeling occurred predominantly along the astrocytic processes and outer cytoplasmic layers of the oligodendrocytes surrounding the myelinated axons. No labeling was observed on the axolemma, even at the level of the nodes of

Ranvier (Fig. 6, *d* and *g*, *arrowheads*), where only the overlying astrocytic processes appeared labeled. Interestingly, brain ankyrin antibody displayed a very weak cross-reaction with peripheral nerve (not shown). This difference is likely to reflect the existence of different isoforms of ankyrin_B in the central and peripheral nervous systems. The brain ankyrin antibody used in this study displays a selective cross-reactivity with restricted domains of ankyrin (Fig. 2), does not cross-react with the ankyrin_B of kidney (not shown) and thus may distinguish between slightly different isoforms of ankyrin_B. In support of the presence of ankyrin_B in peripheral nerve is that sciatic nerve contains a polypeptide of $M_r = 220,000$ that cross-reacts with a more general antibody against brain ankyrin (not shown).

These results indicate that the ankyrin_R isoform is highly concentrated at the nodes of Ranvier in central and peripheral myelinated axons, whereas the ankyrin_B isoform displays a mainly glial distribution along myelinated axons of the central nervous system.

Localization of the Ankyrin_R Isoform on the Axonal Plasma Membrane of the Node of Ranvier by EM

The subcellular distribution of the ankyrin_R isoform within myelinated and unmyelinated fibers was studied by EM using immunogold-labeling of ultrathin cryosections of rat peripheral nerve. The unadsorbed erythrocyte ankyrin antibody was chosen for these experiments because it combines a high immunoreactivity with a satisfactory specificity for the ankyrin_R isoform, as demonstrated by immunofluorescence in Figs. 3 and 4 (see also Fig. 5).

In electron micrographs of longitudinally oriented myelinated fibers, gold particles were concentrated on the cytoplasmic side of the nodal axolemma and in cytoplasmic sites restricted to nodal areas. In some fibers the subaxolemmal staining was restricted to the nodal axolemma (Fig. 7 *A*), but in others it extended beneath the first two to three paranodal loops and stopped abruptly (Fig. 7 *B*). Nodal axoplasm and Schwann cell processes that oppose the nodal axolemma were also labeled. Axoplasmic staining was distributed randomly and in some sections occurred in rows that were oriented parallel to the nodal axolemma. Labeling of Schwann

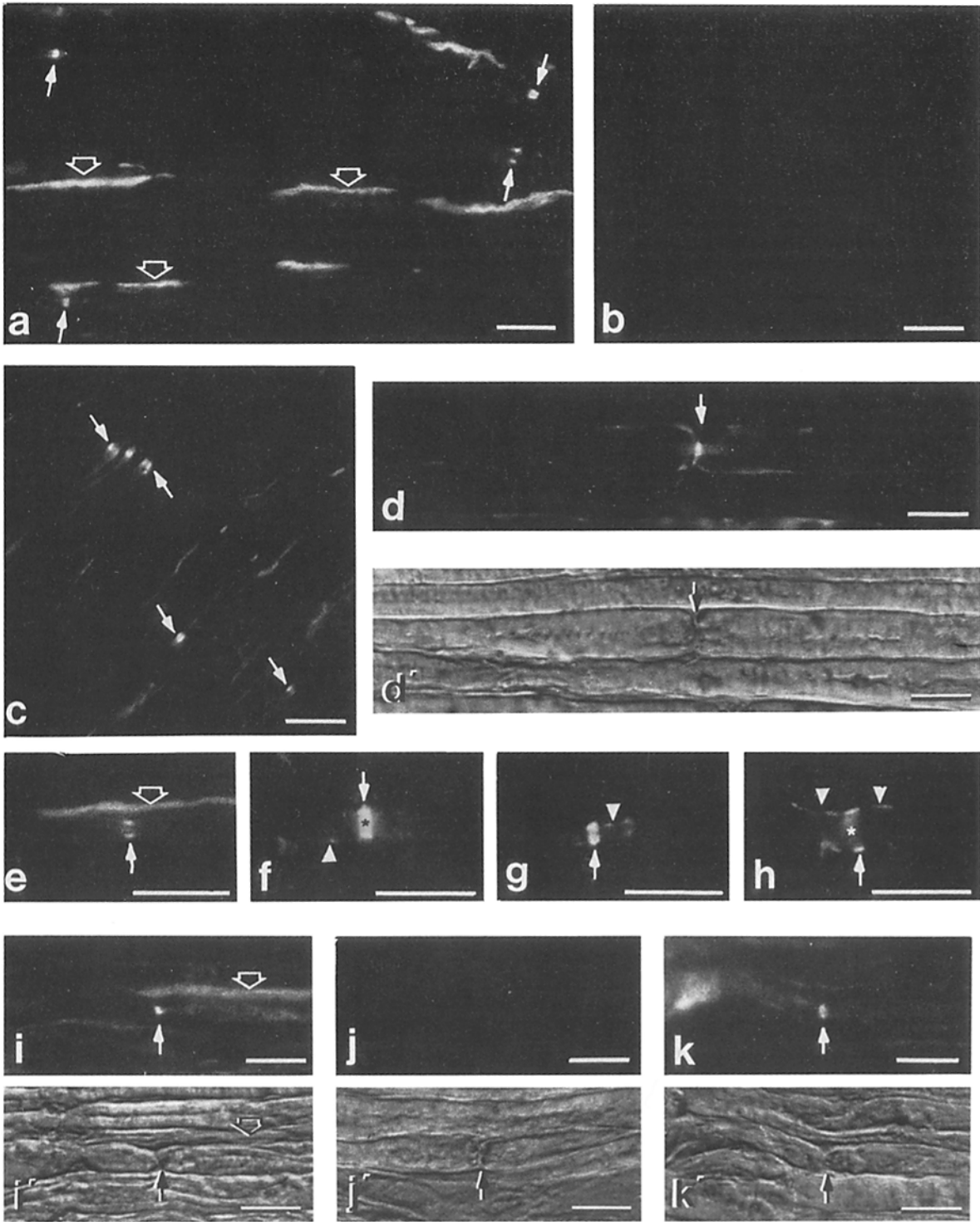


Figure 5. Localization by immunofluorescence of the erythrocyte ankyrin antibody in rat central and peripheral nerve. Longitudinal cryosections of rat peripheral nerve (Fig. 5, *a* and *e-h*, spinal root; *d* and *i-k*, sciatic nerve) and central nerve (*b* and *c*, white matter of spinal cord) were labeled with human erythrocyte ankyrin antibody before and after immunoadsorption with brain ankyrin_B (*a-h* and *i-k*, respectively). The unadsorbed antibody stains the nodes of Ranvier (*arrows*) but not the internodal axolemma (compare labeling on a single myelinated axon (*d*) with corresponding DIC light micrograph, *d'*). *e-h* show labeled nodes of Ranvier on peripheral myelinated axons of different diameter. *Arrowheads* point to the paranodal axolemma and asterisks are placed within the nodal cytoplasm. Heavy staining indicated by asterisk in *f* most likely originates from a tangential section of the nodal axolemma rather than from cytoplasm. Labeling was displaced (*b*) when the unadsorbed antibody was preincubated with a 20-fold molar excess of antigen (215,000 *M_r*, human erythrocyte ankyrin polypeptide). The adsorbed antibody still stained the node of Ranvier (*i*). Labeling was displaced by preincubating the antibody with a 20-fold molar excess of 215,000 *M_r* ankyrin (*j*), but not with the same excess of bovine brain ankyrin_B (Figure 5*k*). Figures 5*i'-k'* are the corresponding DIC light micrographs of nodes of Ranvier. Labeling indicated by open arrows may correspond to bundles of unmyelinated fibers or other cell types different from myelinated axons (compare *i* and *i'*). *a-c* and *e*: 1- μ m-thick sections; *d*, *d'*, and *i-k'*: 4- μ m-thick sections. Bars, 10 μ m.

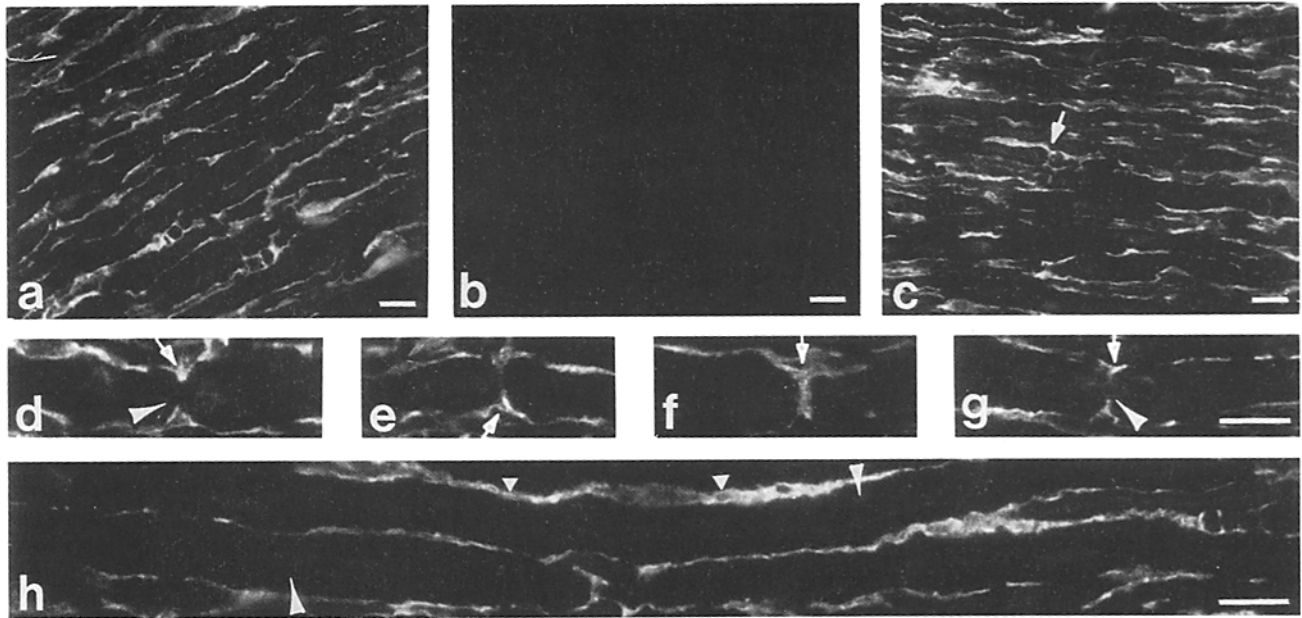


Figure 6. Localization by immunofluorescence of the brain ankyrin antibody in rat central nerve. Longitudinal 1- μm -thick cryosections of rat central nerve (white matter of spinal cord) were labeled by indirect immunofluorescence with the bovine brain ankyrin antibody. Arrowheads point to the axolemma, small arrowheads show the glial processes surrounding the myelinated axons and arrows show nodes of Ranvier. Arrowheads in *d* and *g* point close to the nodal axolemma. In control experiments, the antibody has been incubated with a 20-fold molar excess of purified bovine brain ankyrin_B protein (*b*) and human erythrocyte ankyrin polypeptide of $M_r = 215,000$ (*c*) before incubation with sections. Bars, 10 μm .

cell cytoplasm was most intense in processes that covered nodal axolemma and less intense in paranodal loops. However, at least partial contribution of the brain ankyrin immunoreactivity of the erythrocyte ankyrin antibody to the above glial and paranodal staining cannot be excluded.

To determine whether unmyelinated axons contained ankyrin_R, 1 μm -thick and ultrathin cryosections of dorsal root ganglion were immunostained with antibody against erythrocyte ankyrin (Fig. 8). In 1 μm -thick sections, small dots and short lines of immunoperoxidase reaction product that had the distribution of unmyelinated fibers were scattered among larger unstained myelinated fibers (Fig. 8 *A*). These structures viewed in cross-section are likely to be the same as the elongated immunofluorescent labeling observed in longitudinal sections. This interpretation was supported by our electron microscopic studies. Gold particles were concentrated on the cytoplasmic side of axolemma that were part of unmyelinated axons (Fig. 8 *B*, arrowheads). In contrast, internodal axolemma of myelinated fibers was not labeled at a detectable level (Fig. 8 *B*). Occasional gold particles were found within internodal axoplasm of myelinated fibers and may represent axoplasmic translocation of the ankyrin_R isoform.

Discussion

This report demonstrates that an ankyrin isoform (ankyrin_R), distinguished by antibodies against erythrocyte ankyrin, is highly concentrated at the nodes of Ranvier in myelinated nerve axons of the central and peripheral nervous systems. This protein is also found at the plasma membrane of neuron cells and initial axonal segments, as well as beneath the axolemma of unmyelinated axons. Within myelin-

ated axons, EM revealed that the ankyrin_R isoform is concentrated at the cytoplasmic face of the nodal axolemma within the characteristic subaxolemmal coating. This labeling sometimes extended to the initial segment of the paranodal area, underneath the first two to three paranodal loops of the myelin internode. Ankyrin_B, another isoform of brain ankyrin which is the major form of ankyrin isolated from brain tissue, displays a different distribution in central nervous tissue where it is associated with glial and neuronal cells. Recently, cDNAs encoding ankyrin isoforms have been isolated from a human brain library using antibodies as probes (Otto, E., T. McLaughlin, and V. Bennett, manuscript in preparation.) The sequence of these cDNAs confirms that these proteins are indeed closely related to erythrocyte ankyrin, although they are products of distinct genes.

Both forms of ankyrin are not detectable in the internodal regions of myelinated axons with antibodies utilized in this study. Ankyrin has been reported to be present in axons in other studies. In one case (16), sections were from a small area of the cerebellum and axonal staining may, in retrospect, have resulted from the initial axonal segments which we also have observed (Fig. 3). The other study concerned the chicken optic nerve (30). The apparent absence of ankyrin in internodal axons should be viewed with some skepticism, since our antibodies may not recognize all isoforms of ankyrin. However, if ankyrin actually is not present, the spectrin in internodal regions may be linked to the membrane by an ankyrin-independent protein interaction (39).

A hypothesis for the role of the ankyrin_R isoform at the nodal membrane is to link specialized membrane proteins of the node of Ranvier to a spectrin-actin membrane skeleton and thus restrict the distribution of these proteins in the membrane. Ankyrin associates *in vitro* with both ion chan-

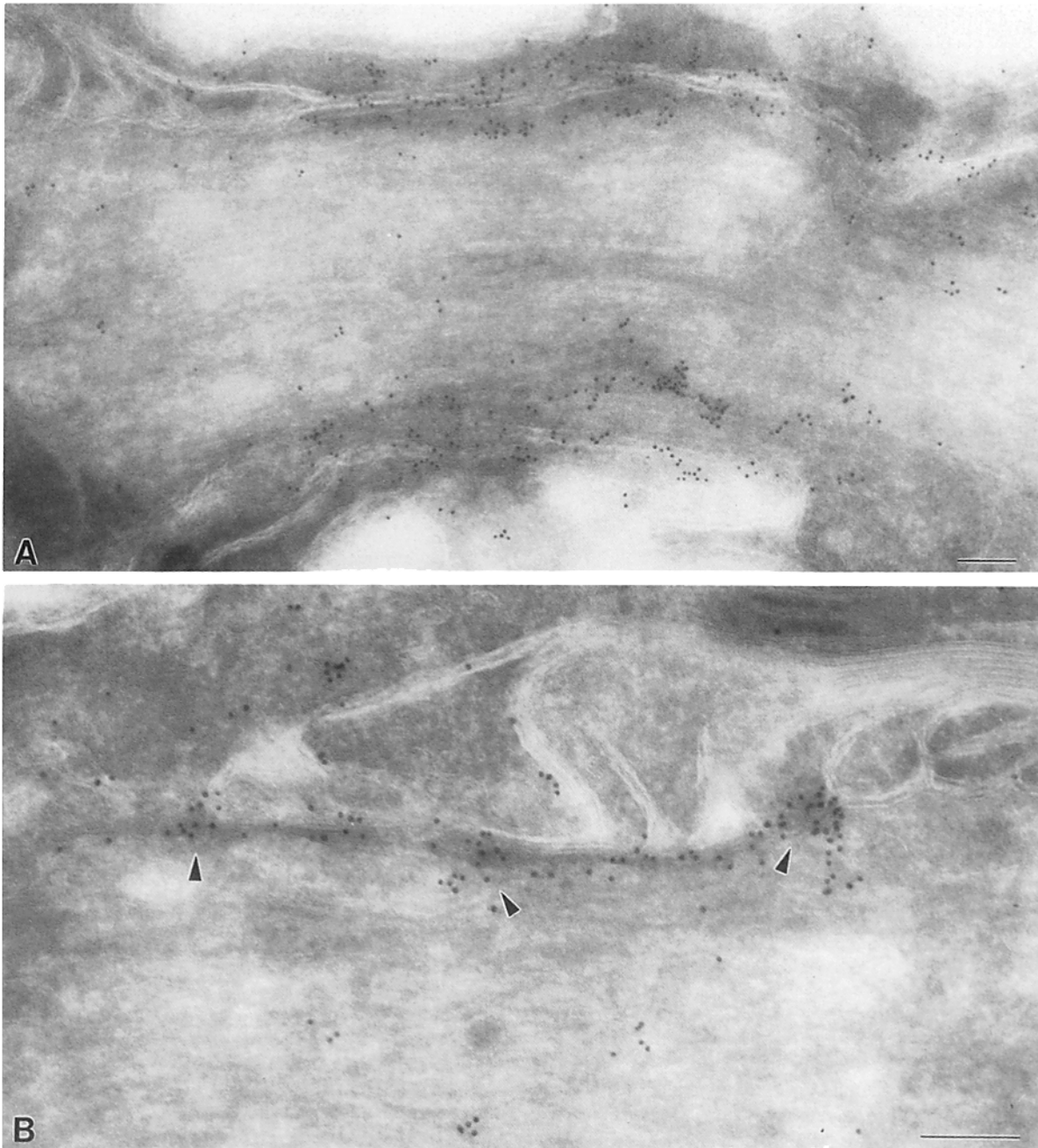


Figure 7. Ultrastructural localization by immunogold of brain erythroid ankyrin (ankyrin_R) in rat peripheral nerve. Longitudinal ultrathin cryosections of rat dorsal spinal root were labeled with the erythrocyte ankyrin antibody by the immunogold technique (see Materials and Methods). Arrowheads point on the axonal plasma membrane of the nodal (a) and paranodal area (b) of a myelinated nerve fiber. Bars, 0.2 μ m.

nels of the nodes, the voltage-dependent sodium channel of brain (38) and the alpha 1 isoform of Na⁺/K⁺ ATPase isolated from kidney tissue (23, 28, 31) that is also expressed in brain (40). Ankyrin_R codistributes with the sodium channel not only at the node of Ranvier of myelinated axons, but also at the initial axonal segments of the neuron cells (43; this paper) and along the axolemma of unmyelinated axons (10, 35, this paper). It has been proposed that the sodium channel is segregated at the nodes either because of the presence of the axoglial junctions at the paranodal area (36), or

by interaction with components of the cytoskeleton (45). The latter hypothesis is more likely, since initial segments of axons and heminodes of dystrophic mice (17) are bounded on only one side by the axoglial junction, but still have a local concentration of sodium channels in the membrane regions lacking adjacent glial contacts. Further evidence for association of the sodium channel with the cytoskeleton is the observation that the channel displays a reduced mobility in the axonal hillock of neuron cells cultured in the absence of glial cells (1).

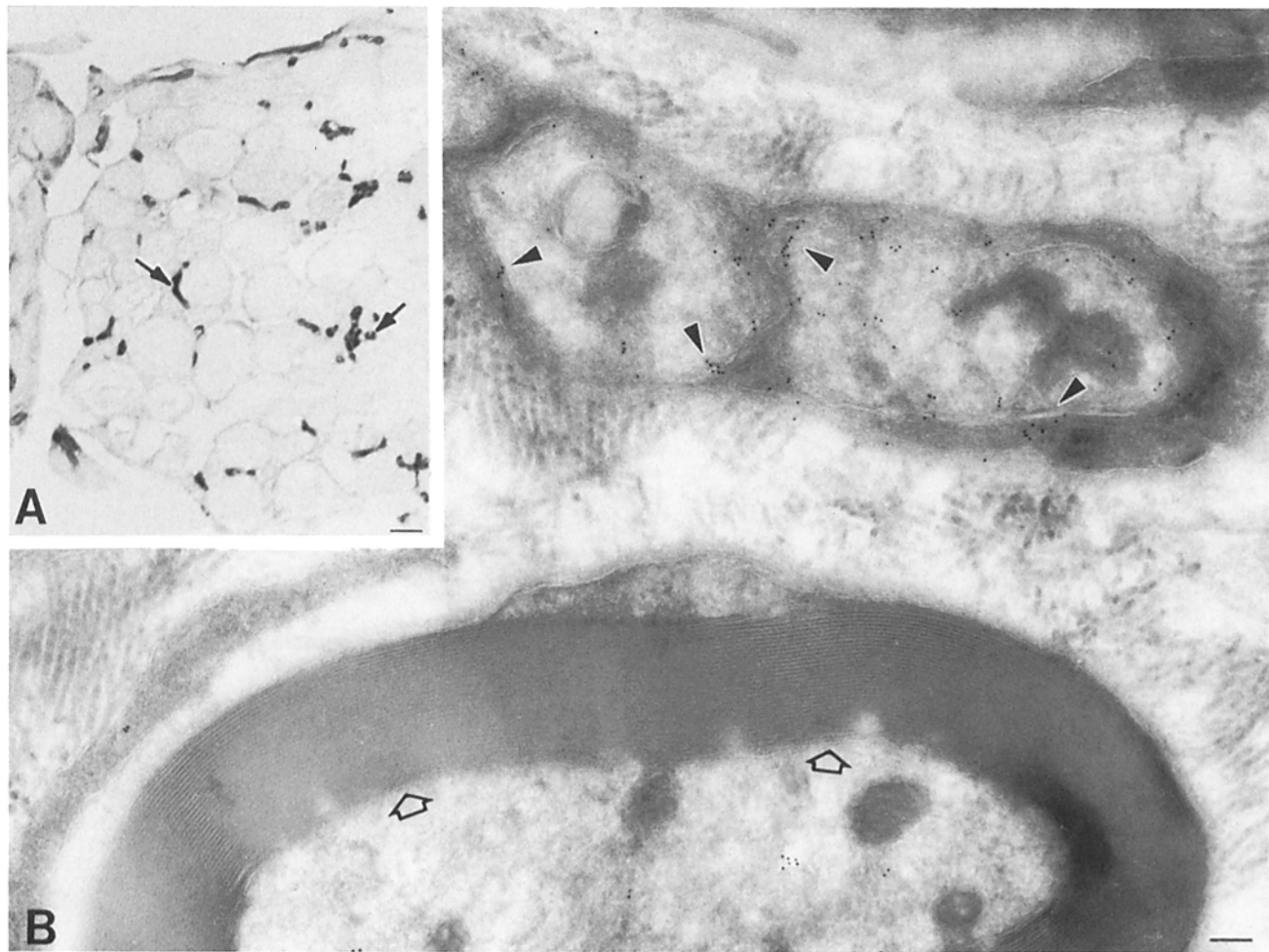


Figure 8. Localization of brain erythroid ankyrin (ankyrin_R) by light and electron microscopy using the erythrocyte ankyrin antibody on cryosections of unmyelinated nerve fibers. 1- μ m-thick cross sections of rat dorsal root ganglion were labeled by immunoperoxidase with the human erythrocyte ankyrin antibody (a). Arrows point on selectively labeled, relatively small rings and short lines. In ultrathin cryosections of the same tissue (b) labeled by the immunogold technique, gold particles accumulate on the axonal plasma membrane of the unmyelinated nerve fibers (arrowheads). No labeling is observed on the internodal plasma membrane of the myelinated axons (open arrows). Bars, 5 μ m (light microscopy) and 0.2 μ m (electron microscopy).

Ankyrin_R and the sodium channel are not exclusively associated with each other in brain. Ankyrin_R, as well as another erythroid ankyrin isoform previously described, is enriched at sites where sodium channels are absent or at least not concentrated such as nerve cell bodies and along dendrites (16, 30, this paper). Moreover, other integral membrane proteins such as the Na⁺/K⁺ATPase and the L₁ glycoprotein (27) are localized at the node of Ranvier and around the neuronal cell bodies, and are candidates for interaction with ankyrin_R. Ankyrin_R may be functionally heterogeneous due to posttranslational modifications, alternative splicing of mRNA or encoding by distinct genes. Differences in the level and occasional absence of staining of spinal neuron cell plasma membranes with the ankyrin_R-specific antibody have been consistently observed in this study, suggesting the existence of multiple ankyrin_R isoforms expressed by morphologically and structurally distinct neuron cells. In this case, each subpopulation of ankyrin_R molecules could selectively interact with distinct integral membrane proteins.

The multiple isoforms of the sodium channel (21, 32) and

the Na⁺/K⁺ATPase (reviewed in reference 40) may interact differently with different ankyrin isoforms. Diversity in associations with ankyrin could play a role in placement of different isoforms of these ion channels at specialized membrane domains. For example, the preferential localization of the ankyrin_B isoform detected with the present antibody in the glial membranes of central myelinated axons, versus a mainly neuronal distribution of ankyrin_R might be related to the existence of glial and neuronal forms of the sodium channel (3) and the Na⁺/K⁺ATPase (40). Also, a neuronal isoform of the Na⁺/K⁺ATPase is present in high concentrations in the internodal axolemma of the myelinated axons (18), and presumably does not interact with ankyrin_R and ankyrin_B.

An interesting observation in this study was that the ankyrin_R isoform is also enriched in the nodal cytoplasm and was present at lower levels in the internodal cytoplasm. Because axonal proteins are synthesized in the neuron cell bodies, ankyrin_R is presumably axonally transported to the nodes. Ankyrin_R may reach the nodes by slow axonal trans-

port along with other soluble cytoplasmic proteins and may associate with the sodium channel (and other proteins) after they are inserted in the nodal membrane, providing stabilization. Cytoplasmic labeling then could represent a cytoplasmic pool of free ankyrin in the nodal cytoplasm. Another possibility is that ankyrin_R associates with the cytoplasmic surface of sodium channels immediately after biosynthesis. The two proteins would then be axonally transported in vesicles by a fast, kinesin-driven mechanism along microtubules to the nodes of Ranvier. In this case, cytoplasmic labeling would represent accumulation of protein-carrying vesicles at nodes and ankyrin would have the potential to target the sodium channel to the specialized nodal axolemma. It is pertinent in this regard that sodium channels have been observed to undergo fast axonal transport (25) and ankyrin can interact in vitro with tubulin (8, 13). It will be important for the understanding of the role of ankyrin_R at the nodes to determine the rate of axonal transport of this isoform.

The concentration of the ankyrin_R isoform at the node of Ranvier raises the question of which isoform of spectrin is present at these sites: the generally expressed form of spectrin (fodrin), or erythroid spectrin. Erythrocyte ankyrin binds preferentially in in vitro assays to erythrocyte spectrin rather than fodrin (9, 20). Based on the immunological relationship of ankyrin_R with erythrocyte ankyrin it might be anticipated that ankyrin_R also would bind with higher affinity to erythroid spectrin, and that these proteins would be segregated with each other. However, axons are believed to contain fodrin rather than erythroid spectrin (24, 34). Fodrin has been observed to be concentrated at the nodes of Ranvier of isolated desheathed peripheral axons (22) and in paranodal loops of myelinated fibers in rat peripheral nerve (42). It is conceivable that the ankyrin_R isoform is a chimeric ankyrin with certain features in common with erythrocyte ankyrin, but a binding preference for fodrin at the nodes of Ranvier.

In summary, local segregation of an apparently minor isoform of ankyrin at the nodes of Ranvier provides indirect evidence suggesting a role for ankyrin in the formation and/or the maintenance of this axonal structure which displays a high degree of structural and functional differentiation. An experimental challenge in the future will be to resolve the activities of ankyrin at the node as well as at other specialized membrane domains.

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