The Subcellular Distribution of Chromosome 6–encoded Dystrophin-related Protein in the Brain

Tejvir S. Khurana, Simon C. Watkins,* and Louis M. Kunkel

Program of Neuroscience and Department of Pediatrics, Harvard Medical School and The Howard Hughes Medical Institute, Division of Genetics, Children's Hospital, Boston, Massachusetts 02115; and *Department of Neurobiology, Anatomy and Cell Science, University of Pittsburgh, Pennsylvania 15261

Abstract. Chromosome 6–encoded dystrophin-related-protein (DRP) shows significant structural similarities to dystrophin at the carboxyl terminus, though the two proteins are encoded on different chromosomes. Both DRP and dystrophin are expressed in muscle and brain and show some similarity in their subcellular localization. For example, in skeletal muscle both are expressed at neuromuscular and myotendinous junctions. However, while dystrophin is absent or severely reduced in Duchenne/Becker muscular dystrophy, DRP continues to be expressed. Within the brain, dystrophin is enriched at the postsynaptic regions of specific subsets of neurons, while the distribution of DRP is yet to be described. In this study we demonstrate a distinct though highly specific pattern of distribution of DRP in the brain. DRP is enriched in the choroid plexus, pia mater, intracerebral vasculature, and ependymal lining. Within the parenchyma proper, DRP is located at the inner plasma face of astrocytic foot processes at the abluminal aspect of the blood-brain barrier. The distribution of DRP is conserved across a large evolutionary distance, from mammals to elasmobranchs, suggesting that DRP may play a role in the maintenance of regional specializations in the brain.

DYSTROPHIN is a large cytoskeletal protein belonging to the superfamily of spectrin-like proteins. It is closely associated with the inner face of the plasmalemma in muscle and postsynaptic region of neurons in the brain (Hoffman et al., 1987; Koenig et al., 1988; Arahata et al., 1988; Bonilla et al., 1988; Watkins et al., 1988; Zubrzycka-Gaarn et al., 1988; Miike et al., 1989; Lidov et al., 1990). The role of dystrophin in muscle or neurons remains unclear, however mutations in the gene encoding dystrophin result in Duchenne/Becker Muscular Dystrophy (D/BMD), a disease characterized by progressive muscle wasting, cognitive impairment, and death by the third decade of life in humans (Duchenne, 1868; Engel, 1986; Hoffman and Kunkel, 1989).

The spectrin superfamily includes a class of proteins sharing limited structural similarity and organizational motifs including an actin-binding amino terminus and a 'rod' like central domain, predicted to be composed of triple α-helical repeat motifs (Davison and Critchley, 1988; Koenig et al., 1988; Byers et al., 1989). While the carboxyl terminus of dystrophin bears no homology to either the spectrins or α-actinins, it shares considerable homology with chromosome 6 encoded transcript of the DMDL gene (Love et al., 1989). The protein product of this autosomal transcript, dystrophin-related protein (DRP)[1] was identified by expressing the cloned transcript as a recombinant bacterial protein and generating antibodies against the purified fusion protein (Khurana et al., 1990). DRP and dystrophin are similar in size and present at relatively low abundance in normal skeletal muscle (Hoffman et al., 1987; Khurana et al., 1990). However, DRP is more widely distributed than dystrophin, being detected in brain, blood vessels, nerves, kidney, spleen, liver, testis, stomach, and muscle. Additionally, the expression of DRP is unaltered in age matched D/BMD skeletal muscle, in which dystrophin is either not detectable or severely reduced (Hoffman et al., 1988; Khurana et al., 1990).

The subcellular distribution of DRP in skeletal muscle has recently been elucidated using immunohistochemistry (Khurana et al., 1991; Man et al., 1991; Ohlendieck et al., 1991; Takemitsu et al., 1991; Cartaud et al., 1992). DRP is detected in intramuscular nerves, blood vessels, and found to be enriched at the neuromuscular and myotendinous junctional areas of the adult dystrophic myofiber (Khurana et al., 1991). Using antibodies of varying sensitivity and specificity, other investigators have detected DRP at some but not all these sites (Man et al., 1991; Ohlendieck et al., 1991; Takemitsu et al., 1991). Minimal sarcolemmal labeling of dystrophic muscle has also been noted when the sensitivity of DRP immunolabeling is optimized (Khurana et al., 1991; Man et al., 1991; Tanaka et al., 1991; Voit et al., 1991). However, during muscle regeneration intense and extended sarcolemmal immunolabeling is seen to completely encircle

1. *Abbreviation used in this paper*: DRP, dystrophin-related protein.

the myofibers. Additionally, greater amounts of DRP are found during embryonic and perinatal development than in adult dystrophic muscle. Developmental studies suggest that DRP may play a role similar to that of dystrophin during certain developmental stages in the *mdx* mouse muscle (Khurana et al., 1991; Takemitsu et al., 1991).

Whereas recent studies surveying the distribution of dystrophin and DRP have found some similarity in their distribution in myofibres (Byers et al., 1991; Khurana et al., 1991), the distribution of DRP in the brain is as yet unknown. However, anomalous immunolabeling of the cerebral vasculature and pial surfaces of the *mdx* brain using some dystrophin antisera has been reported and provides hints about the localization of DRP in the brain (Ishiura et al., 1990; Yoshioka et al., 1992), in much the same way that such cross reactivity did in skeletal muscle (Fardeau et al., 1990; Samitt and Bonilla, 1990). To address basic questions of the physiological role of DRP it is important to determine the subcellular distribution of DRP in the brain. We have therefore used a combined approach of immunoblotting, immunohistochemistry, immunoelectron microscopy, and tissue culture to define the distribution of DRP in the brain of *mdx* mice.

Materials and Methods

Antibody Specificity

cDNA clones encoding the carboxyl termini of dystrophin (clone dl1 encompassing nucleotide position 9,786–11,555 of the dystrophin sequence, Koenig et al., 1988) and DRP (nucleotide position 690–1,353, Love et al., 1989: homologous to 10,457-11,518 of the dystrophin sequence, Koenig et al., 1988) were expressed as bacterial fusion peptides and purified as previously described (Koenig and Kunkel, 1990; Khurana et al., 1990). To determine the specificity of DRP antibodies on immunoblotting, equivalent amounts of purified fusion proteins were electrophoresed alongside each other and electro-transferred to nitrocellulose. The immunoblots were processed using affinity purified antibodies to the carboxyl terminus of DRP according to previously described methods (Hoffman et al., 1987; Khurana et al., 1990). (Note: for sake of brevity in this manuscript, we will refer to the dystrophin and DRP fusion peptides described above, as dl1 fusion or DYS-pep and DRP fusion or DRP-pep, respectively).

Peptide Competition

An excess (~1 mg) of either DRP peptide (DRP pep- encoding the carboxyl terminus of DRP) or dystrophin peptide (DYS pep- encoding the carboxyl terminus of dystrophin) was resuspended in 20 μl of water. This was added to 100 μl of affinity purified DRP antibodies at a concentration of 1 μg/ml (final antibody concentration ~800 ng/μl) and the mixture used for immunohistochemistry on cryostat sections of *mdx* brain. For comparison, 10-μm-thick serial sections were incubated in parallel with (*a*) preimmune sera from the same rabbit and (*b*) affinity purified DRP antibodies without the DRP peptide at ~1 μg/ml. Photographs were taken on Kodak Tri-X Pan film (Eastman Kodak Co., Rochester, NY) using the same camera settings and processed in parallel.

Immunoblotting

The methods used for immunoblotting have been previously described (Hoffman et al., 1987). Briefly, 20-μm cryostat sections, aliquots of tissue, or pellets of mechanically harvested tissue culture cells were placed in preweighed 0.5- or 1.5-ml tubes and chilled on dry ice. ~20 mg of tissue were solubilized in 20 volumes of sample buffer containing 10% SDS, 0.1 M Tris, pH 8.0, 10 mM EDTA, bromophenol blue, and 50 mM DTT. Tissue lysates were made using a metallic or teflon-coated tissue homogenizer to thoroughly crush the sample inside the tube. The sample was boiled for 2 min and cooled to room temperature. Protein concentration was measured using a colorimetric assay by staining an aliquot of the sample with Amido black (Nakamura et al., 1985). Appropriate dilutions were loaded in each well to achieve uniform loading. These aliquots were fractionated on a 3.5–12.5% gradient SDS–polyacrylamide gel. Electrophoresis of the 1.5-mm-thick gel was for 3 h at 100 V or in some cases 60 V for 12 h (constant voltage).

After electrophoresis, proteins were electro-transferred onto nitrocellulose filters, and the filters dried overnight. To control for uniformity of loading, the posttransfer gels were stained with Coomassie blue dye for residual myosin. In addition the filters were stained with Ponceau S solution (Sigma Chemical Co., St. Louis, MO), which together with the transfer of prestained protein molecular weight standards served to control the efficiency of transfer. DRP and dystrophin antibody complexes were detected using alkaline phosphatase staining as previously described (Hoffman et al., 1987; Khurana et al., 1990). Multiple filters were tested for each experiment. For quantification, the DRP band was subjected to computerized laser densitometric analysis by scanning on an LKB Ultroscan XL (LKB Instruments Inc., Bromma, Sweden) machine and results plotted as previously described (Khurana et al., 1991).

Immunohistochemistry

Immunohistochemistry was performed using previously described methods (Watkins et al., 1989). *mdx* mice were anaesthetized and sacrificed by cervical dislocation. *Raja erinacea* fish were obtained from the Marine Biological Laboratories (Woods Hole, MA). Fish were anaesthetized with chloral hydrate and sacrificed by cervical transsection. Brains were dissected out and various orientations of the brain placed on a marked piece of cardboard and plunged into isopentane cooled in liquid nitrogen and stored at −70°C. After mounting on cold metal chucks with Tissue-Tek O.C.T. Compound (Miles Laboratories Inc., Elkhart, IN), 5–10-μm sections were cut and lifted onto gelatin or polylysine-coated slides. Sections were fixed with cold methanol for 5 min and washed with PBS before being labeled with affinity purified DRP antibody in PBS with 10% calf serum (final concentration ~1 μg/ml) for 1 h at room temperature. After washing in PBS, sections were incubated with FITC or TRITC anti-sheep or anti-rabbit antibody (Sigma Chemical Co.) for 1 h. Washed sections were mounted in Gelvatol (Monsanto Chemical Co., St. Louis, MO) or Aqua-mount (Lerner Laboratories, Pittsburgh, PA). In some cases immunolabeling of serial sections was done with antibodies against the vascular marker, von Willebrands Factor (Sigma Chemical Co.) as described above. Sections were examined using either a Nikon FXA (Nikon Inc., Melville, NY) or Zeiss Axiophot microscope (Carl Zeiss, Inc., Thornwood, NY) equipped with epi-fluorescence optics.

Immunoelectron Microscopy

mdx mice brains were fixed by cardiac perfusion with 2% paraformaldehyde, 0.01% glutaraldehyde in PBS. Brains were removed from the cranial cavity and the cerebrum and cerebellum were dissected out and further fixed in the same buffer for a total of 1 h. Samples were cut into 1-mm cubes and immersed in 2.3 M Sucrose in PBS overnight. Blocks were mounted on cutting slabs, shock frozen in liquid nitrogen, and stored under liquid nitrogen. 70-nm sections were cut using a Reichert Ultracut microtome fitted with a FC4D cryo-attachment (Reichert Scientific Instruments, Buffalo, NY). Sections were mounted on 200 mesh carbon-formvar grids, labeled with antibodies to DRP and revealed with a 5-nm gold conjugate directed against the rabbit primary antibodies as previously described (Byers et al., 1991). EM was performed with a Jeol 100-CX II (JEOL USA Inc., Peabody, MA). Micrographs were taken at a magnification of 29,000.

Tissue Culture

Anaesthetized *mdx* mice were sacrificed by cervical dissociation. For neuronal cultures embryonic day 18 mice were used and for glial cultures 1-d-old mice were used. Cells were cultured by standard protocols, as described by Freshney (1987). Briefly, brains were removed from the cranium and the meninges dissected out under a Zeiss stereo microscope (Carl Zeiss, Inc.). The cerebral hemispheres were minced, trypsinized in 10 volumes of PBS and dissociated by forced passage through flame-polished glass pipettes. The supernatant pools were decanted, and the separated tissue clumps discarded. The supernatant was spun at low speed and pellet resuspended in culture medium. Viable cells in the cell suspension were counted using trypan blue exclusion. ~10^5 cells were plated on polylysine-coated cover slips. Neuronal cultures were enriched by treatment with the pyrimidine analogue cytosine arabinoside for 4–8 d before use. Antibodies against glial fibrillary acidic protein (GFAP) (Sigma Chemical Co.) were used as control for glial contaminants. Glial cultures were stained to evaluate the purity of cultures which were found to be >90% positive.

Results

Specificity and Sensitivity of Affinity-Purified DRP Antibodies

The domain of DRP used for expression of fusion proteins and antibody production is highly conserved between dystrophin and DRP. We have previously demonstrated that DRP antibodies are specific for DRP since they recognize DRP but not the internally truncated, smaller dystrophin in biopsies of Becker muscular dystrophy patients (Khurana et al., 1990; Khurana et al., 1991). To strengthen these original controls we performed experiments to determine whether DRP antibodies could recognize extremely small amounts of DRP fusion peptides and distinguish DRP peptides from the structurally similar dystrophin fusion peptides. DRP antibodies were used to probe an immunoblot containing increasing amounts of fusion protein representing the carboxyl termini of DRP and dystrophin. As demonstrated in Fig. 1, DRP antibody detected DRP fusion proteins at extremely low concentrations while not recognizing equivalent or greater amounts of fusion protein containing the corresponding carboxyl terminus of dystrophin. This observation coupled with our previous patient studies demonstrates that, on immunoblots, the affinity-purified DRP antibodies used in this study were sensitive and specific for DRP.

Having established that the DRP antibodies were specific for DRP on Western blots we wished to extend these findings and characterize the specificity of the DRP antibodies by immunofluorescence microscopy. This was especially important in light of the possible existence of an alternative, shorter transcript of the dystrophin locus, that is predicted to encode the carboxyl terminus of dystrophin alone (Bar et al., 1990; Rapaport et al., 1992). This short transcript and encoded protein are predicted to be present in *mdx* mouse brain though full length dystrophin is absent in the *mdx* mouse (Hoffman et al., 1987). To demonstrate specificity by immunofluorescent microscopy, we competed the binding of DRP antibodies by either DRP or dystrophin carboxyl-terminal fusion peptides. Serial cryostat sections of *mdx* mouse brain were incubated with DRP antibody either with an excess of DRP carboxyl-terminal fusion protein or with excess of a dystrophin carboxy-terminal fusion protein. As shown in Fig. 2, DRP immunolabeling was successfully competed by the presence of DRP fusion protein but not by dystrophin fusion protein. This suggests that the affinity-purified DRP antibodies used were specific for DRP and capable of recognizing DRP-peptide despite the presence of the peptides encoding the carboxyl terminus of dystrophin, by immunofluorescence.

Enrichment of DRP in Blood Vessels and the Choroid Plexus

Having ensured that the DRP antibodies were specific and sensitive enough to distinguish DRP from dystrophin, we used these affinity-purified antibodies to quantitate regional differences in the amount of DRP in the (full length) dystrophin null mutant *mdx* mouse brain. Various regions of adult *mdx* mouse brain were dissected out, protein content quantitated, and equivalent amounts fractionated by SDS-PAGE, electro-transferred and immunoblotted with DRP antibodies. As shown in Fig. 3, DRP was detected in blood vessels, choroid plexus, cerebral cortex, cerebellum, internal capsule, spinal cord, and leptomeninges. The amount of DRP in vascular tissue and the highly vascularized choroid plexus of the brain was observed to be significantly greater than elsewhere. To quantitate the relative amount of DRP in various regions of the brain, the immunoblot was subjected to densitometric scanning and the level of DRP was found to be approximately threefold greater in cerebral blood vessels and choroid plexus compared with other regions of the brain. Similar results were obtained on three independent trials using the brains from different mice (data not shown).

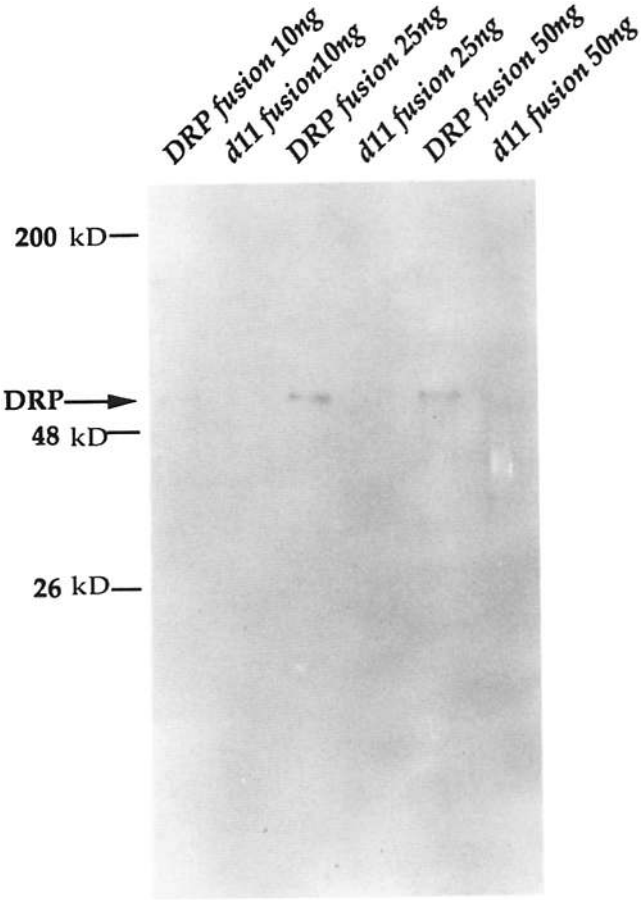


Figure 1. Specificity of affinity purified DRP antibodies by immunoblotting. Equivalent amounts of purified fusion peptides containing the carboxyl termini of DRP (DRP fusion: nucleotide position 690–1,353, Love et al., 1989; homologous to 10,457–11,518 of the dystrophin sequence, Koenig et al., 1988) and dystrophin (d11 fusion: nucleotide position 9,786–11,555 of the dystrophin sequence, Koenig et al., 1988) were fractionated by SDS-PAGE and electrotransferred to nitrocellulose. Immunoblots were processed using affinity purified antibodies to the carboxyl termini of DRP. As shown above, DRP antibodies recognized minute amounts of DRP fusion peptides (~60 kD) but not fusion peptides expressed by the d11 clone encoding the carboxyl terminus of dystrophin (predicted weight ~100 kD).

Immunolocalization of DRP in the mdx Mouse Brain

Having determined by Western blotting areas of the brain that were enriched in DRP, we extended these findings using immunofluorescent microscopy. A survey of cross sections of brain tissue revealed intense DRP immunolabeling at the pia mater, choroid plexus, and intracerebral vasculature,

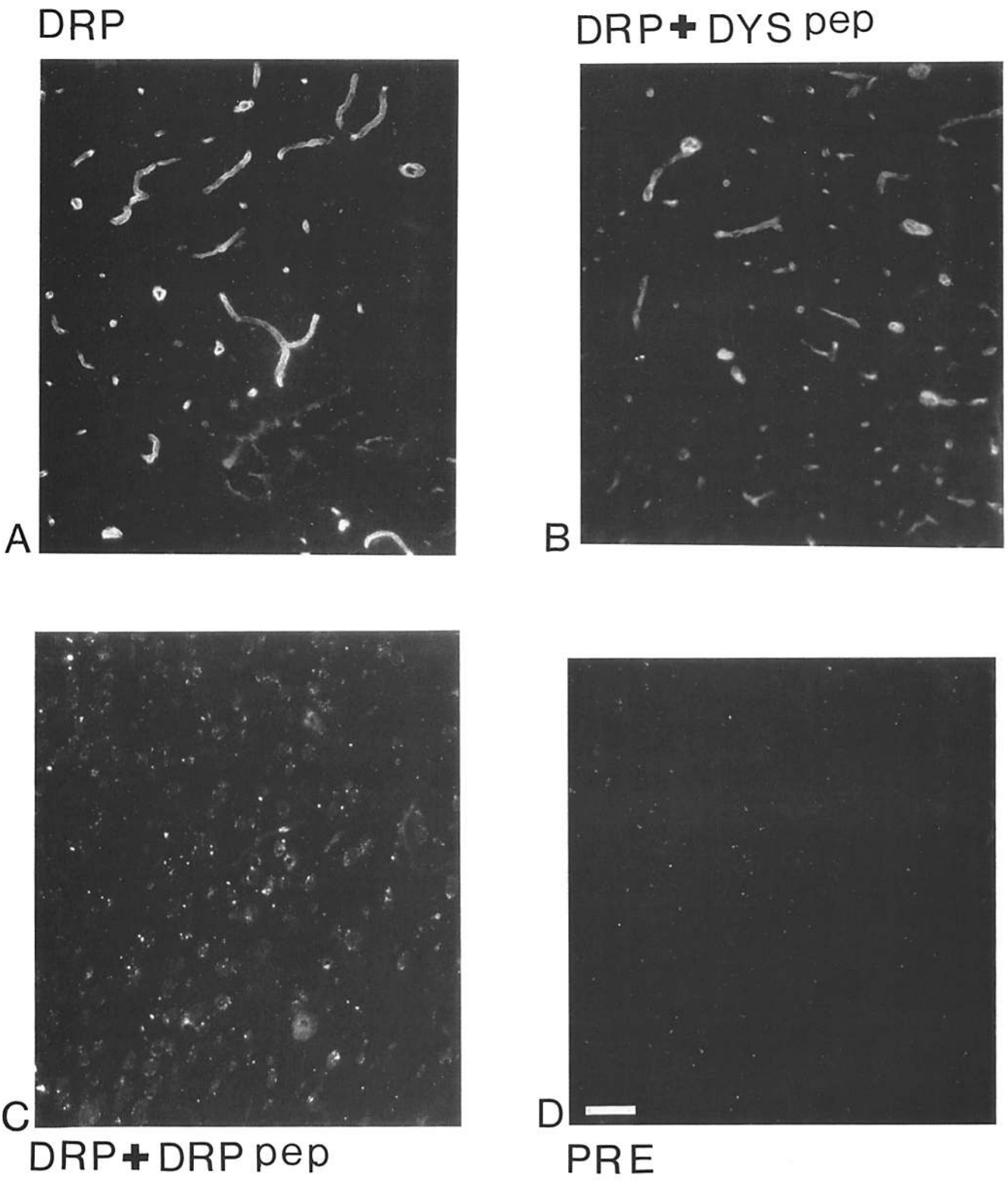


Figure 2. Specificity of affinity purified DRP antibodies by immunofluorescence. 10-μm-thick serial sections of *mdx* mouse brains were incubated for 1 h with (*A*) DRP antibodies alone, (*B*) DRP antibodies + 1 mg dystrophin fusion peptide (DYS-pep: d11 fusion: nucleotide position 9,786–11,555 of the dystrophin sequence, Koenig et al., 1988), (*C*) DRP antibodies + DRP fusion protein (DRP-pep: nucleotide position 690–1,353, Love et al., 1989; homologous to 10,457–11,518 of the dystrophin sequence, Koenig et al., 1988) and (*D*) Preimmune sera (*PRE*). Slides were processed as described in Materials and Methods and photographed. The immunolabeling pattern was unchanged despite the presence of DYS-pep. The immunolabeling was competed with DRP-pep itself as evidenced by precipitated complexes, consistent with previous results obtained while affinity purifying the DRP antibodies against the DRP-pep (Khurana et al., 1990). Bar, 50 μm.

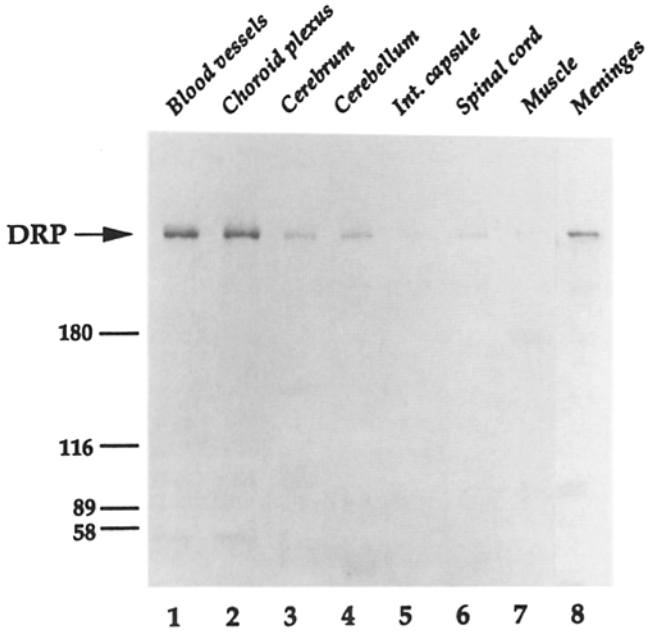


Figure 3. Immunoblot analysis of DRP content in various regions of the *mdx* mouse brain. Various regions of the brain were dissected, solubilized, and proteins quantified as described in Materials and Methods. Equivalent amounts were fractionated by electrophoresis and immunoblotted with DRP antibodies. Different regions analyzed were (*1*) cerebral blood vessels, (*2*) choroid plexus, (*3*) cerebral cortex (*4*) cerebellum, (*5*) internal capsule, (*6*) spinal cord (cervical level), (*7*) skeletal muscle (control), and (*8*) leptomeninges (separate blot, same quantity of total protein). As demonstrated the DRP content (marked DRP; 427 kD) of cerebral blood vessels and choroid plexus is greater than other regions of the brain. The experiment was repeated three times and one representative blot subjected to densitometric analysis. DRP content of vessels and choroid was ~3 times greater than other regions.

however, only minimal DRP immunolabeling was detected in neuronal or glial elements of brain parenchyma (Fig. 4 *A*). The pia mater was immunolabeled, both at the surface of the brain and within infolding of the sulci. Intracerebral vascular structures such as veins, arteries, smaller arterioles and capillaries were immunolabeled uniformly with the antibody (Fig. 4 *B*). The choroid plexus was found to label intensely, as were the specialized vessels forming the plexus, vessels associated with the ependyma and the ependyma itself (Fig. 4 *C*). The immunolabeling associated with the pial surfaces on the exterior surface of the brain was seen to follow invaginations of vasculature into the brain parenchyma along the anatomical space of Virchow-Robin (Fig. 4 *D*), however, it was not possible to distinguish, at the level of light microscopy if the leptomeningial immunolabeling was at the outer pial layer or associated with the inner, closely associated glia limitans. The identity of immunolabeled structures was verified by analyzing their characteristic morphology with phase–contrast microscopy. In some cases serial sections were also stained with haematoxylin and eosin (H&E), crystal violet or labeled with antibodies against the endothelial marker, von Willebrand's factor to further validate the nature of identified structures. Additionally, similar immunolabeling results were obtained using normal B10 mice (data not shown). Preimmune sera from the same rabbit was used as a negative control to exclude nonspecific cross-reactivity in all experiments for both immunolabeling (Fig. 4 *E*), and immunoblots of dissected *mdx* cerebral blood vessels (data not shown).

DRP Immunolabeling Across Species

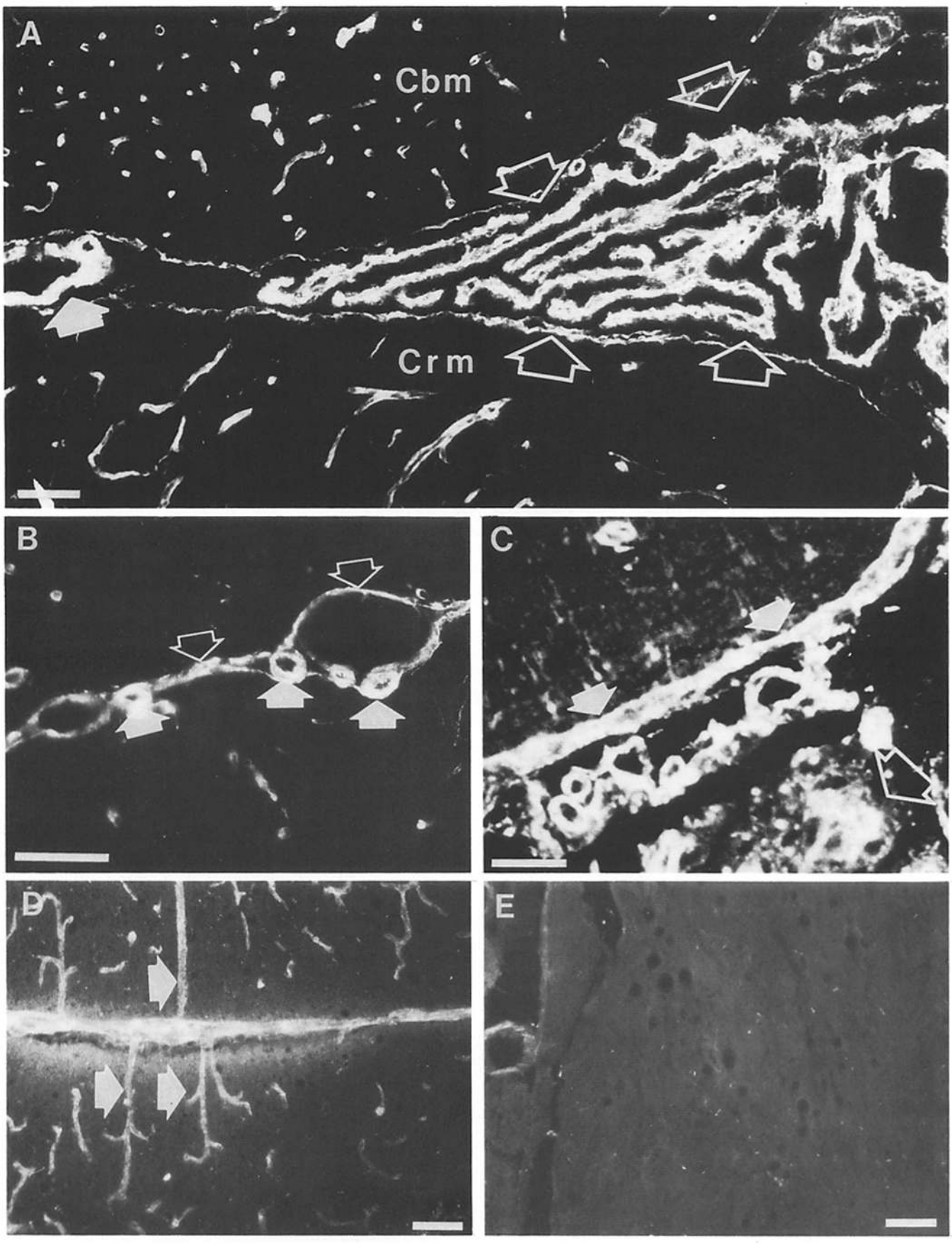
After definition of DRP distribution in the mammalian brain by immunofluorescence we addressed the question of whether the distribution of DRP in the brain was conserved across species, since such conservation is often indicative of significant physiological function. Previously it has been demonstrated that the gene encoding DRP, the protein and the subcellular distribution of DRP in the muscle is conserved across species (Love et al., 1989; Khurana et al., 1990; Cartaud et al., 1992). We therefore analyzed by immunofluorescent microscopy, the distribution of DRP in cryosections of brain from *Raja erinacea*, an elasmobranch fish that is evolutionarily quite divergent from mammals. Significant, uniform immunolabeling was visualized at intracerebral vasculature with DRP antibodies (Fig. 5 *A*). Since the *Raja erinacea* intracerebral vessels possess a fenestrated endothelial lining rather than a relatively impermeable one, an ideal comparison would entail using the few rare regions of the mammalian brain parenchyma proper that have fenestrated endothelium as well. We therefore analyzed the area postrema from the *mdx* mouse brain (an area with fenestrated endothelium) for this comparison, and found similar patterns of immunolabeling with DRP antibodies as well (Fig. 5 *B*). The overall pattern of DRP immunolabeling in both regions was similar to that seen in other regions of the *mdx* mouse brain (Fig. 4).

DRP Is Expressed in Tissue Culture

While DRP immunolabeling was consistently associated with intracerebral capillaries, owing to the limited resolution by immunofluorescence microscopy, it is possible that DRP is distributed with components of the brain that lie in close proximity to the capillaries rather than the capillaries themselves. To address the possibility of immunolabeling either perivascular glia or neurons, these two major components of the brain were cultured in relative isolation from each other and analyzed for DRP content. Thus, we generated primary cultures of neurons and glia from *mdx* mouse brains, quantified proteins and analyzed equal amounts by immunoblotting with DRP antibodies. As shown in Fig. 6, both neurons and glial cultures expressed DRP however, glial cultures expressed significantly greater amounts of DRP than neuronal cultures. The lower expression of DRP in neurons is consistent with the minimal immunolabeling of identified neurons observed by immunofluorescence. The high levels of DRP expression in glial cultures may reflect the increased expression of DRP in the pool of perivascular glia, consistent with the possibility that perivascular regions of the brain were immunolabeled by DRP antibodies (Fig. 4).

DRP Is Selectively Expressed at the Foot Processes of Perivascular Astrocytes

Both immunofluorescent microscopy and tissue culture experiments using DRP antibodies coupled with the anatomical structure of the intracerebral vasculature suggest the possibility that perivascular glia are enriched in DRP. To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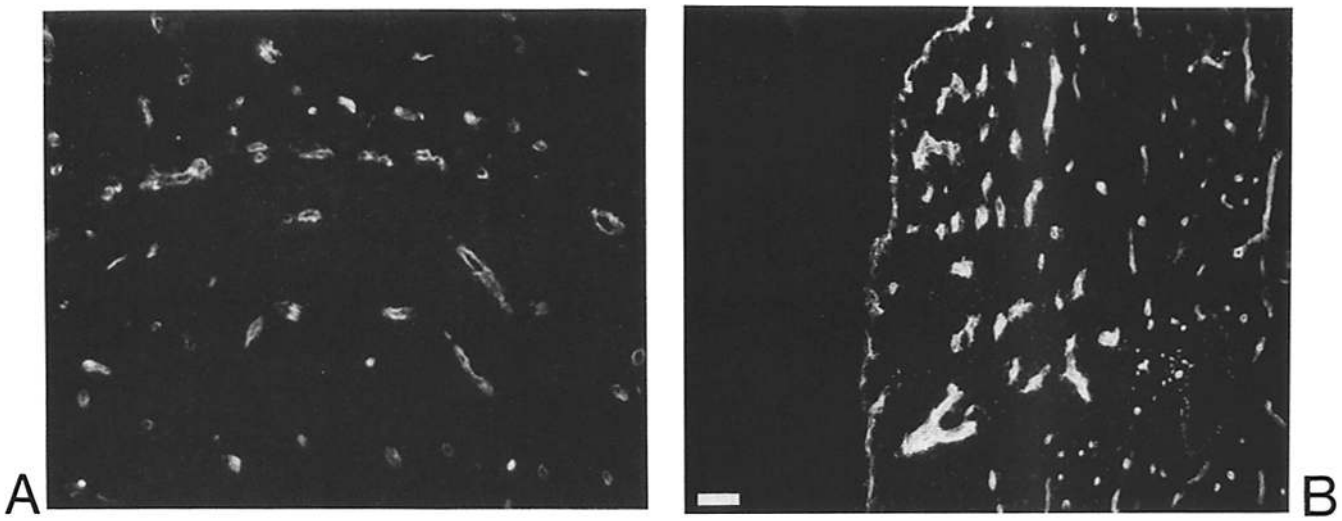

Figure 5. Immunolocalization of DRP in Elasmobranch fish brain and the area postrema of *mdx* mouse brain. 10-μm cryosections of brain tissue from *Raja erinacea* (*A*) and *mdx* mouse brain (*B*) were labeled with DRP antibodies. (*A*) Vascular labeling was similar to that seen in murine brain (compare with Fig. 4). (*B*) Transverse section at the level of the area postrema shows immunolabeled blood vessels, the overall pattern of both panels is similar to that seen in the rest of the *mdx* mouse brain. Bar, 50 μm.

address this issue directly and define the subcellular localization of DRP we used the higher resolution offered by immunogold EM to examine the distribution of DRP in *mdx* brain sections. DRP was seen to selectively label the end-feet or foot processes of astrocytes closely apposed to intracerebral blood vessels (Fig. 7). Astrocytes were identified by their characteristic morphology and presence of glial filaments within the foot process (Fig. 7). The immunolabeling was seen to be maximal at the inner plasma face of astrocytes, regions forming the glio–vascular interface. Since the perivascular glia completely surround capillaries, this location of DRP is consistent with the uniform perivascular immunolabeling seen by fluorescent microscopy using DRP antibodies (Fig. 4). No immunolabeling was noted at the luminal aspect of basal lamina surrounding the endothelial cells. Sections labeled with either no primary or control antibodies were negative (data not shown).

Discussion

Using complementary immunoassays, cell biological, and microscopic techniques, we have determined the distribution of the chromosome 6 encoded DRP in the *mdx* mouse brain. DRP is expressed in neuronal, glial, and vascular cells of the brain and is enriched in neuroglial cells. DRP is localized at the end-feet of astrocytes closely apposed to capillaries, pia mater, intracerebral vasculature, the ependyma, and choroid plexus. Since proteins structurally similar to DRP are known to exist (e.g., dystrophin), it is of crucial importance to exclude their inadvertent visualization in studies such as this. To avoid anomalous cross-reaction with dystrophin we used the *mdx* mouse. This mouse strain harbors a point mutation in the dystrophin gene that precludes the translation of dystrophin, hence analysis of brain tissue from this mouse excludes the possibility of inadvertent visualization of full length dystrophin (Sicinski et al. 1989). Importantly, while no full length dystrophin is made in the *mdx* mouse, an alternative, smaller transcript encoding dystrophin's carboxyl terminus alone has recently been suggested to exist and to be expressed in nonmuscular tissues of this mouse (Bar et al., 1990; Rapaport et al., 1992). However, the antibodies used here only recognize the full length DRP and do not detect the 71-kD, smaller, alternative DMD gene product in the brain of *mdx* mice (Lederfein et al., 1992) (Fig. 3). Furthermore, peptide competition experiments clearly demonstrate that DRP antibodies can recognize DRP even in the presence of an excess of dystrophin carboxyl-terminal epitopes, both on immunoblotting and immunohistochemistry (Figs. 1 and 2). The use of specific and sensitive DRP antibodies, correlation of immunohistochemical and immunoblot results and tissue culture experiments strongly suggest that we are describing the distribution of DRP itself rather than dystrophin or its known isoforms at the aforementioned sites, although this possibility cannot be formally ruled out in the absence of a null mutant for DRP.

In mammals the term "blood-brain barrier" refers to the endothelial cell–based permeability barrier that prevents the

Figure 4. Immunolocalization of DRP in the *mdx* mouse brain. 7–10-μm cryosections of *mdx* mouse brain were labeled with DRP antibodies and visualized by indirect immunofluorescence. (*A*) DRP antibodies label the choroid plexus and pia mater (*open arrows*). A muscular artery is also labeled (*closed arrow*). All calibers of cerebral vessels were labeled and visualized in different orientations (i.e., lengthwise, tangentially, and end-on) within the substance of both the cerebral (*Crm*) and cerebellar (*Cbm*) hemispheres. (*B*) Immunolabeling of arterioles (*closed arrows*) and invaginations of the pia mater around the vessels (*open arrows*). (*C*) Intense immunolabeling is seen in the choroid plexus (*open arrows*) and ependyma lining the ventricular cavity (*closed arrows*). (*D*) The immunolabeling associated with blood vessels is seen to be colocalized with investments of the pia mater from the surface of the cerebellum. *E* demonstrates a lack of immunolabeling with preimmune antisera from the same rabbit. (*C*) 100 μm; (*A*, *B*, and *D*) 50 μm.

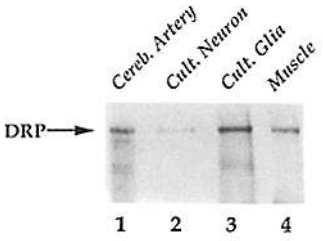

Figure 6. DRP expression in tissue culture. Primary cultures of neurons and glia were made from *mdx* mouse brains. The cells were harvested, proteins quantified, and equivalent amounts electrophoresed and immunoblotted with DRP antibodies. DRP was detected in (*1*) *mdx* cerebral vessels (control), (*2*) *mdx* cultured neurons, (*3*) *mdx* cultured glia, and (*4*) *mdx* skeletal muscle (control). Note: background staining for various lanes with these DRP antibodies was dependent on the complexity of tissue type rather than loading differences (compare lanes *1* and *4*; see Khurana et al., 1990). As shown, DRP expression was greater in cultured glia as compared with neurons.

entry of many blood-borne molecules into the brain parenchyma, thus helping maintain the milieu interieur necessary for neuronal function. Additionally, vascular pericytes and perivascular neuroglia, while not forming the actual barrier, have been ascribed a supportive role in the maintenance of blood-brain barrier integrity (Peters et al., 1991; Risau and Wolburg, 1990; Cserr and Bundgaard, 1984). Indeed, cocultured astrocytes are considered crucial for the induction of polarity and barrier properties among endothelial cells in murine models (Beck et al., 1984; Janzer and Raff, 1987; Tao-Cheng et al., 1987). Interestingly, in submammalian vertebrates (Elasmobranchs, e.g., Torpedo fish, skates and sharks) and invertebrates the endothelial cell layer is "leaky" and the barrier function is carried out exclusively by the end-feet of perivascular astrocytes (Bundgaard and Cserr, 1981). Such comparative studies have led to the suggestion that the mammalian (endothelial) blood-brain barrier may have evolved from an ancient one based solely on glial end-feet ensheathing blood vessels as they coursed through the primitive brain (Cserr and Bundgaard, 1984). Our demonstration that DRP is located within these perivascular astrocytes and conservation of its distribution over 400 million years of evolution strengthens the suggestion that perivascular astrocytes and proteins present in them (such as DRP) may play a role in maintaining the cellular specializations associated with the blood-brain barrier, perhaps by influencing the spatial regulation of intramembranous proteins such as ion channels or the orthogonal array of particles (OAPs) that cluster in this region of the astrocyte (Risau and Wolburg, 1990). Other members of the spectrin superfamily of cytoskeletal proteins have previously been demonstrated to interact with and stabilize integral membrane proteins, thus helping to form and maintain regional cellular specializations (Srinivasan et al., 1988; Coleman et al., 1989; Bennet 1990).

Ohlendieck and coauthors drew an analogy between the localization of DRP and certain isoforms of laminin in the adult skeletal muscle (Ohlendieck et al., 1991). The laminins are constituents of the basal lamina which surround mammalian muscle and are known to be particularly well

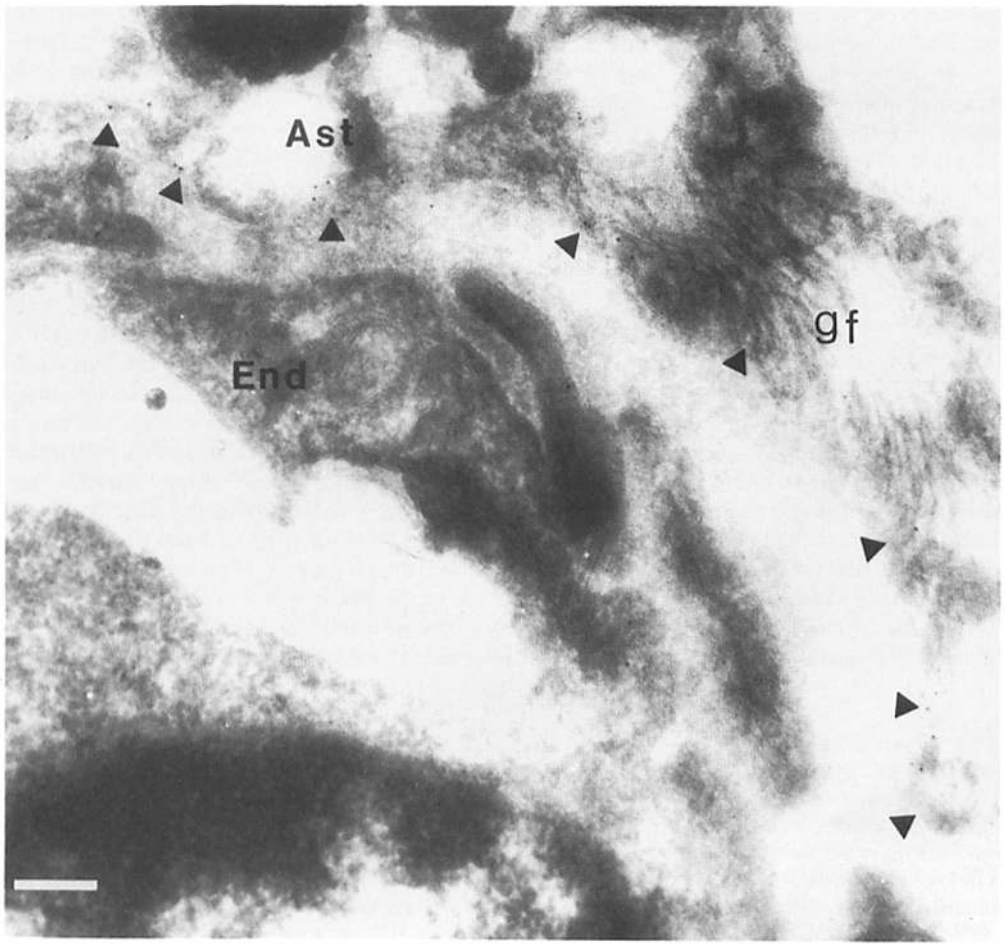

Figure 7. Immunoelectron microscopy of DRP at the blood-brain interface. Ultrathin cryosections of *mdx* mouse brain were incubated with DRP antibodies and distribution of DRP revealed by 5-nm goat anti-rabbit gold conjugates. The figure demonstrates the ultrastructure of the blood-brain barrier. An astrocytic foot process (*Ast*) is seen to be closely apposed to an intracerebral capillary. Glial filaments (*gf*) present in the astrocytic foot process are marked. ~10-fold more gold particles (*arrowheads*) are seen to be distributed within astrocytic end-feet at the glio-vascular interface, than at the endothelial cells (*End*) or lumen of intracerebral capillaries. Bar, 0.2 μm.

defined and somewhat distinct, at intramuscular nerves, vessels, and neuromuscular and myotendinous junctions of the muscle (Sanes, 1986; Sanes et al., 1990). The overall similarity of distribution of laminin(s) with DRP and dystrophin in the skeletal muscle is quite striking (Sanes and Hall, 1979; Sanes et al., 1990; Byers et al., 1991; Khurana et al., 1991; Man et al., 1991; Ohlendieck et al., 1991). In the brain, the basal lamina and laminin are known to be well defined at the distinct locales of the ependymal lining, choroid plexus, glia limitans, and the blood-brain barrier (Risau and Wolburg, 1990; Peters et al., 1991; Hagg et al., 1989; Chiu et al., 1991). We find it noteworthy that both the laminin(s) and DRP are similarly distributed in the brain as well. However, since DRP immunolabeling is wholly intracellular and laminin isoforms are distributed extracellularly, our study suggests that their interaction, if any, is likely to be indirect as has been demonstrated in case of dystrophin (Ibraghimov-Beskrovnaya et al., 1992).

In conclusion, we have used a variety of techniques to describe the distribution of DRP in the *mdx* mouse brain. Consistent with predictions based on cross-reactivity of dystrophin antisera in *mdx* mice (Ishiura et al., 1990), we detected DRP in all regions of the brain tested including the pia mater, choroid plexus, cerebral vasculature, and spinal cord, a distribution which is quite different from that described for dystrophin (Miike et al., 1989; Lidov et al., 1990). The distribution of DRP in the brain was conserved over a significant period of evolution, from elasmobranch to mammals, suggesting that DRP plays an important physiological role. DRP was located by immunoelectron microscopy to the end-feet of perivascular astrocytes, thus its function is likely to be related to the maintenance of blood-brain barrier integrity. In view of the possible structural role(s) for DRP, we believe that further cell biological and tissue culture experiments are needed to address the issue of possible interactive/inductive properties of DRP and other proteins located at the blood-brain barrier.

Thanks to Drs. Manuel Graeber, Hart Lidov, Helen Cserr, and Kiichi Arahata for invaluable advice and insightful comments on neuroanatomy, neuropathology, and the blood-brain barrier. To Drs. A. H. Beggs, T. J. Byers, and M. S. Anderson for constructive criticism, to Alan Frederikson for technical support, and to Dixon Yun for photography.

This work was supported by grants from the National Institutes of Health (L. M. Kunkel), Muscular Dystrophy Association of U.S.A. (L. M. Kunkel), the National Science Foundation (S. C. Watkins) and the Division of Medical Sciences, Harvard Medical School (T. S. Khurana). L. M. Kunkel is an investigator of the Howard Hughes Medical Institute.

Received for publication 10 April 1992 and in revised form 6 July 1992.

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