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IDENTIFICATION OF A NOVEL MEMBRANE-ASSOCIATED PROTEIN EXPRESSED IN NEURONS OF THE SQUID AND RODENT NERVOUS SYSTEMS

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Abstract—In a previous communication, we reported the isolation of a novel cDNA clone (pA6) from a library constructed from squid axonal mRNAs. The partial cDNA clone contained a unique open reading frame that encoded 84 amino acids and was complementary to a moderately abundant mRNA approximately 550–600 nucleotides in length [Chun et al., J. Neurosci. Res. 49 (1997) 144–153]. In this report, we identify the pA6 gene product, and characterize its expression in the squid and rodent brain. Results of immunoblot analyses conducted in squid, using a polyclonal antibody raised against a synthetic peptide corresponding to the C-terminus of the putative protein, established the presence of two pA6 immunoreactive proteins of approximately 14 kDa and 26 kDa in size. In contrast, mouse brain contained only a single 26-kDa immunoreactive species. In both the squid and mouse brain, the expression of pA6 appears highly selective, being detected in certain neurons but not in non-neuronal cells, as judged by both *in situ* hybridization and immunocytochemistry. Findings derived from light microscopic, double-label immunohistofluorescence studies indicate that pA6 protein co-localizes with prohibitin, a mitochondrial marker protein. Consistent with these results, electron microscopy localized pA6 immunoreactivity to several membrane compartments to include the outer membrane of mitochondria, as well as to the smooth endoplasmic reticulum and tubulovesicles in dendrites, axons, and axon terminals of neurons in the rat brain.

Taken together, these findings indicate that pA6 is a novel, membrane-associated protein, which is expressed in the distal structural/functional domains of neurons in both the invertebrate and vertebrate nervous systems.
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Key words: novel mRNA, axon, synaptosome, mitochondrial-associated protein, squid, mouse brain.

Although the majority of mRNAs are transcribed and translated in the neuronal cell soma, some transcripts are selectively transported to the distal structural/functional domains of large asymmetric neurons to include the dendrites, axon, and presynaptic nerve terminal. The subcellular compartmentation of these mRNAs is thought to regulate the local synthesis of proteins that play a key role in the establishment of nerve cell polarity, development and maintenance of the axon, and in the remodeling of the nerve terminals in response to neural activity (for review, see Koenig and Giuditta, 1999; Alvarez et al., 2000).

Early support for the selective axonal targeting of mRNA was obtained from studies conducted in the squid giant axon, where it was found that a small subset of the mRNAs present in the cell bodies were also located in the axon. The axonal mRNA population was comprised of approximately 100–200 different mRNA species many of which were capable of programming protein synthesis in a cell-free translation system (Giuditta et al., 1986; Perrone Capano et al., 1987). To date, β-actin, β-tubulin, kinesin heavy chain, neurofilament proteins, microtubule-associated protein H1, enolase, 70-kDa heat shock protein, and several nuclear-encoded mitochondrial mRNAs have been identified in the axonal mRNA population of the squid giant axon (Kaplan et al., 1992; Gioio et al., 1994, 2001; Chun et al., 1995). Besides the mRNAs known to encode metabolic, organelle and structural proteins, as well as molecular motors, molluscan axons also contain mRNAs encoding several neuropeptides (van Minnen et al., 1988; Landry et al., 1991; Dirks et al., 1993).

Interestingly the relative abundance of the mRNAs present in the axon differs markedly from that manifest

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Abbreviations: AP, alkaline phosphatase; DIG, digoxigenin; EDTA, ethylenediaminetetra-acetate; EGTA, ethylene glycol-bis(2-aminoethyl-ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; FITC, fluorescein isothiocyanate; KLH, keyhole limpet hemocyanin; PAGE, polyacrylamide gel electrophoresis; PB, phosphate buffer; SSC, saline sodium citrate; SDS, sodium dodecyl sulfate; TBS, Tris-buffered saline.

in the parental cell bodies, suggesting that these mRNAs are differentially transported into the axonal domain (Chun et al., 1996). In contrast, the mRNA encoding the sodium channel is completely absent in the axon, as judged by quantitative reverse transcription polymerase chain reaction methodology, although these sequences are relatively abundant in the perikaryon (Gioio et al., 1994; Chun et al., 1996). These findings indicate that the mechanism governing the axonal transport of mRNA is highly selective.

In a previous study, we reported the isolation of a novel mRNA (pA6) from a cDNA library prepared from the squid axonal mRNA population (Chun et al., 1997), and subsequently confirmed its axonal localization by *in situ* hybridization histochemistry. In this communication, we employ a polyclonal antiserum prepared against a pA6 synthetic peptide to identify the protein encoded by pA6 mRNA and document its expression in the squid and rodent nervous systems. In addition, preliminary evidence is provided to indicate that pA6 is a novel, nuclear-encoded, membrane-associated protein that is relatively abundant in axons and presynaptic nerve terminals.

EXPERIMENTAL PROCEDURES

Preparation of tissue

Squid (*Loligo pealei*) were obtained during the summer months at the Marine Biological Laboratory (Woods Hole, MA, USA). Squid were kept in a large tank with running seawater and used within 24–48 h after capture. The giant fiber lobe was dissected from the stellate ganglion, and the optic lobe was removed quickly from the decapitated head. Squid tissues used for immunocytochemistry were washed twice with filtered seawater, followed by a wash in ice-cold filtered seawater containing 2% paraformaldehyde. Tissue was subsequently fixed in 2% paraformaldehyde plus 0.1% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) and embedded in paraffin. Tissues used for *in situ* hybridization were kept in fixative (4% paraformaldehyde plus 0.1% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2) for at least 3 h before paraffin embedding.

A synaptosomal fraction from squid optic lobe was prepared as described by Crispino et al. (1993), and synaptosomal mitochondria isolated by sucrose density gradient centrifugation (Gioio et al., 2001).

Generation of pA6 antipeptide antiserum

A rabbit polyclonal antiserum to pA6 was generated to a unique peptide sequence located at the C-terminus of the putative protein (amino acid residues 76–89; [C]RDTDVKDPQSDRYSCOOH). The selection of the peptide was based upon computer algorithms which predicted its hydropathy index, accessible surface probability, and favorable secondary structure. Peptide synthesis, peptide–keyhole limpet hemocyanin (KLH) conjugation, and rabbit antibody production were performed by Zymed Laboratories (San Fransisco, CA, USA). The peptide was conjugated to KLH through the N-terminal cysteine, using *N*-succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate as the KLH activating agent. New Zealand White rabbits were immunized with peptide–KLH conjugate emulsified in Freund's adjuvant at 3-week intervals and antiserum was collected 10 days after the fourth immunization. The antibody titer of all antisera was determined with an enzyme-linked immunosorbent assay with free peptide used as coat.

Immunoblot analysis

Tissues from mouse and squid were minced and homogenized in dissecting buffer (0.3 M sucrose, 25 mM Tris–HCl, pH 7.2, 1 mM EGTA, and protease inhibitor cocktail; Sigma, St. Louis, MO, USA; diluted 1:5000), with a motor-driven glass/Teflon homogenizer. Tissue homogenates were cleared of nuclei by centrifugation at $3000\times g$ for 5 min at 4°C in an Eppendorf microfuge. The supernatant was subsequently centrifuged at $20\,800\times g$, at 4°C for 30 min, and the resultant pellet was suspended in Tris-buffered saline (TBS; 25 mM Tris–HCl, pH 7.5, 150 mM NaCl). Samples were placed in Laemmli buffer containing 2% sodium dodecyl sulfate (SDS) and were fractionated on 12% or 15% polyacrylamide gels using the Mini Protean II Electrophoresis system (Bio-Rad, Hercules, CA, USA). Electrophoresis was conducted at 100 V for 90 min in the presence of Kaleidoscope polypeptide standards (Bio-Rad). After transfer by electroelution to nitrocellulose membranes (350 mA for 60 min), blots were blocked with 5% non-fat dry milk in TBS-T (25 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20) for 1 h, and incubated with rabbit anti-pA6 serum (diluted 1:5000) or rabbit anti-prohibitin antibody (Research Diagnostics; diluted 1:1000) in 5% non-fat dry milk in TBS-T at 4°C overnight or at room temperature for 2 h, followed by incubation (1 h) with alkaline phosphatase (AP)-conjugated secondary antibody (Bio-Rad) in TBS-T (diluted 1:3000). Colorimetric detection of the immunoreactants was effected using AP conjugate substrate kit (Bio-Rad).

In situ hybridization histochemistry

Digoxigenin (DIG)-labeled riboprobes used for *in situ* hybridization were prepared from recombinant pBluescript vectors containing pA6 cDNA using a DIG labeling kit (Boehringer Mannheim, Indianapolis, IN, USA). Hybridization experiments conducted on mouse brain were performed using 15-μm sections as previously described (Torp et al., 1997; Wen et al., 1999). In brief, the sections were incubated in a hybridization solution containing 50% formamide, 10% dextran sulfate, 1 mM EDTA, 0.3 M NaCl, and 20–25 ng of DIG-labeled RNA probe, and were subsequently hybridized at 50°C for 16–18 h. After hybridization, the sections were immersed sequentially in 2×saline sodium citrate (SSC; 0.3 M NaCl and 0.03 M Na-citrate, room temperature, 45 min), 2×SSC containing 50% formamide (50°C, 30 min), and 2×SSC (room temperature, twice for 10 min each). Unhybridized RNA was removed by RNase A. DIG-labeled probes were detected using anti-DIG antibodies labeled with fluorescein isothiocyanate (FITC; Boehringer Mannheim). Negative control experiments were conducted using both sense strand RNA probes and pretreatment of tissue sections with RNase A (50 μg/ml in 25 mM TBS, pH 7.6, for 10 min at 37°C).

Hybridization with squid tissues was performed as described by Chun et al. (1997). In brief, paraffin-embedded tissue sections (15 μm) were pretreated with proteinase K (10 μg/ml) and 2% HCl (10 min each) and were hybridized overnight at 55° in 2×SSC, 50% formamide, 1 mM EDTA, 10% dextran sulfate, 0.5 mg/ml calf thymus DNA, and 20–25 ng DIG-labeled riboprobe. After hybridization, the sections were washed and reaction products visualized as described above.

Immunocytochemistry

Immunochemistry was performed essentially as described by Nielsen et al. (1995). Paraffin-embedded tissue sections (8 μm) were deparaffinized, rehydrated and blocked with 2% non-fat dry milk for 1 h at room temperature. Incubation with pA6 antiserum (diluted 1:1000 with TBS-T) was performed at 4°C overnight, followed by two washes with TBS. Sections were subsequently incubated with FITC- and/or rhodamine-labeled secondary antibody (goat anti-rabbit IgG, Fab′). In double-label experiments, an additional 1-h incubation with 2% non-fat dry milk was conducted, followed by incubation (1 h) with a rabbit immunopurified polyclonal antibody to prohibitin (1:100

dilution), a mitochondrial marker protein. Prohibitin immunoreaction products were subsequently visualized using rhodamine-labeled secondary antibody.

Electron microscopic analysis

All methods employed in the ultrastructural localization of pA6 have been previously described (Pickel et al., 1997). Adult male Sprague–Dawley rats (200–300 g) were deeply anesthetized with sodium pentobarbital (100 mg/kg, i.p.), and the brains were fixed by aortic arch perfusion with 3.8% acrolein in 2% paraformaldehyde followed by 2% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). The animal protocols used in this study were approved by the Animal Care Committee at Cornell University Medical College and strictly adhere to the NIH Guidelines on the Care and Use of Animals in Research. After perfusion, the brains were cut into 4–5-mm coronal slabs of tissue and sectioned at 30–40 μm on a vibratome. The sections were subsequently cryoprotected in 25% sucrose and 3.5% glycerol in 0.05 M PB, rapidly frozen in chlorodifluoromethane followed by liquid nitrogen.

Sections were processed for immunoperoxidase labeling of pA6 using the avidin–biotin peroxidase complex method (Hsu et al., 1981). Sections were incubated (12–18 h) in TBS containing 0.1% bovine serum albumin and pA6 antiserum at a dilution of 1:2000, and were subsequently reacted against a biotinylated goat anti-rabbit immunoglobulin (1:400, Vector Laboratories, Burlingame, CA, USA). Bound peroxidase was visualized by reaction with 3,3′-diaminobenzidine in the presence of hydrogen peroxide. Sections for electron microscopy were postfixed in 2% osmium tetroxide counterstained with uranyl acetate and lead citrate and examined using a Philips CM-10 electron microscope.

RESULTS

Identification of pA6 gene products

To identify the product of the pA6 gene, a polyclonal antiserum was generated against the last 14 amino acids of the C-terminus of the putative protein. In protein obtained from the squid optic lobe, western blot analysis

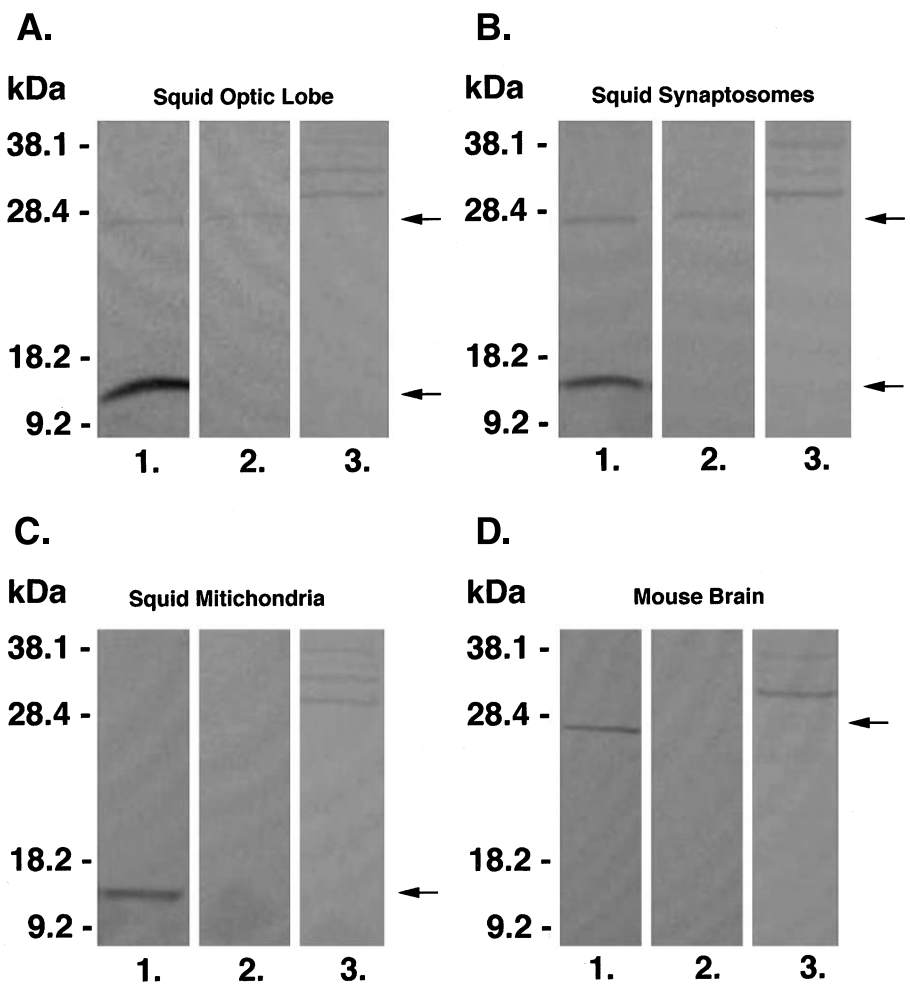

Fig. 1. Immunoblot analyses of pA6 in squid optic lobe and mouse brain. Protein (25 μg/lane) was fractionated by SDS–polyacrylamide gel electrophoresis (PAGE), transferred to nitrocellulose membranes and reacted with pA6 antiserum as described in Experimental procedures. (A) Squid optic lobe protein. A major band of immunoreactivity is clearly visible at approximately 14 kDa and a second minor band of immunoreactivity is observed at 26 kDa. (B) Immunoreaction conducted with protein isolated from a squid optic lobe synaptosomal fraction. (C) Protein isolated from purified synaptosomal mitochondria. Note the appearance of a single immunoreactive species at 14 kDa. (D) Mouse brain protein isolated from a mitochondrial-enriched fraction. A single band of pA6-like immunoreactivity is present at 26 kDa. Arrows indicate the position of pA6 immunoreactivity. Lanes: 1, pA6 antiserum; 2, immunoreactions conducted with pA6 antiserum preadsorbed with the synthetic peptide used as antigen; 3, immunoreactions conducted with non-immune serum.

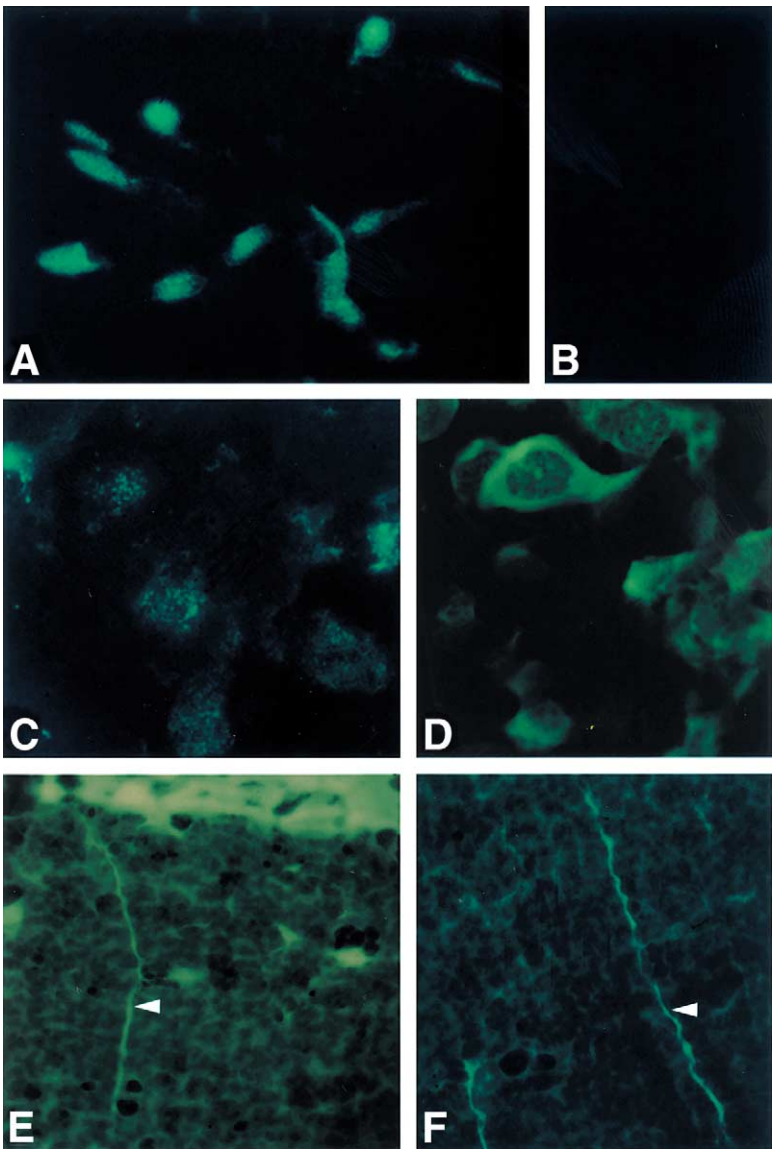

Fig. 2. Expression of pA6 mRNA in squid optic lobe by *in situ* hybridization histochemistry. Tissue sections were hybridized using a DIG-labeled, antisense pA6 riboprobe and hybridization signal visualized using an anti-DIG antibody conjugated to FITC. A punctate, granular histofluorescence signal was typically observed over neuronal perikaryon in the granular cell layer (A and C). No signal was observed in tissue sections hybridized to a sense strand pA6 riboprobe (B). Hybridization was also conducted on identical tissue sections using a riboprobe to squid elongation factor-2 as a positive control (D). Note the uniformity in the appearance in the hybridization signal obtained with this probe. Significant pA6 hybridization signal was also observed throughout the axonal processes in the plexiform layer of the optic lobe (E and F, arrowheads). Magnification in A–C, 400×; E–F, 1000×.

revealed a major immunoreactive product which migrated with an apparent electrophoretic mobility of approximately 14 kDa and a second minor species approximately 26 kDa in size (Fig. 1A, lane 1). It bears mention that the relative intensity of the minor 26-kDa band differed between tissue preparations. Neither the 14-kDa nor 26-kDa signal was present when the reactions were conducted with non-immune serum (Fig. 1A, lane 3), although a few non-specific, higher molecular weight bands were evident (Fig. 1A–D, lanes 3). Identical 14-kDa and 26-kDa pA6 immunoreactive signals were also observed in a synaptosomal fraction prepared from squid optic lobe (Fig. 1B, lane 1), a subcellular fraction enriched for the presynaptic nerve terminals of photoreceptor neurons. In control experiments, the use of pA6 antiserum preadsorbed with the cognate synthetic peptide totally eliminated the 14-kDa form of immunoreactivity and greatly reduced (50–60%) the 26-kDa immunoreaction product (Fig. 1A, lane 2). Similar results were obtained in control experiments in which synaptosomal protein was reacted against preadsorbed pA6 antiserum or non-immune serum (Fig. 1B, lanes 2 and 3, respectively). Interestingly, only the major 14-kDa immunoreactive signal was observed in the mitochondria isolated from the same synaptosomal fraction (Fig. 1C). These results raised the possibility that the 14-kDa form

of pA6 was a nuclear-encoded mitochondrial protein which was present in the nerve endings of the photoreceptor neuron.

In contrast to the findings in squid, a single 26-kDa immunoreactive product was observed in total protein isolated from mouse brain (data not shown), as well as in a brain mitochondrial-enriched fraction (Fig. 1D, lane 1). In accord with the above findings, the pA6-like immunoreactivity was not observed in mouse brain protein reacted against pA6 antiserum preabsorbed with the antigen or non-immune serum (Fig. 1D, lanes 2 and 3, respectively).

Visualization of pA6 gene products in squid nervous tissue

Hybridization of a FITC-labeled antisense pA6 riboprobe to tissue sections prepared from squid optic lobe revealed an intense granular or punctate fluorescence in neuronal cell bodies located throughout the tissue, whereas little signal was observed in the surrounding glia (Fig. 2A, C). Hybridization with a sense pA6 probe eliminated the signal (Fig. 2B). Similar negative results were obtained in tissue sections pretreated with RNase (data not shown). In contrast to the granular appearance of the hybridization pattern observed with the pA6 probe, a more typical, uniform fluorescence was obtained in similar tissue sections hybridized with an antisense probe to squid elongation factor-2 (Fig. 2D). Consistent with the previous report that pA6 mRNA was located in the squid giant axon (Chun et al., 1997), intense pA6 immunoreactivity was frequently observed in the large axonal processes of second order visual cells located in the outer plexiform layer of the optic lobe (Fig. 2E, F).

In the squid giant fiber lobe, pA6 protein was revealed in neurons by immunostaining using pA6 antiserum and a rhodamine-conjugated secondary antibody (Fig. 3).

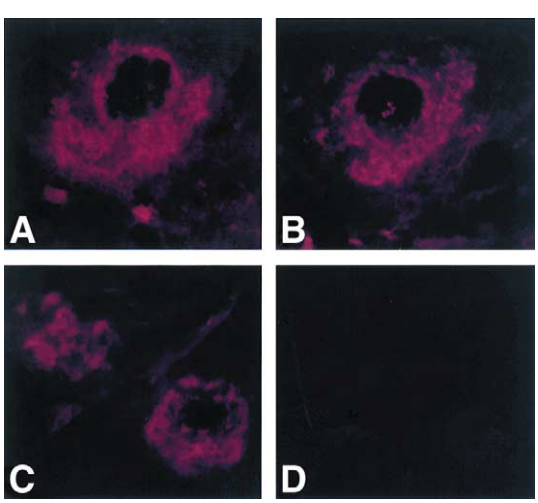

Fig. 3. Immunocytochemistry of pA6 in squid giant fiber lobe. Paraffin-embedded tissue sections were reacted with pA6 antiserum (A–C) or non-immune serum (D) and immunoreaction products visualized using a rhodamine-conjugated secondary antibody. Note the punctate appearance and polar distribution of the immunoreactivity. Magnification, 1000×.

The large, punctate immunofluorescence was preferentially expressed in neurons and was concentrated about the nucleus (for example, see Fig. 3A, B). Often, the immunoreactivity was observed in an asymmetric orientation in the neuron. This labeling pattern was reminiscent of the preferential distribution of mitochondria manifest in some cell types (Nie and Wong-Riley, 1996; Murata et al., 2000; Lindenau et al., 2000). The immunolabeling was abolished when the reactions were performed with either non-immune serum (Fig. 3D) or pA6 antiserum preabsorbed with the peptide antigen (data not shown).

pA6 gene products are expressed in mouse brain

Hybridization of mouse brain tissue sections to a DIG-labeled pA6 riboprobe yielded strong signals in neuronal perikarya of the Purkinje cells of the cerebellum, and in both the cochlear and vestibular nuclei (Fig. 4A-D). Strong hybridization signals were also observed in the facial motor and trigeminal nuclei with moderate levels of signal detected in the cerebral cortex and hippocampal formation. Similar to the findings in the squid optic lobe, the hybridization signal was punctate in appearance in all neurons, and was not detected in non-neuronal cells. The hybridization signal was eliminated after pretreatment of the tissue sections with RNase (Fig. 4E) or after hybridization with a sense pA6 riboprobe (Fig. 4F).

In the cerebellum, little hybridization signal could be observed in the granule cell layer or molecular layer. In the cochlear nucleus, the strongest labeling was observed in the large, multipolar neurons in the anterior cochlear nucleus, although signal was readily detected in fusiform cells in both the posterior and dorsal aspects of the region. In the vestibular nucleus, intense signal was observed in neurons located throughout the region.

The cellular distribution of pA6 protein in the vestibular nucleus and cerebellum was investigated using a double-immunolabeling procedure which employed pA6 antiserum and an antiserum to prohibitin, a mitochondrial marker protein. To assess the specificity of the prohibitin antibody, an immunoblot analysis was conducted using protein from a mitochondrial-enriched fraction from mouse brain. As shown in Fig. 5, anti-prohibitin antibody yielded a single immunoreactive protein that migrated with a molecular weight of approximately 30 kDa (Fig. 5, lane 3). In this experiment, pA6 immunoreactivity served as a positive control (Fig. 5, lanes 1 and 2).

In the light microscopic immunocytochemical studies, the pA6 and prohibitin immunoreaction products were subsequently visualized using FITC- and rhodamine-labeled secondary antibodies, respectively. As shown in Fig. 6A–C, both antibodies yielded similar patterns of immunoreactivity in vestibular neurons. In both cases, the immunofluorescence was punctate in appearance and was distributed in an asymmetric fashion about the cell nucleus. Similar results were obtained in the Purkinje cell (Fig. 6D–F). In both regions, a significant amount of the cellular dual-label immunohistofluores-

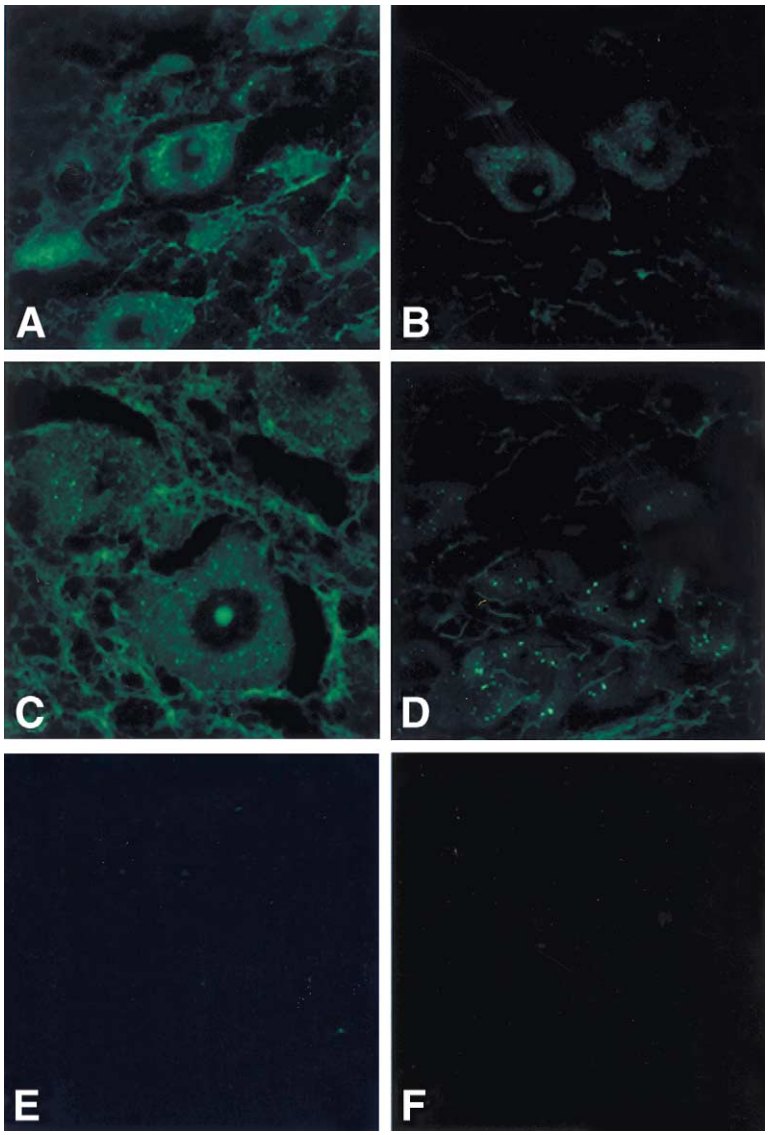

Fig. 4. Expression of pA6 mRNA in mouse brain. Hybridization of tissue sections was conducted using a DIG-labeled squid pA6 riboprobe and hybridization products visualized with a FITC-conjugated anti-DIG antibody. Typical tissue sections are shown from cerebellum (A and C, Purkinje cells), vestibular nucleus (B), and cochlear nucleus (D). The punctate appearance of the hybridization signal is noteworthy. Negative control experiments were conducted with tissue sections pretreated with RNase (E) or hybridized to a sense strand pA6 riboprobe (F). Magnification, 1000×.

cence signal could be colocalized using a double filter set (Fig. 6C, F). Replacement of the pA6 antiserum and prohibitin antiserum with non-immune serum and non-specific mouse IgG, respectively, completely eliminated the immunofluorescence signal from the cerebellar tissue slices (Fig. 6G–I).

Subcellular localization of pA6 immunoreactivity

Electron microscopic analysis established the presence of pA6 immunoperoxidase reaction product in select neurons in the rat brain. For example, in the caudate–putamen nucleus, pA6 labeling was prominently observed on or near mitochondrial membranes in both axons and dendrites (Fig. 7A). In addition, labeled tubulovesicles resembling saccules of smooth endoplasmic reticulum were observed near outer nuclear membranes in somata (Fig. 7A). In dendrites, dense accumulations of pA6 immunoreactivity were also distributed along membranous structures that were juxtaposed to labeled mitochondria and often appeared to be in continuity with the mitochondrial and/or plasma membrane (Fig. 7B).

In both the caudate–putamen and cerebellum, pA6 immunoperoxidase reaction product was also observed in axon terminals (Fig. 8). In striatal terminals, diffuse labeling was seen juxtaposed to the plasma membrane and membranes of nearby small synaptic vesicles of excitatory-type terminals forming asymmetric synapses with dendritic spines, which also occasionally showed intense pA6 immunoreactivity (Fig. 8A). Large cerebellar mossy fiber axon terminals showed a pattern of mitochondrial labeling more comparable to that seen in dendrites (Fig. 8B).

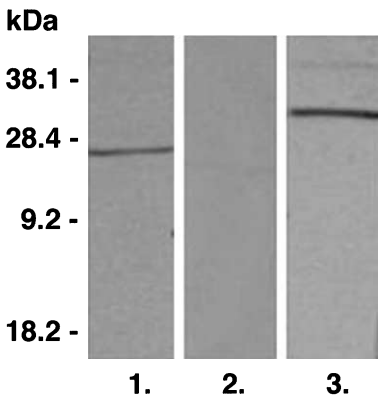

Fig. 5. Immunoblot analysis of pA6 and prohibitin in mouse brain. Protein (35 μg/lane) was isolated from a brain mitochondrial-enriched fraction and was fractionated by SDS–PAGE as described in Experimental procedures. Lanes: 1, pA6 antiserum; 2, pA6 antiserum preadsorbed with the synthetic peptide used as antigen; 3, anti-prohibitin antibody.

DISCUSSION

The pA6 clone was initially isolated during the characterization of a squid axonal cDNA library (Kaplan et al., 1992). The cognate polyadenylated mRNA was approximately 500–600 nucleotides in length, and the partial cDNA clone contained a unique open reading frame that encoded 84 amino acids (Chun et al., 1997). To identify and characterize the distribution of the pA6 gene product in this study, a peptide corresponding to the C-terminal 14 amino acids of the putative protein was synthesized and used as an antigen to generate a rabbit polyclonal antiserum.

Results of western blot analyses of total brain protein revealed the existence of two immunoreactive forms of pA6 in squid nervous tissue (14 and 26 kDa) and a single 26-kDa protein in rodent brain. Preliminary data derived from two-dimensional gel electrophoretic analyses indicate that the 14-kDa form of pA6 is the protein encoded by the axonal cDNA clone previously reported by Chun et al. (1997). For example, this immunoreactive protein had a molecular weight and basic isoelectric focus point consistent with that predicted for the putative amino acid sequence derived from the cDNA clone. Whether the different forms of pA6 in the squid represent different gene products or alternatively spliced forms of a single gene transcript remains to be determined. Importantly, the pA6 immunoreactivity is relatively abundant in the synaptosomal fraction prepared from the squid optic lobe. This cellular fraction has been demonstrated to consist predominately of the large presynaptic nerve terminals of retinal photoreceptor neurons that are highly enriched in mitochondria (Crispino et al., 1997). The finding that pA6 immunoreactivity was present in mitochondria purified from the synaptosomal fraction was consistent with the mitochondrial localization of the pA6 protein. Additional support for this hypothesis was obtained from immunocytochemical studies. The punctate nature and cellular distribution of pA6 immunoreactivity in neurons of both the squid and mouse are consistent with a mitochondrial localization (Nie and Wong-Riley, 1996). The results of the double-label immunohistofluorescence experiments provide independent evidence to support this hypothesis. In this experiment, the distribution of immunoreactivity generated by antibodies to both pA6 and a mitochondrial marker

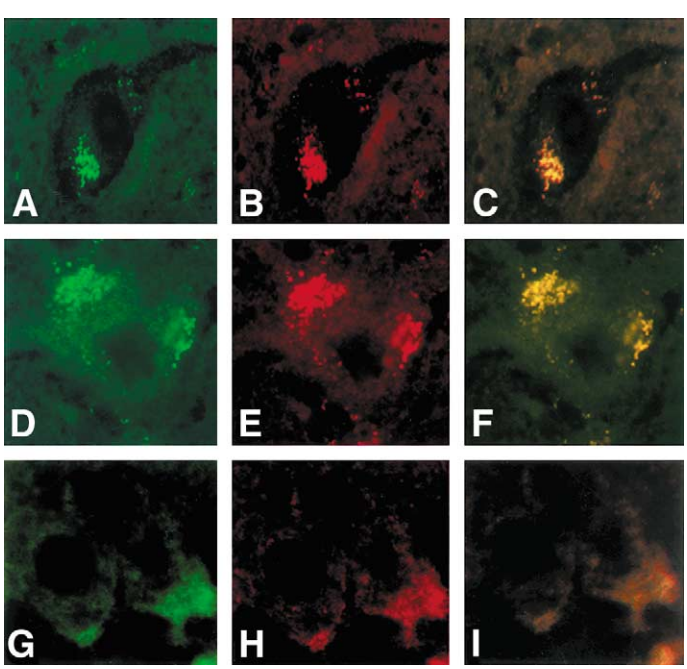

Fig. 6. Colocalization of pA6 and mitochondrial gene products in mouse brain. Double-label immunocytochemical experiments were conducted using antisera to pA6 and prohibitin, a mitochondrial marker protein. pA6 and prohibitin immunoreaction products were visualized with rhodamine- and FITC-conjugated secondary antibodies, respectively. Vestibular neurons (A–C), and Purkinje cells (D–F). Results of negative control studies conducted in cerebellum using non-immune serum are also shown (G–I). Magnification, 1000×.

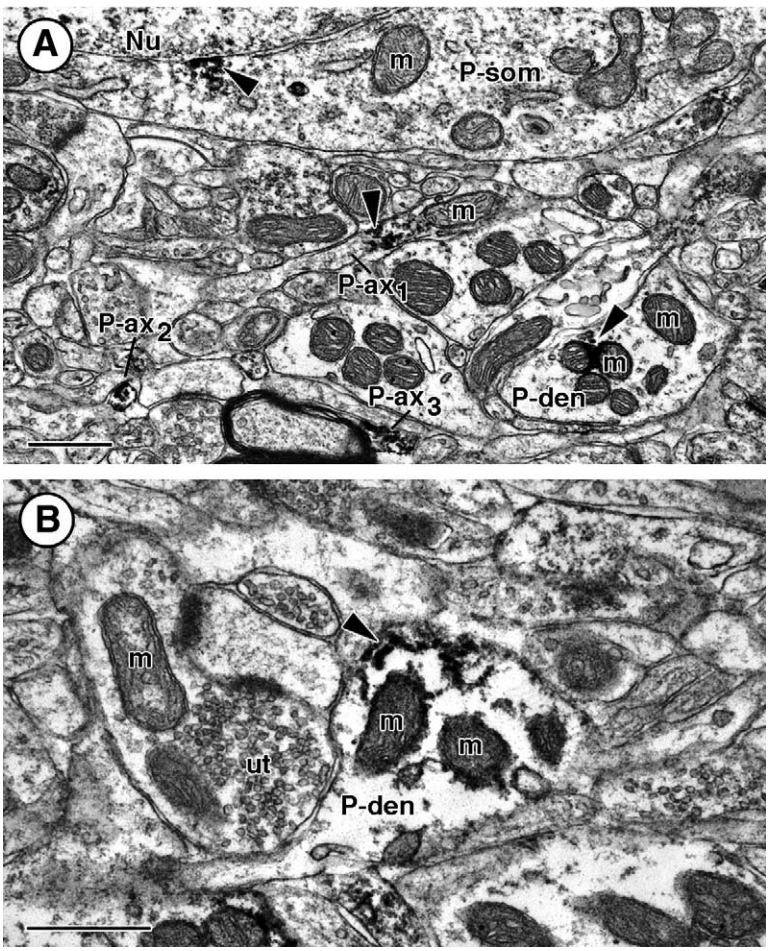

Fig. 7. Electron micrographs showing immunoperoxidase labeling for pA6 (arrowhead) in the rat caudate–putamen nucleus. pA6-like immunoreactivity is associated with the outer membranes of selected mitochondria (m) in dendrites (P-den) that are seen in both A and B. In the micrograph of A, immunoreactivity is also localized (1) to membranous vesicles near the outer membrane of a nucleus (Nu) within the soma of a spiny neuron (P-soma), and (2) diffusely distributed in several small axons (p-ax1–3). In the longitudinally sectioned axon (p-ax1), the reaction product is near a mitochondrion and is juxtaposed beneath the axolemma. In B, dense labeling (arrowhead) has a punctate distribution along membranous structures in the region between the mitochondrial surface and the plasma membrane. No labeling is seen on or near the mitochondrion (m) in a nearby unlabeled terminal (ut). Scale bar = 0.5 μm (in A and B).

protein were strikingly similar in several neuronal cell types.

Direct evidence for the mitochondrial localization of pA6 was gleaned from electron microscopic immunohistochemical analyses. At the ultrastructural level, pA6 immunoreactivity was clearly localized to the outer membrane of mitochondria, as well as tubulovesicular organelles resembling the smooth endoplasmic reticulum. Immunoperoxidase activity was most readily apparent in axons and pre- and postsynaptic nerve terminals, where it was often visualized beneath the plasma membrane. In this regard, the subcellular distribution of pA6 resembles that of sorcin, a 22-kDa calcium binding protein that is associated with the ryanodine receptor in both muscle and brain (Pickel et al., 1997). Interestingly, pA6 immunoreactivity was observed in only a subset of mitochondria. In transverse sections of the organelle, pA6 immunoreactivity often appeared as a cap-like structure apposed to other mitochondria or smooth endoplasmic reticulum (for example, see Figs. 6A and 7B). In view of the intermittent distribution of pA6 immunoreactivity along the outer mitochondrial membrane, one would tend to underestimate the number of these organelles that associate with this nuclear-encoded protein at the electron microscopic level.

Surprisingly, both the *in situ* hybridization and immunocytochemical signals for pA6 were punctate in appearance and were often distributed in a asymmetric fashion in the cell. The uniform distribution and agranular nature of the hybridization signal obtained for squid elongation factor-2 argues against the possibility that the pA6 signal derived from a tissue preparation and/or fixation artifact. In this regard, there is evidence to suggest that ribosomes are directly attached to the outer membrane of mitochondria and that nuclear-encoded mRNA translation and protein import are coupled and occur at the surface of the organelle (Kellems et al., 1975; Fujiki and Verner, 1993; Crowley and Payne, 1998; Ni et al., 1999). The subcellular distribution and appearance of the pA6 hybridization and immunocytochemical signals are consistent with this postulate. Whether pA6 is synthesized locally in the axon

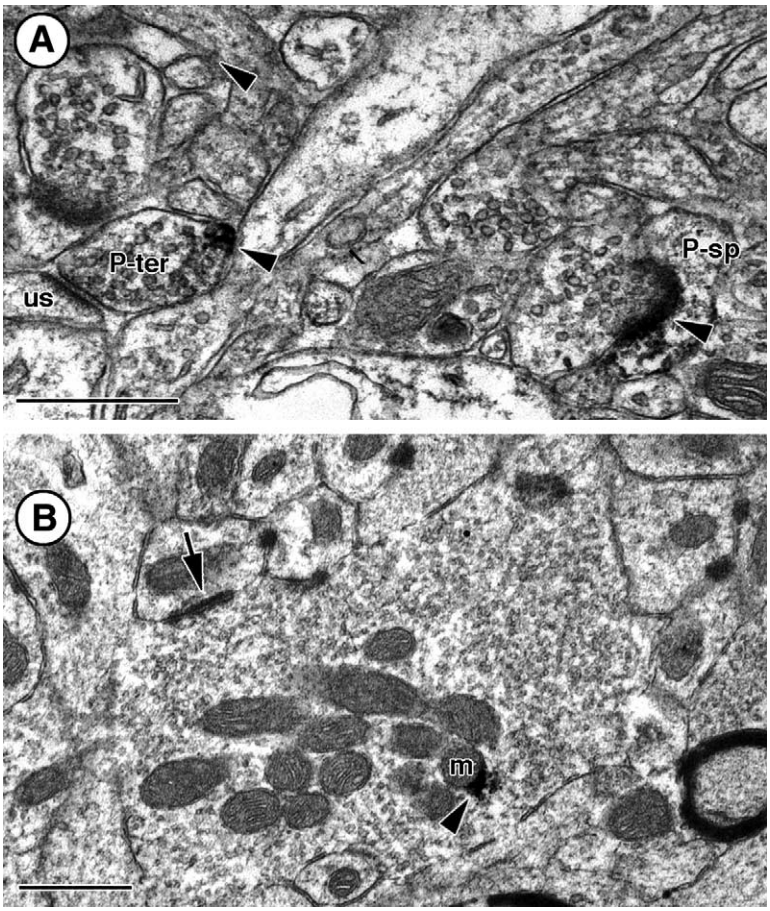

Fig. 8. Ultrastructural immunoperoxidase labeling in the rat caudate–putamen nucleus (A) and cerebellum (B) reveals that pA6 (arrowheads) is present in axon terminals (P-ter). In the micrograph of A, the labeling is seen near the plasma membrane and nearby vesicles in an axon terminal forming an asymmetric, excitatory-type synapse (P-ter) with an unlabeled dendritic spine (us). Within the field, the reaction product is also seen within and near an asymmetric synapse onto a labeled spine (P-sp). In the large mossy fiber axon terminal in B, the immunoreactivity is discretely associated with the membrane of a single mitochondrion (m). The terminal forms multiple contacts with unlabeled dendrites, one of which shows a clear asymmetric junction (arrow). Scale bar = 0.5 μm (in A and B).

and nerve terminal, as suggested by the subcellular distribution of its mRNA, remains an open question.

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