Immunocytochemical Localization of Coated Vesicle Protein in Rodent Nervous System

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ABSTRACT Immunocytochemistry has been used to study the distribution of the major 180,000-mol wt protein of coated vesicles in rodent cerebellum. An antibody to the coat protein was prepared in rabbits and characterized by immunodiffusion and immunofixation of polyacrylamide gels. At the light microscope level the protein was primarily localized in punctate profiles surrounding Purkinje cells and within the cerebellar glomeruli. At the electron microscope level the punctate distribution was confined to presynaptic terminals of basket and Golgi II neurons as well as mossy fiber terminals of the glomeruli. This label was heaviest on the lattice coat of coated vesicles but, in addition, label was found within the presynaptic axoplasm and along the cytoplasmic surface of the plasmalemma. Coated vesicles in cell somata were labeled as well as the cytosol around groupings of these vesicles. These data suggest that there may be two forms (or more) of coated vesicle protein in neurons, a lattice form associated with coated vesicles and a soluble form associated with the cytoplasmic matrix.

Coated vesicles, consisting of a central membrane vesicle surrounded by a lattice of protein, have been identified in many cell types. Their biological roles, although dependent on the functions of the cells in which they occur, seem to be concerned with uptake of exogenous macromolecules (2, 3, 18, 19) and perhaps retrieval of axolemmal membrane in presynaptic terminals (11). In addition, coated vesicles may contribute to the transport of macromolecules from synthetic sites within cells to sites of secretion or insertion of the macromolecules into the cell membrane (9, 17). Although these potential roles for coated vesicles have been documented, there is now little evidence concerning the type of stimulus and the mechanisms leading to formation of the coat in vivo. Anderson et al. (3) have shown that low density lipoprotein (LDL) receptors in fibroblasts are initially localized in pitted indentations along the plasma membrane and, shortly thereafter, the LDL are seen within coated vesicles. These investigators demonstrated that the indentations at the cell surface are coated with the major protein of coated vesicles, clathrin (16). They and others (10–12, 18) have suggested that coated pits can transform into coated endocytotic vesicles which eventually lose their coats at some distance from the site of endocytotic activity. An important question that remains unanswered is the mechanism of initial assembly of the coat protein(s) during endocytosis; it is not known, for example, whether the protein exists in a nonlattice form which is stimulated by the ligand-receptor interaction to polymerize preferentially around absorptive sites. The presence of coated pits at sites of LDL binding to fibroblasts would support such a concept, but it is important to note that coated indentation will form in mutant fibroblasts which do not bind LDL (2). One approach to study this problem is through the immunocytochemical localization of the major coat protein. This should identify its cellular distribution and its location in the formed coat or other subcellular components. We have used this approach to study the distribution of coat protein in rodent cerebellum, a brain region of well-documented cytoarchitecture.

MATERIALS AND METHODS

Coat Protein Purification

A subcellular fraction enriched in coated vesicles was obtained from the gray matter of cerebral hemispheres of fresh pig brain by minor modifications of the sucrose density centrifugation method of Pearse (16) or by the method of Blitz et al. (5) modified by the inclusion of GTP (Sigma Chemical Co., St. Louis, Mo.) in all solutions. The purified coated vesicle fraction was solubilized in sample buffer containing 1.0% sodium lauryl sulfate (Bio-Rad Laboratories, Richmond, Calif.), 40 mM dithiothreitol (DTT, Bio-Rad Laboratories) in 0.1 M Tris buffer, pH 6.8, and subjected to preparative polyacrylamide gel electrophoresis (PAGE) using a slab gel apparatus (Bio-Rad Laboratories). Strips of gels from several

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runs containing the major 180,000-dalton coat protein (clathrin) were homogenized 0.5 M Tris buffer, pH 6.8, containing 0.08% sodium dodecyl sulfate (SDS) and 0.125% DTT. The homogenate was stirred for 2 h at 37°C and centrifuged for 15 min at 8,000 *g* to remove gel fragments. The supernate was homogenized with an equal volume of Freund's complete adjuvant (Difco Laboratories, Detroit, Mich.) for 2 min at top speed of a VirTis 23 homogenizer (VirTis Co., Inc., Gardiner, N.Y.) and used to immunize rabbits.

Preparation of Antisera

Female New Zealand white albino rabbits (2.5–3.0 kg) (Mecklenburg Rabbitry, Janesville, Wis.) were bled to collect preimmune serum, and 1 wk later, they were injected subcutaneously and intramuscularly at several sites along the back and limbs. Each animal received ~ 250–350 μg of antigen in a total of 2 ml of emulsion. This injection schedule was repeated at 30 d and the animals were bled 1 wk after the booster injection. The serum was clotted at 4°C overnight and the clot was discarded. After centrifugation at 2,000 rpm for 15 min, small volumes of the serum were rapidly frozen on dry ice and stored at −70°C until use.

Characterization of Sera

The immune sera were characterized by Ouchterlony immunodiffusion and by immunofixation of polyacrylamide gels.

IMMUNODIFFUSION: 50 μl of undiluted preimmune or immune sera was placed in the peripheral wells of Agarose Rheophoresis plates (Abbott Laboratoreis, North Chicago, Ill.) and 15 μl of the coated vesicle fraction or coat protein extract was dissolved in 0.5 M Tris buffer, pH 6.8, containing 0.08% SDS and 0.125% DTT and placed in the central well. The immunodiffusion plates were developed overnight at 25°C in a dessicator and photographed unstained using Kodak Plus-X film.

IMMUNOFIXATION: Polyacrylamide slab gels (7.5 or 12.5% acrylamide) containing duplicate groupings of the coated vesicle fraction and the extended 180,000-dalton protein were treated to remove SDS as described by Fairbanks et al. (7). The gel was sliced vertically, and a portion stained with Coomassie brilliant blue dye. Other portions were treated with 1:10 dilutions of either preimmune or immune serum in phosphate-buffered saline (PBS) for 8 h (all reactions at 25°C). The gels were washed for 20 h with PBS and treated for 6 h with either goat anti-rabbit Fab-horseradish peroxidase (HRP) conjugate or by the peroxidase-anti-peroxidase unlabeled antibody method of Sternberger (20). In one experiment the gels were treated with the rabbit sera followed by the ^{125}I-staphylococcal protein A (SPA) autoradiography method of Adair et al. (1).

Preparation of Tissue

Mature C57Bl/6J mice or Sprague-Dawley albino rats were anesthetized by intraperitoneal injections of Nembutal (Abbott Laboratories) and perfused through the heart with a fixative containing freshly prepared 4.0% paraformaldehyde and 0.1% glutaraldehyde in 0.12 M Millonigs phosphate buffer, pH 7.2 (21). After perfusion the cerebellum was removed and stored overnight at 4°C in 4.0% phosphate-buffered paraformaldehyde. The fixed cerebellum was cut parasagittally into 40–50-μm slices using an Oxford Vibratome (Ted Pella, Inc., Tustin, Calif.). The slices were collected in PBS and the best sections were chosen under a dissecting microscope for immunocytochemistry.

Immunocytochemical Procedures

All steps were performed with constant gentle agitation at 25°C except as indicated. The tissue slices were treated for 30 min with 3% normal goat serum and then incubated for 45–60 min in 1:1,000 dilutions of either preimmune or anti-coat protein sera, or anti-coat protein sera absorbed with an excess of the gel extract of the coat protein. The slices were washed in PBS for 3 h (five to seven changes) and treated for 45–60 min with a 1:1,000 dilution of peroxidase-labeled Fab of purified goat anti-rabbit IgG (13, 14) prepared according to Avrameas and Ternynck (4). The slices were washed again for 3 h in PBS to remove the excess second antiserum and then incubated for 15–20 min at 4°C in 50 ml of PBS containing 30 mg 3,3'-diaminobenzidine (DAB, Sigma Chemical Co.) and 10 μl of 30% hydrogen peroxide. The slices were washed with PBS for 30 min and appropriate areas were dissected into separate pieces before postfixation with 2.0% osmium tetroxide in 0.12 M Millonigs buffer, pH 7.2. These pieces were dehydrated through ethanol and propylene oxide, and flat-embedded in Epon-Araldite. Ultrathin sections, parallel to the cut face originally exposed to the reagents, were examined in a Philips EM 301 electron microscope with or without heavy metal staining.

RESULTS

Each of three rabbits injected produced antisera that gave a positive response by double immunodiffusion (Fig. 1), but the quality of the antisera differed. Rabbit 1 produced antisera that gave a single intense immunoprecipitin band when tested either against the coated vesicle fraction or the extracted coat protein. Rabbit 6 produced antisera that gave a single, but weaker, immunoprecipitin band. Rabbit 5 produced sera that occasionally gave a second opalescent precipitin band with the antiserum and indications of faint precipitin bands with the preimmune serum. This may have been the result of a nonspecific precipitation caused by the presence of SDS and DTT in the system, or it may have been due to the presence of autoantibodies in this particular rabbit. Because the requirements of our immunodiffusion system were not ideal, we characterized our antisera further by immunofixation of polyacrylamide gels. To circumvent possible confusion in the immunocytochemical experiments, we performed all of the experiments reported here with sera from rabbit 1. In preliminary experiments, antisera from rabbits 5 and 6 gave immunocytochemical results indistinguishable from rabbit 1 antiserum, except that the intensity of labeling was less.

The results of immunofixation of polyacrylamide gels (Fig. 1) indicated that our antisera was directed primarily, if not exclusively, against the 180,000-dalton protein predominate in coated vesicle fractions. Similar results were obtained with either of the HRP methods as well as the SPA method. The pattern of staining of the coated vesicle fraction proteins separated on 12.5% gels was a single sharp line at 180,000 mol wt surrounded by a much lighter diffuse staining immediately adjacent to this line (data not shown). The immunofixation staining pattern obtained using 7.5% gels was a broader line at 180,000 mol wt that approximated the width of the Coomassie brilliant blue stain of this band, but was still slightly narrower (Fig. 1).

The immunocytochemical localization of coated vesicle protein at the light microscope level in rat cerebellum is shown in Fig. 2. In the cerebellar cortex the most conspicuous staining is a punctate distribution of reaction product surrounding the Purkinje cell somata (Fig. 2). The Purkinje cell cytoplasm is lightly stained and the cell nucleus is completely unstained. Punctate reaction product is also seen throughout the molecular layer. In the granular cell layer the cerebellar glomeruli are prominently stained. In this region the antibody labeling pattern is in the form of small punctate deposits (Fig. 2). The staining profile of cerebellar cortex is similar regardless of whether or not the tissue is pretreated with Triton X-100 before immunocytochemical labeling, but the detergent-treated slices stain more intensely (Fig. 2). Those sections treated with preimmune serum (data not shown) or anti-coat protein serum which is preadsorbed with coat protein do not reveal specific localization of peroxidase reaction product (Fig. 2).

At the electron microscope level the punctate profiles surrounding Purkinje cell somata are identified as immunolabeled presynaptic terminals (Fig. 3). The labeled profiles of the cerebellar glomeruli are the presynaptic mossy fiber and Golgi II axon terminals; the granule cell dendrites are unlabeled (Fig. 4). Although presynaptic terminals are the primary structures labeled by anti-coat protein antibody, less conspicuous label is also observed on the coat portion of coated vesicles within neuronal cell bodies. This labeling is discrete but readily apparent in Purkinje cell somata (Fig. 3). Many of these labeled

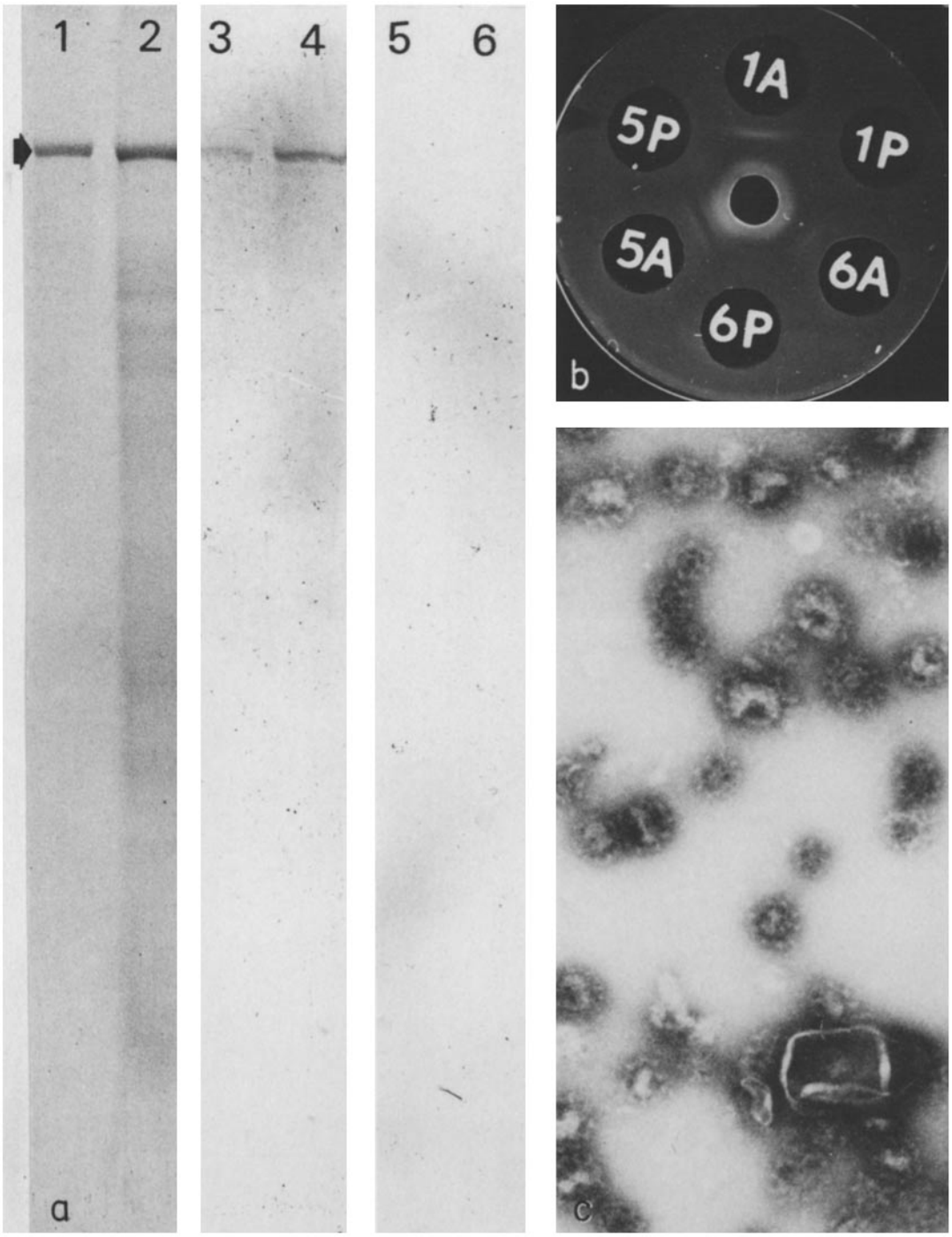

FIGURE 1 Immunofixation (*a*). After electrophoresis the slab gel (7.5% acrylamide) was divided into three sections and stained with Coomassie Blue dye (lanes *1* and *2*) or by an immunoperoxidase method (see text) using either immune serum (lanes *3* and *4*) or preimmune serum (lanes *5* and *6*). The odd-numbered lanes contain the 180,000-mol wt band used as the antigen, and the even-numbered lanes contain all of the proteins in the coated vesicle fraction. The staining in lanes *3* and *4* is the peroxidase-DAB reaction product. Arrow = 180,000 mol wt "coat" protein. Ouchterlony immunodiffusion (*b*). The numbers indicate different animals. The peripheral wells contained 50 μl of preimmune (*P*) or immune (*A*) serum. The central well contained 15 μl of purified coated vesicle fraction in 0.08% SDS-Tris buffer. (*c*) An electron micrograph of the purified coated vesicle fraction negatively stained with 1.0% uranyl acetate. × 93,000.

coated vesicles are in the vicinity of the Golgi apparatus (Fig. 5).

The anti-coat protein label within presynaptic terminals exhibits an interesting distribution. The most intense label is apparent surrounding the coat of coated vesicles (cf. Figs. 4–6). In addition to labeling of the coat, less intense reaction product is distributed throughout the presynaptic cytosol in spaces among coated vesicles, synaptic vesicles, and mitochondria (cf. Fig. 6). Additional staining is apparent on mitochondrial membranes, smooth membrane cisternae within the presynaptic terminal,and along the cytoplasmic face of the presynaptic axolemma (Figs. 4–6). Electron microscopy of those slices

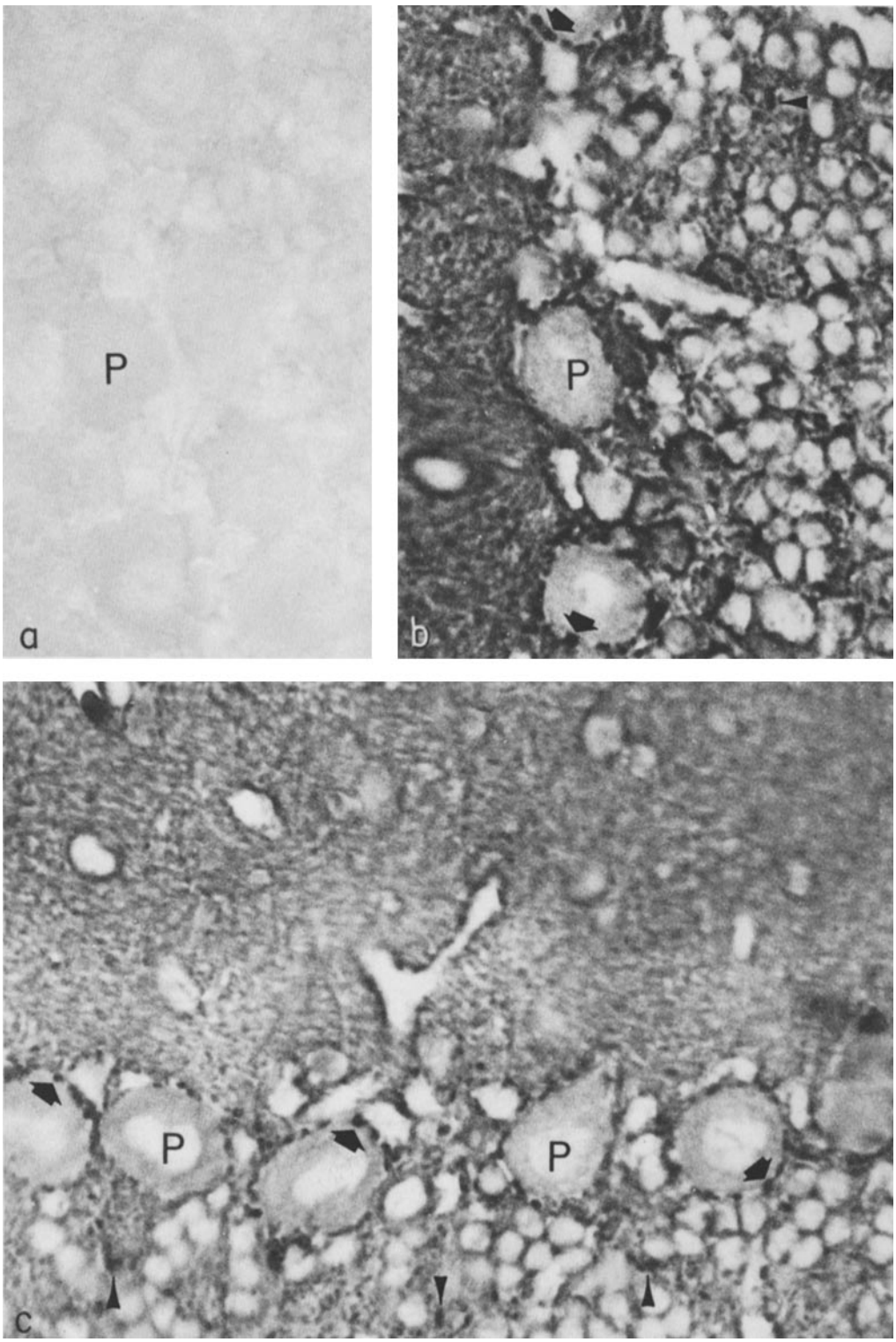

FIGURE 2 Immunohistochemical localization of coated vesicle protein in rat cerebellum in the presence (*b*) or absence (*c*) of 0.1% Triton X-100. Note the punctate label (arrows) distributed around the Purkinje cell somata (*P*) and within the cerebellar glomeruli (arrowheads). Slices treated with immune serum preabsorbed with coated vesicle protein do not show specific reaction product (*a*).

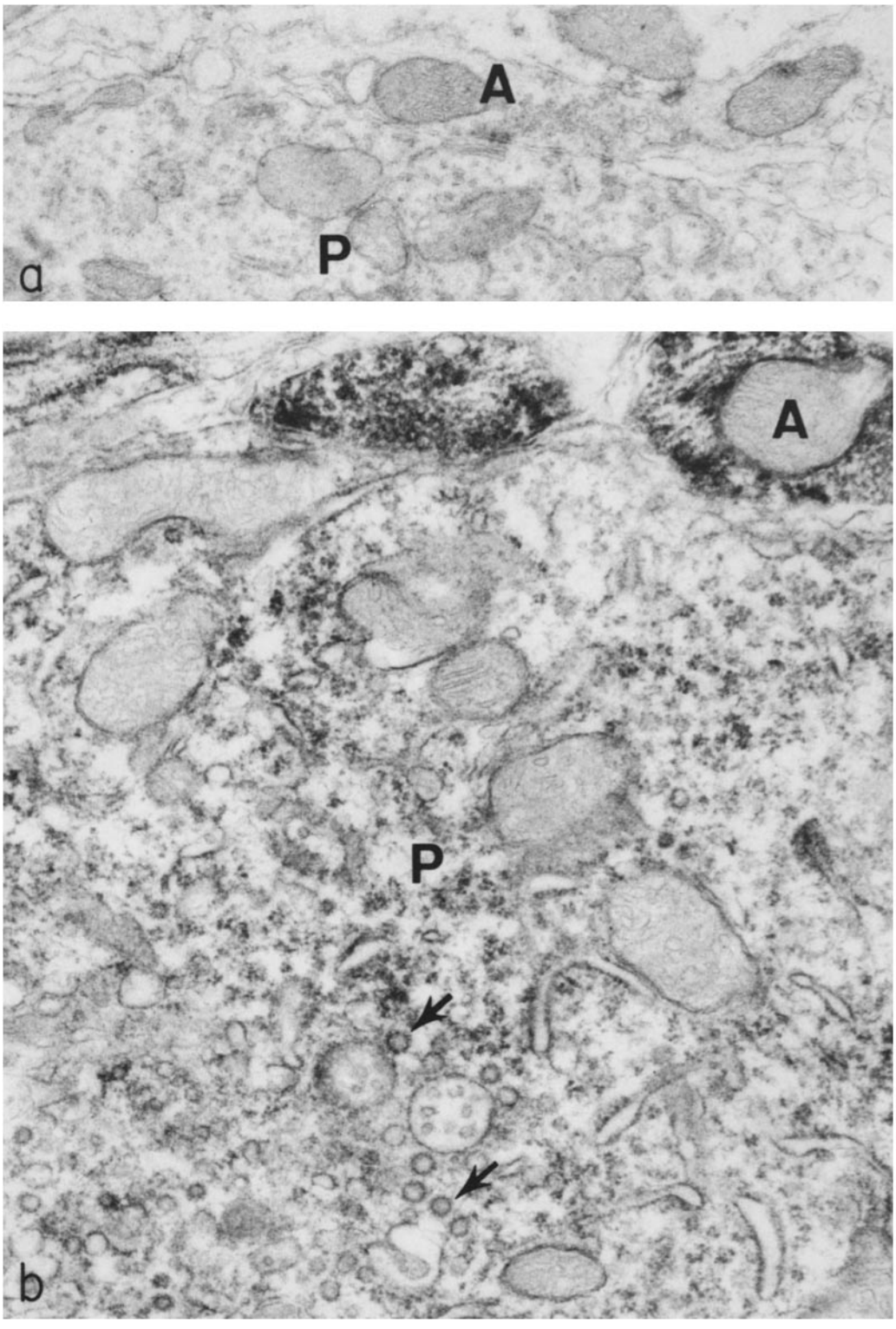

FIGURE 3 Immunocytochemical localization of coated vesicle protein (*b*) in presynaptic terminals (*A*) and Purkinje cell somata (*P*). The presynaptic terminals are heavily labeled. Labeled coated vesicles are seen within the Purkinje cell somata (arrows). Control tissue treated with absorbed immune serum (*a*) is negative. *a*, × 22,500; *b*, × 42,000.

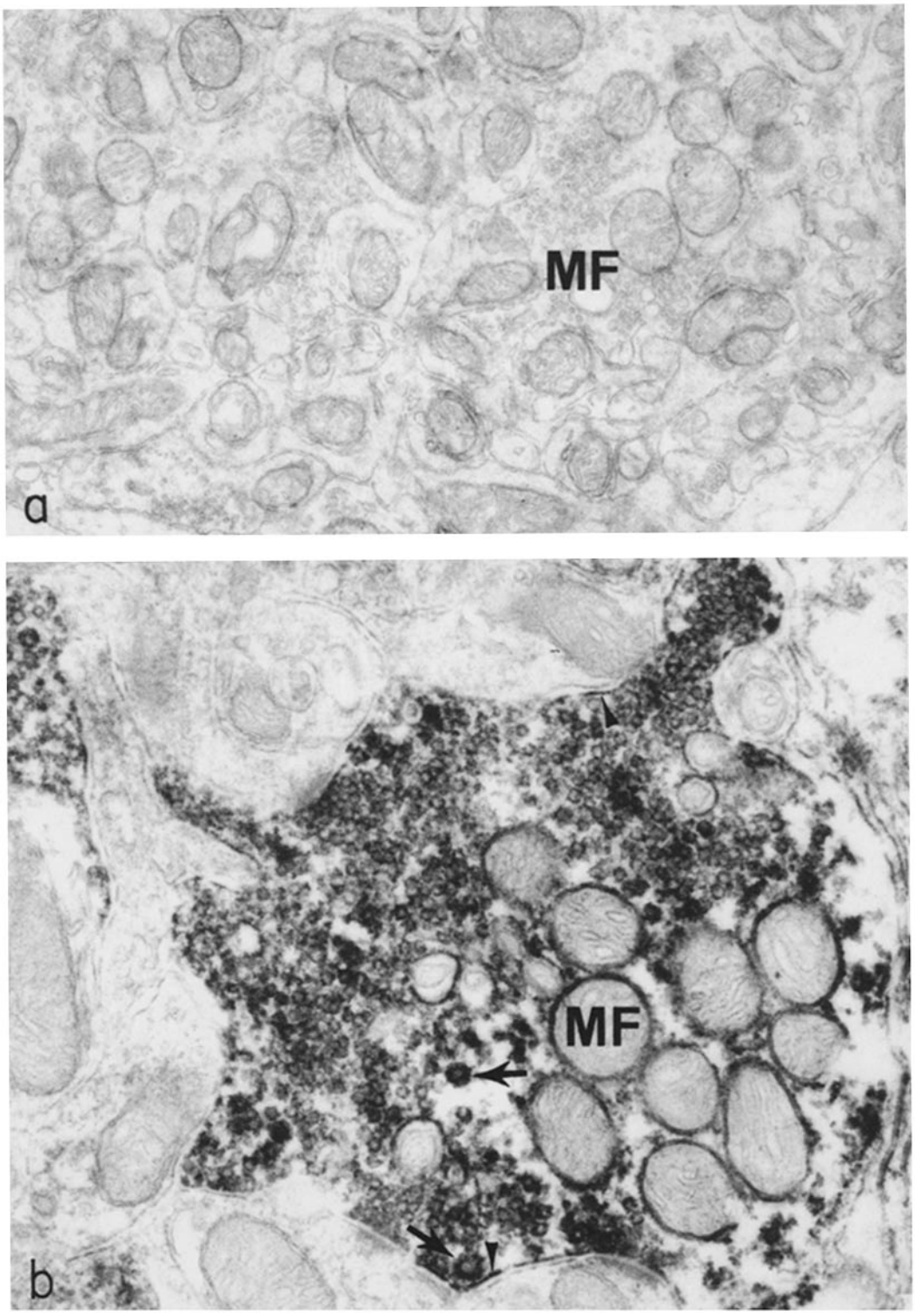

FIGURE 4 Immunocytochemical localization of coated vesicle protein in the cerebellar glomerulus (*b*). The mossy fiber terminal (*MF*) is heavily labeled and the granule cell dendrites are unlabeled. A Golgi II presynaptic terminal is seen in the upper left corner of this micrograph. The coated vesicles (arrows) are heavily labeled but less intense label is seen throughout the presynaptic terminals. In some places the cytoplasmic side of the presynaptic terminal axolemma is labeled (arrowheads). No label is seen in the control tissue treated with absorbed immune serum (*a*). *a*, × 21,300; *b*, × 52,000.

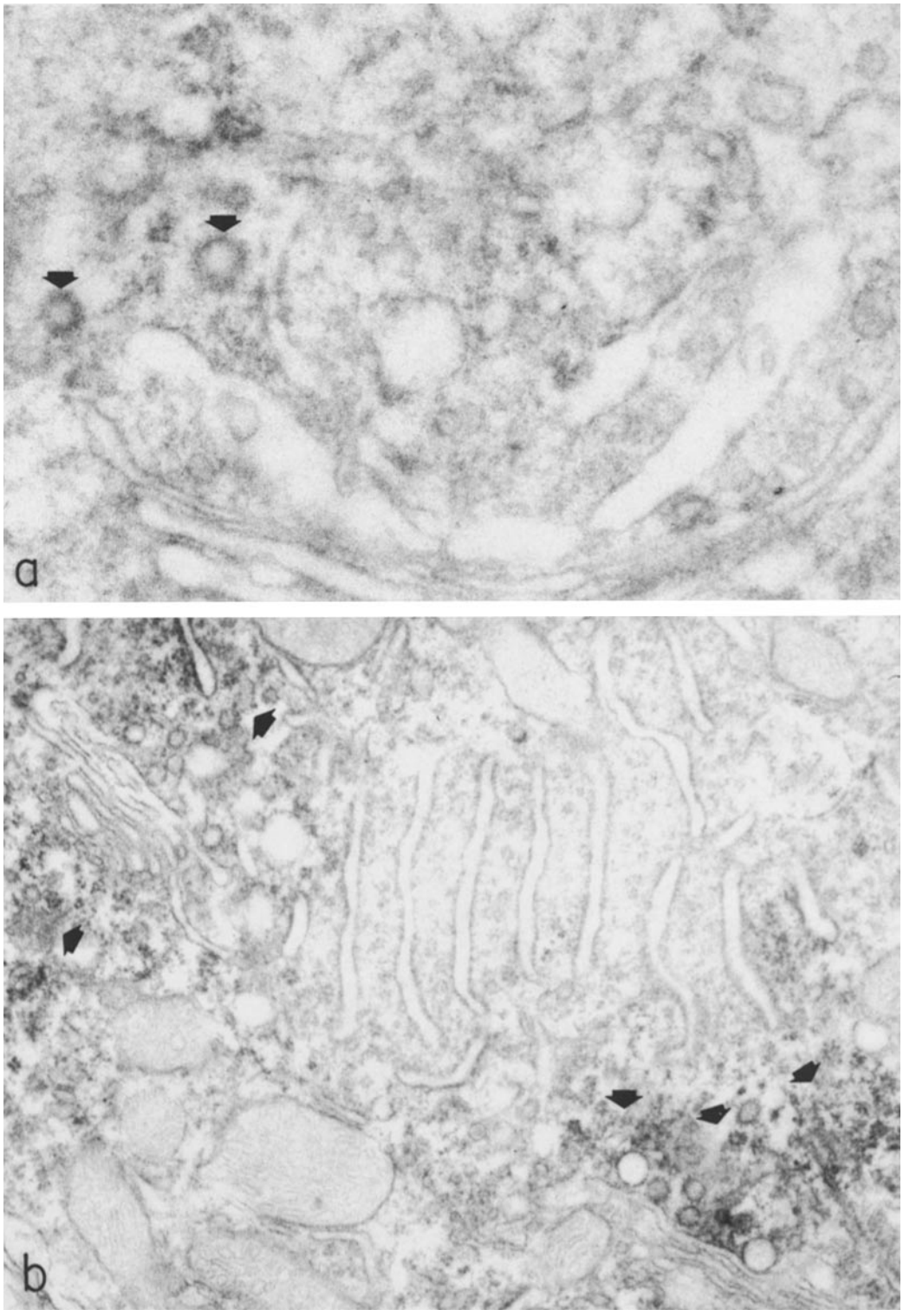

FIGURE 5 Immunocytochemical localization of coated vesicle protein in Purkinje cell somata. The label (arrows) in the Purkinje cell is restricted to regions between stacks of rough endoplasmic reticulum cisternae (*b*) and is seen in the vicinity of the Golgi apparatus (*a*). The cell somata label is either directly on coated vesicles or in the cytosol immeidately around these vesicles. Both panels show tissue unstained with heavy metals. *a*, × 120,000; *b*, × 36,400.

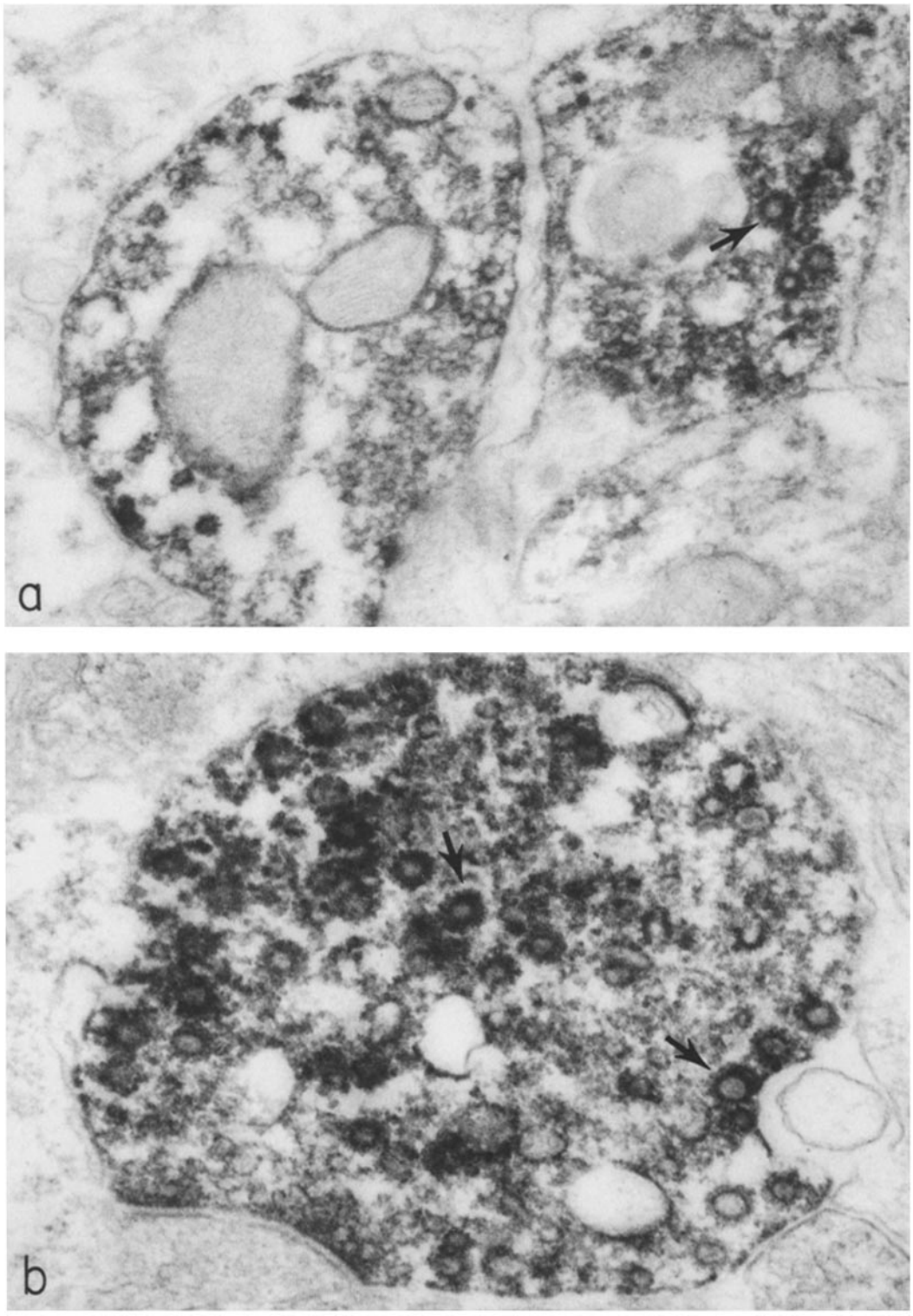

FIGURE 6 Higher magnification examples of coated vesicle protein localization in presynaptic terminals of the cerebellar cortex. The label within presynaptic terminals is prominent around coated vesicles (arrows) but is also seen in the presynaptic axoplasm suggesting the presence of two pools of coated vesicle protein within the presynaptic terminals. *a*, × 42,000; *b*, × 68,000.

treated with preimmune serum (data not shown) or with anti-coat protein antiserum, that had been absorbed with purified coat protein, reveal no specific reaction product for peroxidase (Figs. 3 and 4).

DISCUSSION

Results presented in this communication indicate that extracts of the 180,000-mol wt band obtained upon PAGE of coated vesicles may be used to produce antisera in rabbits to the major coat protein. Other investigators have shown that antisera to the brain coat protein may be obtained using urea extracts of coated vesicle fractions as the antigen (3, 5). Our results of immunodiffusion and, particularly, immunofixation of acrylamide gels indicate that the antiserum only cross-reacts with the 180,000-mol wt protein of coated vesicle fractions. A point of interest regarding the immunofixation data is that the band observed using peroxidase or SPA immunochemistry is not as broad as the Coomassie Blue-stained coat protein band. A similar result is shown in the work of Anderson et al. (3). There could be many reasons for this result including sensitivity of the different staining methods or ease of access of antibody compared to dye stain to the tightly packed protein molecules in the band. The latter would be suggested by our observation that the broadness of the band observed by immunofixation appears to be a function of the percentage of acrylamide in the gel. Because the antisera from both groups react in immunocytochemical tests with the coat of coated vesicles in fibroblasts or brain tissue it seems unlikely that the antisera are directed against a component other than the coat protein, but the results may indicate, in addition to the possibilities mentioned above, that the 180,000-mol wt coat protein is heterogeneous.

Immunocytochemical results at both the light and electron microscope level indicate that the coated vesicle protein is highly concentrated in presynaptic terminals. Within the terminals the antisera heavily labeled the coat of coated vesicles, but, perhaps more interesting, at least one other pool of this protein appears to be distributed throughout the cytosol of the presynaptic terminal. These results suggest that there is a pool of nonlattice form of the protein which may be available for transformation into the lattice form on demand (presumably of increased endocytotic activity). This pool is not distributed throughout the neuron, but rather is restricted to the presumed site of greatest coated vesicle activity, the presynaptic terminal. We consider this another interesting example of a situation whereby a cell can sequester functionally important proteins to the site of greatest need for the cell (22).

The immunocytochemical results also suggest that membrane systems of the presynaptic terminal may contain some antigen sites in common with the 180,000-mol wt coat protein. A major difficulty with this apparent result is that if the nonlattice form coat protein does exist as a cytosolic component, it could easily be precipitated or cross-linked onto these membrane systems during the procedures of fixation and handling of the tissue for immunocytochemistry. In our hands this problem is particularly prominent with soluble antigens leading to potential artefactual staining of mitochondrial membranes (21–23). We are particularly interested in the possibility of a localization of coated vesicle protein lining the cytoplasmic side of the presynaptic axolemma. The immunocytochemical labeling pattern of this structure is not uniform and it may be that coat protein associates with the underside of the membrane only in those regions where formation of a coated vesicle is eminent. To provide more information in this area it will be necessary to separate the various components of the presynaptic terminal and define their chemical, immunochemical, and immunocytochemical features.

Our results indicate a discrete localization of reaction product around coated vesicles in Purkinje cell somata. In addition to labeling the lattice coat, reaction product is apparent in the cytosol in the immediate vicinity of labeled coated vesicles. Although it is difficult to exclude the possibility that the cytosolic label is caused by diffusion of the DAB reaction product (15), this result may indicate that a pool of soluble coat protein exists in the neuronal somata, and the restricted localization may reflect a high demand for conversion of the soluble to the lattice form in regions of the Golgi apparatus where coated vesicles of the somata are most actively formed. It should be noted that, in our hands, short times of DAB incubation at 0°–4°C greatly diminishes the diffusion of reaction product.

The present results allow us to confirm that the 180,000-mol wt protein seen by PAGE of brain-coated vesicle fractions is indeed a "coat" protein of brain-coated vesicles. In addition, this protein is highly concentrated in presynaptic terminals of identified synapses in the cerebellum and appears to exist in at least one nonlattice form in the terminals. The relationship between the coat and nonlattice forms of this immunoreactive material and the biological functions subserved by the protein in these two or other compartments remain undefined.

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