The Phosphorylation of Coated Membrane Proteins in Intact Neurons

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Abstract. To complement studies that have demonstrated the prominent phosphorylation of a 50-kD coated vesicle polypeptide in vitro, we have evaluated the phosphorylation of coated membrane proteins in intact cells. A co-assembly assay has been devised in which extracts of cultured rat sympathetic neurons labeled with [^{32}P]-P_i were combined with unlabeled carrier bovine brain coat proteins and reassembled coat structures were isolated by gradient centrifugation. Two groups of phosphorylated polypeptides, of 100–110 kD (pp100–110) and 155 kD (pp155) apparent molecular mass, were incorporated into reassembled coats. The neuronal pp100–110 are structurally and functionally related to the 100–110-kD component of the bovine brain assembly protein (AP), a protein complex that also contains 50-kD and 16.5-kD components and is characterized by its ability to promote the reassembly of clathrin coat structures under physiological conditions of pH and ionic strength (Zaremba, S. and J. H. Keen, 1983, *J. Cell Biol.*, 97:1337–1348). The neuronal pp155 detected in reassembled coat structures was readily observable in total extracts of [^{32}P]-P_i-labeled neurons dissolved in SDS-containing buffer. A bovine brain counterpart to the neuronal pp155 was also observed when brain coated vesicles were subjected to two-dimensional gel electrophoresis. Phosphoserine was the predominant phosphoaminoacid found in both the pp100 and pp155.

A structural and functional counterpart to the 50-kD brain assembly polypeptide (AP_{50}) was also identified in these neurons. Although the brain AP_{50} is prominently phosphorylated by an endogenous protein kinase in isolated coated vesicle preparations, the neuronal AP_{50} was not detectably phosphorylated in intact cells as assessed by two-dimensional non-equilibrium pH gradient gel electrophoresis of labeled cells dissolved directly in SDS-containing buffers. These results demonstrate that the bovine brain assembly polypeptides of 50 kD and 100–110 kD that we have previously described, as well as a novel 155-kD polypeptide reported here, have structural and functional counterparts in cultured neurons. They also indicate that phosphorylation of the 100–110-kD AP may be involved in the regulation of coated membrane structure and function. The extent of phosphorylation of the AP_{50} in intact cells and in isolated coated vesicles is strikingly different: it has been suggested that the latter process reflects an autophosphorylation reaction (Campbell C., J. Squicciarini, M. Shia, P. F. Pilch, and R. E. Fine, 1984, *Biochemistry*, 23:4420–4426). While other possibilities exist, we speculate that in intact neurons the AP_{50} does not undergo substantial autophosphorylation but rather is active as a protein kinase with other, as yet unidentified, cellular substrates.

CONSIDERABLE evidence has been accumulated to delineate the role that coated membranes play in the processes of receptor-mediated endocytosis, secretory and membrane protein biosynthesis, and membrane recycling in eukaryotic cells (reviewed in references 6, 21, 24). The involvement of clathrin-coated membranes in these processes is well established by morphological criteria; e.g., as coated pits at the plasma membrane or in the Golgi region of cells. However, the molecular interactions that form the basis of these functions, for example in the clustering of receptors and in the formation and intracellular targeting of transfer vesicles (endocytotic and exocytotic), remain unknown. Studies over the past 10 years have defined in considerable detail the polypeptide components of coated membranes (reviewed in references 12 and 18), and attention has more recently been directed toward a description of the factors that regulate coat size, formation, and location within the cell (30, 33), and the interaction of coat components with membranes (32) and with other cellular constituents (11, 29).

In this context, we have previously identified a protein complex derived from coated vesicles (CVs),[1] designated as-

[1] *Abbreviations used in this paper:* AP, assembly protein; CV, coated vesicle; Met, methionine; NEPHGE, non-equilibrium pH gradient electrophoresis; 1-D, one-dimensional SDS; 2-D, two-dimensional isoelectric focusing SDS.

sembly protein (AP), that is essential for the reassembly of clathrin coat structures under physiological conditions of pH and ionic strength (33). AP consists of several polypeptides of 100–110 kD, 50 kD, and 16.5 kD that are stoichiometrically incorporated into reassembled coats. A 50-kD CV polypeptide can be readily phosphorylated by an endogenous CV protein kinase (26) and this phosphorylation has been reported to be increased by the presence of clathrin light chains (25). We have found that this protein corresponds to the AP_{50} and that it comprises one domain within the AP complex, distinct from an assembly-promoting region which is associated with the 100–110-kD components (34). These observations suggest the possibility that phosphorylation is a physiological regulator of coated membrane functions and that a description of phosphorylation events in intact cells is likely to provide clues to their in vivo role.

To study the phosphorylation of coated membrane–associated proteins in intact cells, we have devised a co-assembly assay in which extracts derived from in situ labeled cultured neurons are mixed with unlabeled carrier bovine brain clathrin and AP, and reassembled coats are isolated and characterized. The specificity of the reassembly process is striking: of the entire complement of cellular phosphoproteins, two species are prominently and consistently incorporated into coat structures. These include a set of polypeptides that correspond to the 100–110-kD complex of brain AP and a novel 155-kD CV protein that has not been previously described. A structural and functional counterpart of the AP_{50} has also been identified in these neurons. However, in contrast to results obtained with broken cell preparations, the latter protein is not substantially phosphorylated in intact cells.

Materials and Methods

Materials

Bovine brain CV's, clathrin, and AP preparations were obtained as described previously (33). Reagents for electrophoresis and isoelectric focusing were from Bio-Rad Laboratories, Richmond, CA, [^{32}P]-P_i and gamma [^{32}P]-ATP were products of ICN K&K Laboratories Inc., Plainview, NY, and [^{35}S]-L-methionine (Met) was from Amersham Corp., Arlington Heights, IL. Trayslol (aprotinin), antipain, chymostatin, and leupeptin were obtained from Sigma Chemical Co., St. Louis, MO. Plastic- (100 μm thickness) and glass-backed (250 μm thickness) cellulose plates were obtained from Brinkmann Instruments Co., Westbury, NY, and Analtech, Inc., Newark, DE, respectively.

Cell Culture and Radiolabeling

Dissociated cultures of rat sympathetic neurons were prepared and maintained in culture on 35-mm collagen-coated tissue culture dishes as described previously (28). Cells were labeled with [^{35}S]-L-Met (>800 Ci/mmol, 100 μCi/ml) for 20–24 h in medium containing 5% of the normal L-Met content. For radiolabeling of phosphoproteins, cells were incubated in phosphate-free medium that contained 3% dialyzed human placental serum supplemented with [^{32}P]-P_i (1.0 mCi/ml) for 90–120 min. Qualitatively similar results to those presented in Fig. 1 were obtained when cells were incubated with [^{32}P]-P_i for 8 h instead of 1.5 h; in all experiments reported here, the briefer incubation time was used.

In Vitro Phosphorylation

In experiments for detection of the neuronal pp50 by non-equilibrium pH gradient electrophoresis (NEPHGE), one dish of neurons was washed twice with PBS at 0°C and solubilized with 0.2 ml of 0.1 M NaMES, 1 mM EGTA, 0.5 mM $MgCl_2$, 0.02% sodium azide, pH 6.5 (buffer A) containing 0.5% Triton X-100 and a cocktail of protease inhibitors including 0.5% Trayslol and 10 μg/ml each of leupeptin, antipain, and chymostatin. After homogenization, [^{32}P]-ATP was added (3,500 Ci/mmol, 0.49 mCi/ml final concentration), the sample was incubated for 30 min at 0°C, and the reaction was terminated by addition of ice-cold methanol in preparation for NEPHGE.

For detection of the presence of a reassembly-competent form of the neuronal pp50, a 35-mm dish of rat sympathetic neurons was subjected to hypotonic lysis in 1 mM Tris-HCl, pH 7.4, containing the protease inhibitors noted above. The homogenate was adjusted to 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM NaF, 0.5% Triton X-100, and 10 mM NaMES, pH 6.5, [^{32}P]-ATP was added (3,500 Ci/mol, 0.09 mCi/ml final concentration), and the reaction was allowed to proceed for 20 min at 20°C. Tris-HCl, EDTA, and EGTA were added to terminate the reaction and to extract coat proteins, and the incorporation of radiolabeled pp50 into reassembled coat structures was evaluated as described below.

Co-assembly Assay

After labeling, neuronal cultures were placed on a precooled block and all steps were performed at 0–4°C. Cultures were washed twice with 10 mM Tris-HCl, 20 mM EDTA, 20 mM EGTA, pH 7.4, drained completely, and lysed with two 100-μl aliquots of 0.5 M Tris-HCl, 0.5% Triton X-100, 20 mM EDTA, 20 mM EGTA, 5 mM NaF, 0.02% sodium azide, pH 7.0, containing the protease inhibitors noted above. After homogenization in a Duall glass homogenizer (Kontes Co., Vineland, NJ), the suspension was centrifuged at 70,000 *g* for 30 min and an aliquot of the extract was combined with carrier bovine brain clathrin (60 μg) and AP (40 μg) in a total volume of 0.4–0.7 ml. The ratios of bovine brain clathrin and AP used were chosen to favor complete incorporation of neuronal APs into the reassembled coat structures (33). Assembly was induced by dialysis of this solution in a collodion bag (Schleicher & Schuell, Inc., Keene, NH; UH 100/25) against 0.1 M NaMES, 20 mM EGTA, 5 mM NaF, 0.02% sodium azide, pH 6.5 for 2 h followed by dialysis against buffer A overnight. Reassembled coat structures (250S) were separated from unpolymerized material by ultracentrifugation on a 5–20% glycerol gradient in buffer A in a Beckman SW27.1 rotor at 27,000 rpm for 135 min at 4°C. The gradient was fractionated into 1-ml aliquots as previously described (33).

We ascertained that the phosphorylation events being studied were indeed occurring in intact cells and did not reflect post-homogenization modifications of neuronal proteins or of the bovine brain proteins added subsequently as carrier. Neurons were lysed in a solution containing 20 mM EDTA, 20 mM EGTA, and 0.5 M Tris-HCl supplemented with exogenous [^{32}P]-ATP to yield a specific radioactivity of ~40 Ci/mmol, similar to that estimated to exist in the homogenates used in these studies (8, 9, 19), and subjected to the co-assembly assay described above. No post-homogenization labeling of the AP_{50} was detectable when preflashed films (XAR-5) were exposed for 6 d; a slight band was detectable after a 42-d exposure under the same conditions. In contrast, all films presented in this report were exposed for 5 d or less and were not preflashed. However if the lysis buffer used contained only 10 mM EDTA, a prominently phosphorylated pp50 band was observed, more substantially labeled than all other proteins (also see pages 1330 and 1331).

Electrophoretic Procedures

Unless otherwise noted, in situ labeled cells were dissolved directly in urea–SDS-containing buffers (28) while protein solutions were precipitated with 5–10 vol of ice-cold methanol. One-dimensional electrophoresis in SDS-containing gels was performed as described previously (33) using 3% stacking gels and resolving gels as indicated. NEPHGE (23) or equilibrium two-dimensional isoelectric focusing (2-D) gel electrophoresis (2) were performed according to published procedures. Radiolabeled species were detected by autoradiography with either XAR-5, RP, or MinR X-ray film (Eastman Kodak Co., Rochester, NY) as described. Cronex-Lightning Plus (DuPont Co., Wilmington, DE) intensifying screens and exposure at −70°C were used unless otherwise noted.

Other Procedures

Gel Filtration. Gradient fractions containing reassembled coat structures were pooled and combined with carrier bovine brain clathrin and AP, precipitated with a 50% saturated solution of ammonium sulfate, resuspended in a minimal volume of 1.0 M Tris-HCl:buffer A (1:1, vol/vol) and applied to a 0.9 × 52-cm column containing Sepharose CL-4B (Pharmacia Fine Chemicals, Piscataway, NJ) equilibrated and eluted with the same buffer. Fractions of 0.65 ml were collected by downward elution.

Phosphoaminoacid Determination. Bands to be analyzed were eluted from dried 1-D gels as described by Beemon and Hunter (1). The eluates were hydrolyzed in 5.7 N HCl for 105 min at 110°C and phosphoaminoacids were separated by two-dimensional high voltage electrophoresis using pH 1.9 and 3.5 buffers essentially as described by Cooper et al. (5).

One-dimensional limited proteolysis was performed as described by Cleveland et al. (4).

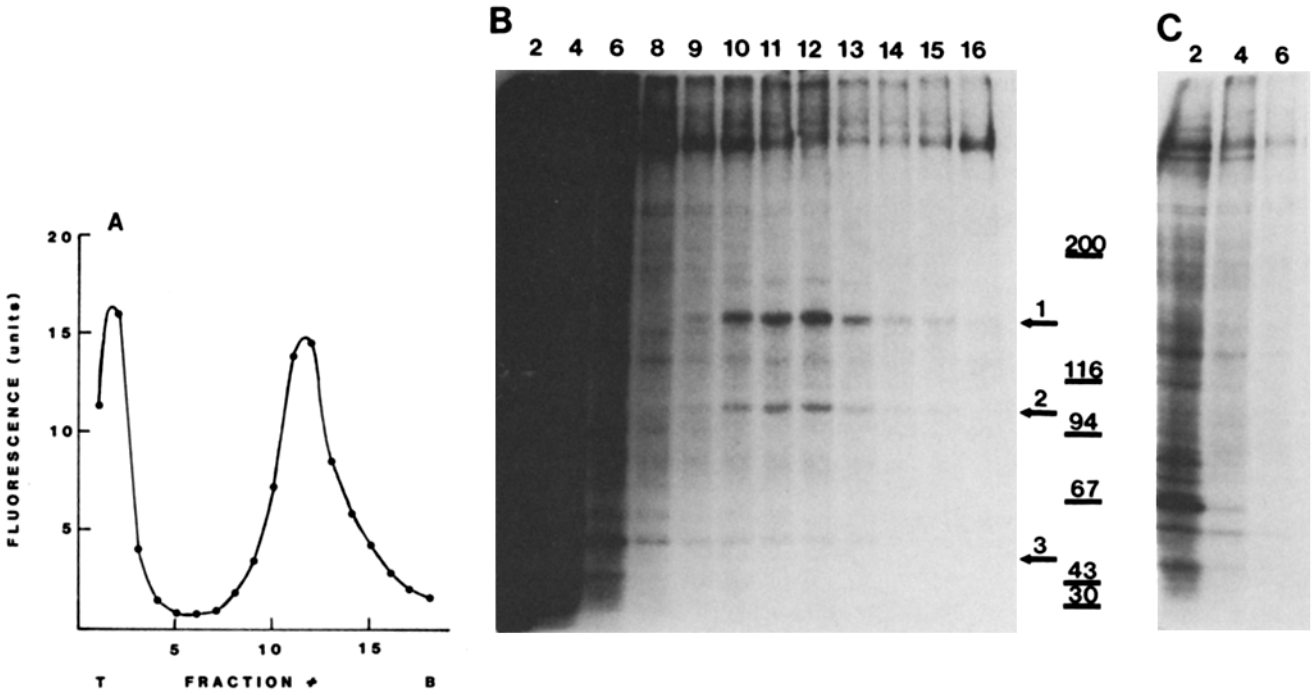

Figure 1. Rat neuronal phosphoproteins are incorporated into bovine brain coat structures. (*A*) Separation of reassembled coats (fractions 10–13) from nonsedimentable material by glycerol gradient centrifugation. The dialyzed reassembly mixture was applied to 5–20% glycerol gradients, centrifuged, and fractionated as described in Materials and Methods. Protein was quantitated in a parallel gradient by fluorescence spectroscopy. (*B*) Gel electrophoresis and autoradiography of indicated glycerol gradient fractions (4–8% gradient resolving gel, 60-h exposure with XAR-5 film). Molecular mass standards ($\times 10^{-3}$) are indicated. (*C*) Autoradiograph of gradient fractions 2, 4, and 6 (RP film, 4-d exposure).

Results

Incorporation of Neuronal Phosphoproteins into Reassembled Coat Structures

Dissociated cultures of pure rat sympathetic neurons were labeled with [^{32}P]-P_i as described in Materials and Methods and then lysed with a solution containing 0.5% Triton X-100 and 0.5 M Tris-HCl in the presence of chelators to block phosphorylation in the broken cell suspension. Under these solution conditions, most coated vesicle components are released in soluble form and the liberated clathrin and AP retain the ability to reassemble into clathrin coat structures on removal of the Tris-HCl (14, 15).

A high speed extract of the cell lysate was prepared and combined with carrier bovine brain clathrin and AP. Coat assembly was then induced by dialysis against buffer A. Reassembled clathrin coat structures ($S_{20,w}$ ~ 250S) were subsequently separated from unpolymerized clathrin and AP (both with $S_{20,w}$ ~ 8S) as well as other nonsedimentable cellular proteins by glycerol gradient centrifugation (Fig. 1 *A*). Analysis of gradient fractions by one-dimensional SDS (1-D) gel electrophoresis indicated that the vast majority of the cellular phosphoproteins remained nonsedimentable. However, two major bands of ~155 kD and 100 kD apparent molecular mass (Fig. 1 *B*, arrows 1 and 2, respectively) consistently cosedimented with authentic clathrin coat structures (compare Fig. 1 *A* and 1 *B*). The appearance of these bands was dependent on coat formation since omission of carrier clathrin from the reassembly mixture blocked their appearance. On briefer autoradiographic exposure of the upper part of the gradient (Fig. 1 *C*), it was also apparent that these two bands did not represent major components of the radiolabeled extract that were nonspecifically incorporated into 250S coats. Several more minor bands, e.g. those of 225, 180, 125, and 54 kD, were also observed but their characterization has not been pursued at present.

We did not detect a phosphorylated polypeptide that co-migrated with authentic bovine brain AP_{50} (Fig. 1 *B*, arrow 3), a protein that has been shown to be prominently phosphorylated in isolated coated vesicle preparations from brain (13, 26, 29). Our studies of the nature of the coat-associated neuronal 155-kD and 100-kD phosphoproteins, and the apparent absence of a phosphorylated 50-kD polypeptide, are described below.

pp100–110

One of the major neuronal phosphoproteins incorporated into reassembled coat structures (Fig. 1 *B*, arrow 2) co-migrated on 1-D gels with a band that we have previously described as part of the 100–110-kD component of bovine brain AP. We have shown that after extraction of coated vesicles and gel filtration, bands of ~100–110-kD, 50 kD, and 16.5 kD, comprising this putative AP complex, elute together and are well resolved from clathrin (15, 33). The polypeptide composition of the 100–110-kD brain AP fraction is complex: on suitable gels, the 100-kD broad band can be resolved into at least four discrete polypeptides of 98–106 kD, while the 110-kD band can be resolved into two bands of ~110–112 kD (Fig. 2 *a*; also see references 14 and 27).

To more precisely define the relationship of the radiolabeled neuronal phosphoprotein(s) to the bovine brain polypeptides, the reassembled coats were dissociated by the addition of 0.5

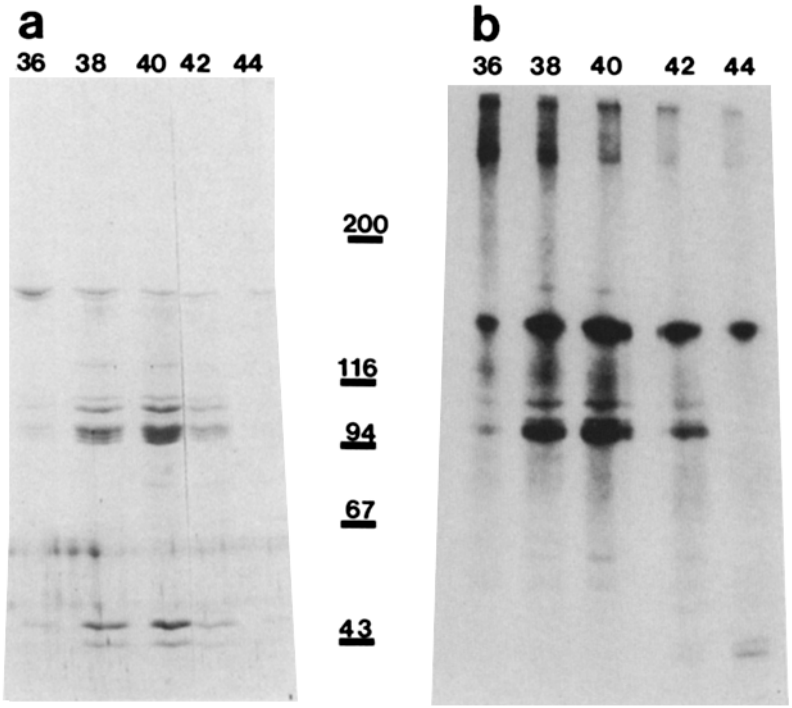

Figure 2. Gel filtration of dissociated coat structures after gradient centrifugation. Reassembled coat structures in gradient fractions 10–13 (Fig. 1 *A*) were dissociated and applied to a CL-4B gel filtration column. Indicated gel filtration fractions were subjected to gel electrophoresis (4–8% gradient resolving gels) and (*a*) Coomassie Blue staining or (*b*) autoradiography (XAR-5 film, 5-d exposure).

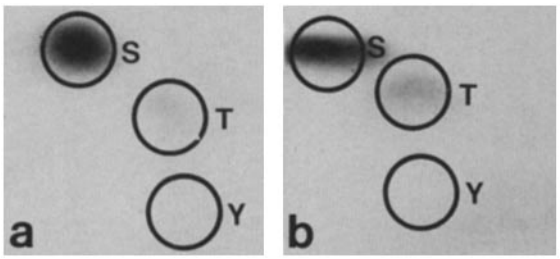

Figure 3. Phosphoaminoacid analysis of the rat neuronal phosphoproteins by two-dimensional thin-layer electrophoresis and autoradiography. *S*, *T*, and *Y* indicate the position of phosphoserine, phosphothreonine, and phosphotyrosine markers, respectively. (*a*) The 100-kD neuronal polypeptide; (*b*) the 155-kD neuronal polypeptide.

M Tris-HCl and then fractionated by Sepharose CL-4B gel filtration. Analysis of these fractions by 1-D gels demonstrated that the neuronal phosphoprotein did co-elute with the carrier cow brain AP (Fig. 2 *b*). Furthermore, on lighter exposure (data not shown) the 100-kD neuronal phosphoprotein could be resolved into a major doublet of ~102–104-kD, a minor band at 105 kD, and a doublet at 112 kD. All of these bands co-migrated exactly with dye-stained bands present in the brain AP preparation. However, radiolabeled (neuron-derived) bands co-migrating with major brain polypeptides at 98–100 kD and at 110 kD were not detectable.

The phosphoaminoacid(s) present in the 100-kD neuronal protein were determined by excising the 100-kD band denoted by arrow 2 in Fig. 1 *b*. Phosphoserine was the predominant phosphoaminoacid found; very little phosphothreonine was observed and phosphotyrosine was undetectable (Fig. 3 *a*).

pp155

The major neuronal phosphoprotein observed in reassembled coat structures (Fig. 1 *b*, arrow 1) had an apparent molecular mass of ~155 kD (pp155). On 2-D gel electrophoresis (Fig. 4 *a*) the pp155 focused as a discrete spot with a relatively acidic pI (~5.1), similar to that of bovine clathrin light chains (see Fig. 4 *d*) (16). Labeled pp155 could be readily detected in 2-D gels of SDS extracts of P_i-labeled cultured neurons (Fig. 4 *c*), thus unambiguously demonstrating that this protein is phosphorylated in situ. Analysis of SDS extracts of [^{35}S]-L-Met–labeled neurons also demonstrated that the pp155 was a prominent biosynthetic product of these cells (Fig. 4 *b*). The pp100–110 were not resolved by this method since they, like the clathrin heavy chain and the AP_{50}, do not focus as discrete spots in 2-D gels (16, 29).

Extraction of the pp155 from 1-D gels followed by acid hydrolysis confirmed the peptide-bound nature of the phosphate label. The phosphoaminoacid composition of this protein was similar to that described above for the pp100: phosphoserine was the predominant phosphoaminoacid found; a slight amount of phosphothreonine was also present but phosphotyrosine was undetectable (Fig. 3 *b*).

Under the extraction conditions used for the co-assembly assay, the vast majority of the pp155 was found in the soluble fraction (data not shown). The solubilized rat neuronal pp155 that had co-assembled with brain clathrin in vitro could be released from the reassembled coats by treatment with 0.5 M Tris-HCl. These properties are similar to those of the brain AP that we have previously described. On subsequent gel filtration the solubilized pp155 also appeared in the AP-containing fractions (Fig. 2 *b*). However, its elution profile did not entirely parallel those of the carrier brain AP and rat neuronal pp100–110. These data indicate that although their molecular dimensions are similar, the pp155 is not stably associated with the neuronal pp100–110 under these conditions.

The rat neuronal pp155 co-migrated on 2-D gels with a polypeptide present in bovine brain–coated vesicle preparations (Fig. 4 *d*). This brain 155-kD polypeptide behaved similarly to the neuronal pp155 in that it was extractable from coat structures with 0.5 M Tris-HCl and on gel filtration was

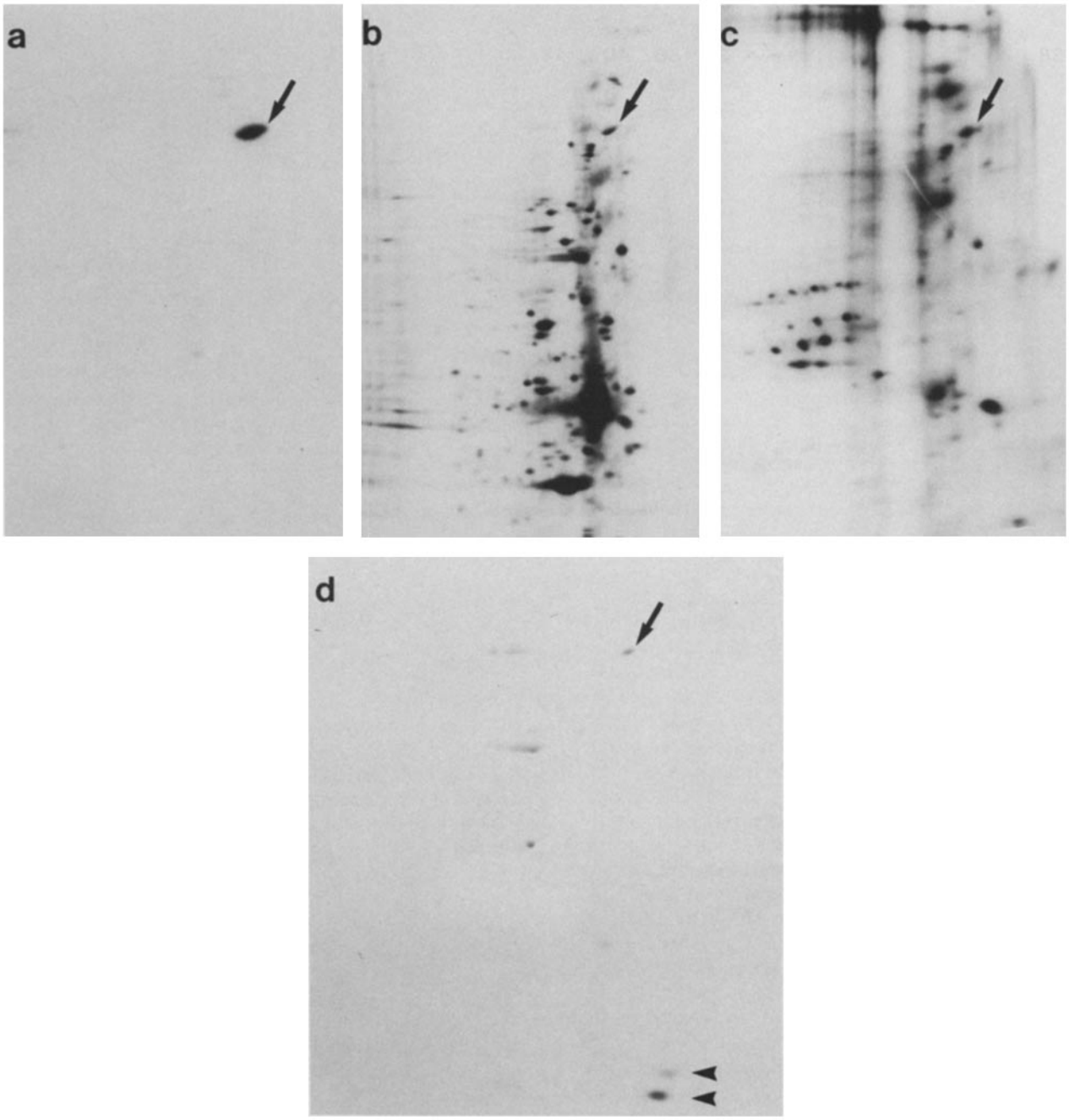

Figure 4. Analysis of the 155-kD coated vesicle protein (arrows) by 2-D gel electrophoresis followed by autoradiography (*a–c*) or Coomassie Blue staining (*d*). (*a*) Autoradiograph of reassembled 250S coat structures (gradient fraction 12, Fig. 1 *a*) prepared from radiolabeled rat neurons and purified bovine clathrin and AP (XAR-5 film, 18-h exposure). (*b*) Autoradiograph of SDS extract of [^{35}S]-L-Met–labeled rat sympathetic neurons (XAR-5 film, 18-h exposure). (*c*) Autoradiograph of SDS extract of [^{32}P]-P_i-labeled rat sympathetic neurons (MinR film, 4-d exposure). (*d*) Bovine brain clathrin (100 μg) and AP (100 μg). The positions of the clathrin light chains are indicated by the arrowheads; the clathrin heavy chain fails to focus in this system.

found in AP-containing fractions (data not shown). Further characterization of the brain polypeptide is in progress and will be reported elsewhere.

pp50

The observation that a 50-kD polypeptide was the predominant phosphorylated product formed when isolated coated vesicle preparations were incubated with radioactive ATP (26) and our finding that this polypeptide was identical to the AP_{50} and could reassemble into coat structures in vitro (13)[2] provided the initial impetus for our asking whether this modification occurred and could be studied in an intact cell system. Thus, it was unexpected that an in situ phosphorylated 50-kD polypeptide was not a conspicuous component of reassembled coats (Fig. 1 *b*, arrow 3) and could not be detected even after purification of an AP fraction by gel filtration (Fig. 2 *b*).

[2] Keen, J. H., K. A. Beck, and M. M. Black, manuscript in preparation.

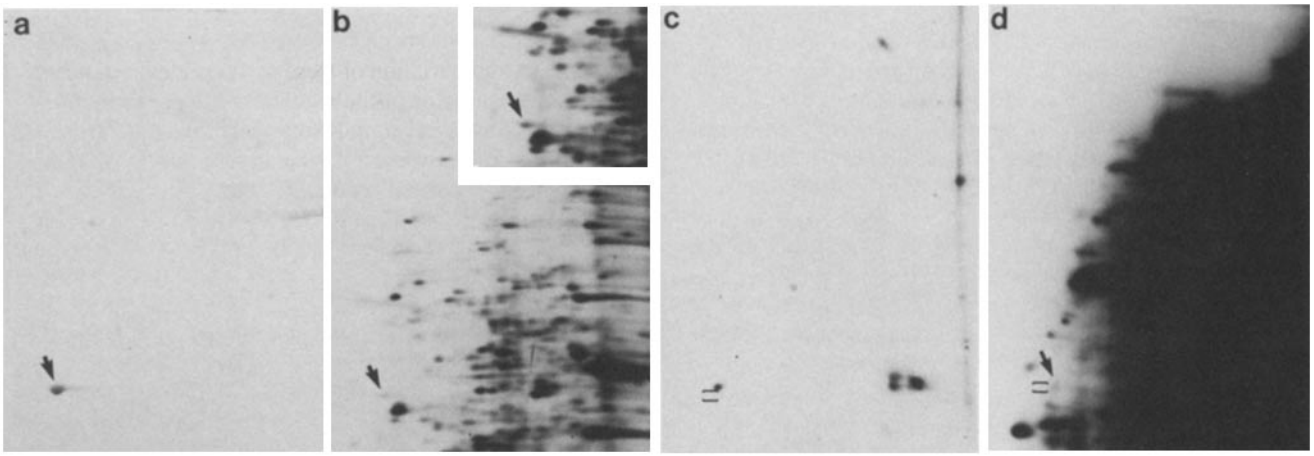

Figure 5. Two-dimensional gel electrophoretic analysis of the AP_{50} using NEPHGE followed by Coomassie Blue staining (*a*) or autoradiography (*b–d*). The position of the stained bovine brain AP_{50} is indicated by the arrows in *a* and *b* and by the horizontal brackets in *c* and *d*. (*a*) Bovine brain AP (100 μg). (*b*) SDS extract of [^{35}S]-L-Met–labeled rat sympathetic neurons (XAR-5 film 18-h exposure without intensifying screen). (*Inset*) Longer exposure (XAR-5 film, 4-d) of lower left region of the gel. (*c*) In vitro labeled rat neuronal pp50 prepared by incubating a 1.0% Triton X-100 lysate of rat sympathetic neurons with [^{32}P]-ATP (XAR-5 film, 18-h exposure). (*d*) SDS extract of [^{32}P]-P_i-labeled rat sympathetic neurons (XAR-5 film, 4-d exposure). A spot that could represent the neuronal pp50 is indicated by the arrow.

This observation could reflect the absence of a phosphorylated rat neuronal counterpart to the brain AP_{50}, or the presence of one which is incapable of co-assembling with cow brain proteins into coat structures. To evaluate these possibilities, we took advantage of the finding that the brain AP_{50} reproducibly migrated as a distinct and strongly alkaline spot (Fig. 5*a*) in 2-D NEPHGE. The results of our experiments using this technique indicate that sympathetic neurons do indeed contain a protein that corresponds to the brain AP_{50}.

First, when SDS extracts of [^{35}S]-L-Met–labeled sympathetic neurons were analyzed on 2-D NEPHGE gels, a labeled spot that co-migrated exactly with brain AP_{50} was observed (Fig. 5*b*). Second, on in vitro incubation of neuronal extracts with [^{32}P]-ATP, a 50-kD neuronal protein was labeled that co-migrated with phosphorylated brain AP_{50} in both 1-D gels (not shown) and in 2-D NEPHGE gels (Fig. 5*c*). The position of both the neuronal pp50 and the brain pp50 (the latter prepared by incubating brain coated vesicles with [^{32}P]ATP; data not shown) was shifted slightly in the acidic direction and to a slightly larger apparent molecular mass, relative to the unphosphorylated carrier AP_{50} included in the gels, consistent with the effects of phosphorylation on the electrophoretic behavior of proteins (22, 31). Third, when these in vitro labeled pp50 bands from bovine brain CVs and rat neurons were excised from NEPHGE gels and subjected to partial proteolysis (4), identical 1-D polypeptide cleavage maps were obtained (data not shown).

If neuronal homogenates that had been incubated with [^{32}P]-ATP were used in the co-assembly assay, a radiolabeled rat neuronal pp50 was incorporated into reassembled coats, indicating that the in vitro generated pp50 is assembly competent (Fig. 6). Under these conditions of cell-free phosphorylation the pp50, rather than a pp155 or pp100–110, was the major coat-associated phosphopeptide observed, consistent with previous studies (13, 26).[2] Finally, when reassembled coats prepared using [^{35}S]-L-Met–labeled cell extracts were analyzed by NEPHGE, a spot that co-migrated exactly with the brain AP_{50} was observed (data not shown). Taken together, these results indicate that rat sympathetic neurons contain a protein that is both structurally and functionally homologous, if not identical, to the phosphorylated brain AP_{50}.

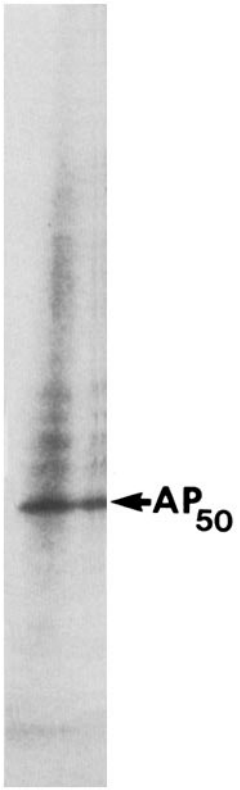

Figure 6. A rat neuronal pp50 is incorporated into reassembled bovine brain coats. A homogenate of rat sympathetic neurons was incubated with [^{32}P]-ATP, centrifuged, and the extract combined with carrier bovine brain clathrin and AP. Reassembled coats, prepared and isolated as in Fig. 1, were analyzed by 1-D gel electrophoresis (5–12% gradient resolving gel) and autoradiography (XAR-5 film, 6-d exposure) with carrier bovine brain AP_{50} added as a marker (arrow).

Although the neuronal AP_{50} could be incorporated into reassembled coats and was readily phosphorylated in broken cell preparations (also see *Co-assembly Assay* in Materials and Methods), we could obtain little evidence of its phosphorylation in intact cells. This was not due to a failure to solubilize the neuronal AP_{50} under our experimental conditions: examination by NEPHGE of the high speed pellet derived from homogenates of [^{32}P]-P_i-labeled cells failed to reveal an insoluble form of the phosphorylated protein (data

not shown). Total SDS extracts of [^{32}P]-P_i-labeled neurons were analyzed directly by NEPHGE to attempt to determine if a phosphorylated form of the neuronal AP_{50} existed in intact cells (Fig. 5 *d*). On lengthy exposure, a possible candidate for a neuronal pp50 is discernible, indicated by the arrow in Fig. 5 *d*, although it is not in exactly the position predicted for a phosphorylated AP_{50} (compare Fig. 5, *c* and *d*). The weak signal and high background in the gel of in situ labeled neurons, combined with the uncertainty introduced by the electrophoretic shift anticipated for the phosphorylated protein relative to the brain standard, precluded a definitive identification of this spot as the phosphorylated form of the neuronal pp50 at this time. In any case, it is clear that the vast majority of the neuronal AP_{50} was not phosphorylated in intact cells, in contrast to results observed with broken cell preparations.

Discussion

Protein phosphorylation is a posttranslational modification that has been shown to regulate numerous cellular functions including enzymatic and binding activities as well as macromolecular associations between structural proteins (7, 10, 17). It was therefore of considerable interest that a 50-kD CV protein was shown to be the predominant in vitro target of phosphorylation by an endogenous protein kinase in preparations of brain (26) or liver (3) CVs. We have subsequently identified this polypeptide as the 50-kD component, designated AP_{50}, of an assembly protein (AP) complex present in preparations of bovine brain CVs (13).[2] The AP is characterized by its ability to promote the in vitro assembly of clathrin into coat structures under physiological conditions of ionic strength and pH (33). These observations prompted us to ask to what extent the striking in vitro phosphorylation of the AP_{50} reflected events that could be shown to occur in intact cells.

To approach this problem, we have developed a co-assembly assay to identify coated membrane-associated proteins of cultured neurons that are phosphorylated in situ. The selectivity of the reassembly process is striking: of the entire phosphoprotein complement that was labeled in these neurons, two groups of phosphoproteins are prominently incorporated into the coat structures (Fig. 1). This specificity has allowed us to identify several polypeptides that correspond to the 100–110-kD brain assembly polypeptides, as well as a novel 155-kD coated membrane polypeptide that has not been previously described, that are phosphorylated in intact cells. Furthermore, a neuronal counterpart of the brain AP_{50} has been identified; however in contrast to the results obtained in in vitro experiments, the neuronal AP_{50} is not substantially phosphorylated in intact neurons.

Phosphorylation of AP in Intact Cells

The brain 100–110-kD AP consists of several species that can be resolved on 1-D gels. We have identified four major and one minor phosphorylated polypeptides in cultured sympathetic neurons that are structurally and functionally homologous to these brain proteins in that they (*a*) co-elute with these proteins on Sepharose CL-4B columns (Fig. 2), (*b*) precisely co-migrate with authentic 100–110-kD AP in 1-D gels (Fig. 2), and (*c*) co-assemble with clathrin into coat structures in vitro (Fig. 1). We have previously shown that intact 100–110-kD AP is required for the in vitro expression of the assembly-promoting activity of the AP complex (34). Thus, the phosphorylation of these polypeptides in intact cells is likely to be a factor in the regulation of coat formation or interaction with other components in the cell.

In vitro phosphorylation of the 100–110-kD CV components has not been consistently observed (3, 20, 25) although phosphate-labeled bands of this apparent molecular weight are visible in some published reports (26, 29, 34). One possible explanation for the difference between the in vivo and in vitro results may be the absence of the appropriate physiological protein kinase in CV preparations. Alternatively, the 100–110-kD AP in CV preparations may already be substantially phosphorylated, and thus incorporate little additional phosphate under in vitro conditions. In this regard, preliminary experiments have indicated the presence of substantial amounts of protein-bound phosphate (30 nm/mg protein) in bovine brain AP preparations (Keen, J. H., and D. Skee, unpublished data).

Since the 50-kD component of bovine brain AP can be readily phosphorylated in preparations of CVs (10) or partially purified AP (13), it is quite unexpected that a phosphorylated 50-kD protein is conspicuously absent from the reassembled coats formed from in situ phosphorylated rat neuronal proteins and bovine brain carrier proteins (Fig. 1). The data demonstrate that a neuronal AP_{50} is indeed present in these cells: it can be detected in SDS extracts although as assayed by L-Met incorporation it is a relatively minor biosynthetic product (Fig. 5, *b* and *c*). The neuronal protein can be readily phosphorylated in broken cell preparations by addition of exogenous ATP, at specific radioactivity (40 Ci/mmol) less than or equivalent to those estimated to prevail in the intact cells under our labeling conditions (8, 9; also Keen, J. H., and M. M. Black, unpublished calculations). The resulting neuronal pp50 is indistinguishable from the phosphorylated form of the bovine brain AP_{50} by 1-D peptide mapping and by 2-D NEPHGE gel analysis. Finally, both the unmodified (i.e. [^{35}S]-L-Met–labeled) and the in vitro phosphorylated rat neuronal polypeptides are incorporated into 250S assembled coat structures, indicating that the neuronal protein is functionally, as well as structurally, homologous to the brain AP_{50}.

Phosphorylation of bovine brain AP_{50} alters its mobility in 2-D NEPHGE gels such that it migrates with a slightly higher apparent molecular mass and acidic pH than its unphosphorylated counterpart (Fig. 5 *c*). Analyses of [^{35}S]-L-Met–labeled neurons indicate that all of the detectable methionine label in this region co-migrates exactly with the unphosphorylated carrier brain AP_{50} (Fig. 5 *b*): we do not detect a Met-labeled spot with the mobility expected of a phosphorylated form of the AP_{50}. Direct analysis of SDS extracts of [^{32}P]-P_i-labeled neurons on 2-D NEPHGE gels does reveal a phosphorylated species in the approximate, although not precise, position anticipated for a phosphorylated form of the neuronal AP_{50} (Fig. 5 *d*). However, the extent of labeling is quite minor and further analysis will be required to unambiguously determine if this species is truly the neuronal AP_{50}.

The co-migration of essentially all of the Met-labeled protein with the (unphosphorylated) brain carrier and the minimal amount, if any, of the phosphorylated protein observed in cells labeled in situ, as compared with those labeled after lysis, leads us to conclude that the extent of phosphorylation

of the neuronal AP_{50} in intact cells must be quite small. This may reflect the existence of a very low steady-state level of phosphorylated AP_{50} in these cells which, though not readily detectable by the methods used here, could be important in the function of coated membranes. However, data has been obtained to indicate that the in vitro formation of pp50 in isolated CVs represents an autophosphorylation reaction (3).[2] Thus, another explanation of the difference in broken and whole cell phosphorylation patterns is simply that the AP_{50} is a protein kinase whose autophosphorylation becomes apparent only in the absence of the appropriate cellular substrates and is not a physiologically relevant reaction.

Identification of a 155-kD Phosphoprotein

The present studies have identified a novel 155-kD rat neuronal protein that is incorporated into in vitro reassembled coat structures with bovine brain proteins. The relatively distinctive behavior of this protein in 2-D gels, with an apparent isoelectric point of ~5.1, provides the basis for the following observations. First, a phosphorylated form of this protein is readily detected in 2-D gels of SDS extracts of [^{32}P]P_i-labeled neurons, thereby unambiguously establishing that it is indeed phosphorylated in situ. The neuronal 155-kD protein can also be detected in SDS extracts of [^{35}S]-L-Met-labeled cells: by this assay, the 155-kD protein is a relatively prominent biosynthetic product in these cells. Finally, CV preparations from bovine brain contain a protein that precisely co-migrates with the neuronal pp155 in 2-D gels. The brain 155-kD component appears to be an extrinsic membrane protein, since it can be released by treatment with Tris-HCl. On gel filtration it partially overlaps the AP peak so that by 2-D gel electrophoresis it is detectable as a minor component of the complete AP fraction (as defined in reference 33). Preliminary results indicate that the bovine brain protein is present in substantial quantities, though it is substoichiometric with respect to the AP, and that it is immunologically related to the rat neuronal pp155 (Chestnut, M. H., M. M. Black and J. H. Keen, unpublished data).

Summary

The data presented here indicate that the bovine brain assembly polypeptides of 50 kD and 100–110 kD that we have previously described, as well as the novel 155-kD protein reported here, have counterparts in rat sympathetic neurons maintained in culture. These proteins are related across species lines not only in their physical properties, as probed by 1-D and 2-D gel electrophoresis and 1-D peptide maps, but also by their ability to co-assemble with bovine brain clathrin into coat structures. This conservation of structure and assembly activity strongly suggests that these coated membrane proteins play critical roles in the membrane dynamics of eukaryotic cells. These may include the modulation of coat size and assembly (33) or of clathrin binding to membranes (32). Thus the functions of the AP may be largely structural, involved in linking of clathrin to receptors or in cytoskeletal–membrane interactions. Since the APs also appear to be more tightly bound to the coated vesicle membrane than is clathrin, it is possible that they remain vesicle associated after the formation of a transfer vesicle, e.g. an endocytic vesicle, possibly fulfilling an active role in the targeting of intracellular membrane components derived either from the plasma or Golgi membranes.

The phosphorylation of the $AP_{100-110}$ and the 155-kD putative coated membrane protein that occurs in intact rat sympathetic neurons is likely to be involved in the regulation of coated membrane functions in vivo. However, our observation that the AP_{50} is not detectably phosphorylated in intact neurons is in striking contrast to its prominent phosphorylation in vitro in isolated CVs. It has been reported that the in vitro reaction may be an autophosphorylation (3).[2] Therefore, we suggest that the AP_{50} is a protein kinase and that in intact cells it does not undergo substantial autophosphorylation, but rather phosphorylates other, as yet unidentified, cellular substrates.

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