Auxilin, a Newly Identified Clathrin-associated Protein in Coated Vesicles from Bovine Brain

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Abstract. We have identified a new coat protein in clathrin-coated vesicles from bovine brain by urea-SDS gel electrophoresis. The protein was purified from Tris-solubilized coat proteins either by combination of hydroxyapatite chromatography and gel filtration or more rapidly in a single step by immunoaffinity chromatography. The purified protein binds to clathrin triskelia and thereby promotes clathrin assembly into regular 50–100-nm cages. We propose for the new protein the name auxilin (Latin *auxilium*, meaning support). Auxilin migrates as a 110-kD polypeptide in standard type SDS-PAGE, but in the presence of 6 M urea shifts to a position corresponding to 126 kD. Gel filtration in 6 M guanidinium hydrochloride gives a molecular weight of ~86,000. The native protein is monomeric in 0.5 M Tris. Antigenic reactivity and two-dimensional peptide maps gave no evidence of gross similarities between auxilin and any of the other known coated vesicle-associated proteins. Since the structural organization of auxilin does not resemble that of the ubiquitous heterotetrameric HA1 and HA2 adaptor complexes, that are believed to connect clathrin to receptors, it is unlikely that it functions as an adaptor. Immunoblotting did not reveal the presence of auxilin in tissues other than brain. If auxilin and AP 180 are indeed both confined to neuronal cells, as the immunochemical evidence suggests, it might be inferred that both serve to adapt clathrin-coated vesicles to an as yet undisclosed function unique to this cell type.

CLATHRIN-COATED pits and vesicles are known to participate in intracellular transport processes such as receptor-mediated endocytosis and in directing lysosomal enzyme receptors from the *trans*-Golgi network to a prelysosomal compartment (for review, see references 7, 21, and 23). One strategy employed for investigating the details of coated vesicle function consists in the identification, purification, and biochemical characterization of coated vesicle components. This approach has led to detailed insight into the structure and properties of the major coat protein clathrin, which self-assembles to form the polygonal framework of coated membranes (12, 20, 34). The interaction between clathrin and receptors in the membrane requires the presence of tetrameric protein complexes (33, 36), which have recently been termed adaptors (22). Two such adaptor complexes, HA1 and HA2, have been identified in bovine brain (24; for different nomenclatures see reference 10). The HA1 adaptor consists of β', γ, 47, and 20-kD subunits, and adapts clathrin to receptors in the *trans*-Golgi network, whereas the HA2 complex, which is made up of α, β, 50, and 16-kD subunits, attaches it to receptors in the plasma membrane (3, 27). The β subunit, which is closely related to the β' subunit of the HA1 adaptor, was recently shown to bind to clathrin, while the other adaptor-specific subunits were conjectured to interact with membranes (1). Moreover, direct interactions between the HA1 and HA2 adaptors and certain cytoplasmic receptor tails have been demonstrated in vitro (6, 22). The protein coat of coated vesicles from bovine brain additionally contain in their coat besides the adaptor complexes the protein AP 180 (Assembly Protein 180) (2, 25) also referred to as AP3 (10). This protein was shown to induce in vitro assembly of clathrin under conditions close to physiological when it does not polymerize by itself. Although it has been claimed that AP 180 binds to coated vesicle membranes (25), it seems unlikely to represent a third class of adaptor protein, because its structural organization bears no relationship to the known adaptor complexes.

Here, we report the discovery of a new coat-associated protein with clathrin assembly promoting properties. In recognition of this property, we propose the name auxilin for the new protein, derived from the Latin *auxilium*, meaning support or help. Auxilin, an 86,000-D protein has probably escaped previous detection, because it behaves anomalously in standard SDS-PAGE and is therefore not readily resolved from the β' subunit of the HA1 adaptor. Auxilin is not related to any of the known coat-associated proteins, but in its main structural features it resembles the protein AP 180 more than the adaptor complexes.

Materials and Methods

Materials

Fresh bovine brains were obtained from a local abattoir and processed

within 1 h of slaughter. Superose 6 gel filtration column, protein standards for SDS-PAGE, CNBr-activated Sepharose 4B, Ficoll 400, and protein-A Sepharose CL-4B were from Deutsche Pharmacia (Freiburg, FRG); EGTA was from Serva (Heidelberg, FRG); reagents for SDS-PAGE and urea were from LKB Instrument GmbH (Gräfelfing, FRG); MES, DTT, and PMSF were from Sigma Chemie GmbH (Deisenhofen, FRG); peroxidase-conjugated IgGs to mouse and rabbit antibodies were from Dakopatts GmbH (Hamburg, FRG); Pansorbin *Staphylococcus aureus* cells were from Calbiochem-Behring Corp. (San Diego, CA); nitrocellulose transfer-membranes (BA 83, 0.2 μm) were from Schleicher & Schüll (Dassel, FRG); Centricon microconcentrators were from Amicon GmbH (Witten, FRG); and the hybridoma cell line CVC.7 was obtained from American Type Culture Collection (Rockville, MD).

Methods

Purification of Auxilin. Coated vesicles from bovine brain tissue were prepared according to Campbell et al. (4). For the purification of auxilin by conventional biochemical techniques, 60 mg total coat protein was extracted with 0.5 M Tris (pH 7.0), containing 0.1 mM PMSF, 2 mM EDTA, and 1 mM DTT (11). To obtain the assembly protein fraction, the extract was clarified by ultracentrifugation and then subjected to gel filtration exactly as described elsewhere (1). The assembly proteins (~12 mg) were then fractionated on hydroxyapatite exactly as described by Ahle and Ungewickell (1). Auxilin desorbed between 0.10 and 0.15 M phosphate. The major contaminant at this stage was AP 180. Fractions that contained auxilin were pooled, concentrated by centrifugation in a Centricon 30 microconcentrator, and then subjected in two to three batches to gel filtration on a 10 × 300-mm Superose 6 gel filtration column. This procedure yielded ~200 μg auxilin.

Alternatively, the protein was purified by immunoaffinity chromatography with the auxilin-directed mAb 100/4. 40 ml of a crude membrane fraction (pellets from the first ultracentrifugation in the standard coated vesicle preparation of Pearse (20) was diluted to 50 ml with 0.1 M MES, 0.5 mM $MgCl_2$, 1 mM EGTA, 0.02% NaN_3 (pH 6.5), and then extracted with an equal volume of 1 M Tris (pH 7.0), 2 mM EDTA, 0.1 mM PMSF at 4°C. Insoluble material was removed by ultracentrifugation for 60 min at 100,000 *g*. The supernatant was passed through a column containing 12 mg of mAb 100/4 coupled to 4 ml Sepharose 4B at a flow rate of 10 ml/h. The column was successively washed with 20 ml of 0.5 M Tris (pH 7.0) (Tris-buffer), 20 ml of 50 mM Tris (pH 8.0), 0.1% Triton X-100, and 20 ml of 50 mM Tris (pH 8.0). The resin was then removed from the column, washed with 5 vol Tris-buffer, and recovered by low speed centrifugation. This procedure was repeated twice before the resin was returned to the column. Auxilin was eluted with 3.5 M $MgCl_2$ at room temperature and immediately desalted on a PD10 (Pharmacia Fine Chemicals, Uppsala, Sweden) G25 gel filtration column. The PD 10 column was equilibrated in 2 mM Tris, 50 mM NaCl, 0.5 mM EDTA (pH 8.0), 1 mg of auxilin resulted from 40 ml crude membranes, corresponding to ~24 mg of total protein in the assembly protein fraction. The protein was stable at −20°C in 50% glycerol.

Determination of Stokes Radius and Sedimentation Coefficient. The Stokes radius of auxilin was determined by gel filtration on a 10 × 300-mm Superose 12 column, equilibrated with 0.5 M Tris-HCl (pH 7.0), 2 mM EDTA, 1 mM DTT. The column was calibrated with apoferritin (64 Å), rabbit immunoglobulin (55 Å), aldolase (45 Å), BSA (35 Å), and ovalbumin (29 Å). The void volume was determined with intact clathrin cages that had been cross-linked with glutaraldehyde and the included volume with ATP. The flow rate was 0.5 ml/min. Elution volumes of the marker proteins were determined by monitoring the eluate at 280 nm. To determine the elution volume of auxilin, 0.2 ml of total coat protein was applied to the column and 0.25 ml fractions were collected. Auxilin was detected by SDS-PAGE and immunoblotting. The Stokes radii were plotted against the inverse error function, $\text{erf}^{-1}(1 - K_d)$, yielding a linear calibration (17). To determine the subunit molecular weight of auxilin total coat protein was denatured by dialysis against 6 M guanidinium chloride, 2 mM EDTA, 1 mM DTT, 20 mM Tris-HCl (pH 8.0), and then applied to a Superose 6 gel filtration column, equilibrated in the same solvent. The column was calibrated with myosin, thyroglobulin, β-galactosidase, and rabbit immunoglobulins heavy and light chains. Elution volumes for the markers and auxilin were obtained as described above. The plot of $K_d^{1/3}$ versus mol wt $^{0.555}$ yields a straight line from which the molecular weight of auxilin was obtained by interpolation (17).

The sedimentation coefficient of auxilin was determined by sucrose density gradient centrifugation in the presence of suitable marker proteins of known sedimentation coefficients according to the procedure of Siegel and Monty (32). 0.5 ml of total coat protein was loaded together with myoglobin (2S), catalase (11.3S), aldolase (7.4S), BSA (4.6S), and clathrin (8.4S) on a 4.5-ml 5–20% linear sucrose gradient. The gradient was centrifuged for 18 h at 45,000 rpm at 4°C in a rotor (SW 60; Beckman Instruments, Palo Alto, CA). The tube was punctured and 0.175-ml fractions were collected manually. The protein composition of each fraction was analyzed by SDS-PAGE and by immunoblotting.

The molecular weight of auxilin was calculated from the equation:

$$M_r = 6\pi N R_s S_{20,w} / (1 - \nu\rho_{20,w});$$

where R_s is the Stokes radius, $S_{20,w}$ the corrected sedimentation coefficient, and N the Avogadro number. The partial specific volume ν of auxilin was taken to be 0.73 ml/g.

Assembly Experiments. 50 μg clathrin triskelia were dialyzed overnight at 4°C either in the absence or presence of auxilin (17 and 34 μg) against 0.1 M MES, 1 mM EGTA, 0.5 mM $MgCl_2$, pH 6.5. The extent of assembly was analyzed by sucrose gradient centrifugation, using 4.5 ml gradients of 5–30% sucrose made up in 0.1 M MES, 0.5 mM $MgCl_2$, 1 mM EGTA, 0.02% NaN_3, pH 6.5. The gradients were centrifuged for 1 h at 38,000 rpm in a rotor (SW 60; Beckman Instruments) at 4°C. Fractions of 0.33 ml were collected manually. The protein composition of each fraction was analyzed by SDS-PAGE, and the extent of assembly was quantified by densitometry of the clathrin zone. Aliquots of the dialysate were also negatively stained with uranyl acetate and viewed in a Zeiss EM 109 electron microscope.

Binding to Preassembled Clathrin Cages. Clathrin triskelia were assembled into cages by dialysis against 0.1 M MES, 1 mM EGTA, 0.5 mM $MgCl_2$, 2 mM $CaCl_2$ (pH 6.5), and then mildly cross-linked with 3,3′-dithiobis(sulfosuccinimidylpropionate) as described in reference 29 to prevent their disintegration upon dilution. Unassembled clathrin was removed by ultracentrifugation. The pelleted cages (preformed cages) were resuspended in 5 mM Hepes, 100 mM potassium tartrate, 0.5 mM EDTA, 0.25 mM $MgCl_2$, pH 7.2 (binding buffer), and incubated on ice for 0.5 h with auxilin. In a typical binding experiment 1.3–8 μg auxilin was incubated either alone, to test for aggregation of auxilin under binding conditions, or with 9 μg clathrin cages in a final volume of 0.13 ml binding buffer. The extent of binding was analyzed by ultracentrifugation for 15 min at 45,000 rpm (~88,000 *g*) in a centrifuge (TL 100, TLA 100 rotor; Beckman Instruments). Pellets and supernatants were analyzed by SDS-PAGE and densitometry.

Antibody Production. The mAb 100/4 used in this paper was obtained from a BALB/c mouse that was immunized three times at intervals of 2 wk with 20 μg of total assembly protein. For three consecutive days before the day of the cell fusion, the mouse was boosted intravenously with 10 μg assembly protein. The fusion routine and tissue culturing were executed exactly as described previously (3). Supernatants were screened by immunoblotting. Colonies of interest were subcloned twice by limited dilution. For large-scale production of monoclonal antibodies, hybridomas were cultured in roller bottles as described before (3).

Protein Concentrations. All concentrations were determined spectrophotometrically. For clathrin, a specific absorbance at 280 nm of $E_{1cm}^{1\%} = 11.9$ was used. The concentration of auxilin was obtained from the absorbance at 205 nm, taking E (1 mg/ml; 1 cm) = 31 (reference 30). The concentrations obtained corresponded to a specific absorbance at 280 nm of $E_{1cm}^{1\%} = 7.9$.

Peptide Mapping. Two-dimensional peptide analysis of ^{125}I-labeled tryptic peptides was performed essentially according to the procedure of Elder et al. (5) and as described in detail elsewhere (2).

Quantitative Immunoprecipitation. 1 g of bovine brain was homogenized in 1 ml of 0.1 M MES, 0.5 mM $MgCl_2$, 1 mM EGTA, 0.02% NaN_3 (pH 6.5), and the suspension clarified by centrifugation for 0.5 h at 7,000 *g*. The supernatant was then centrifuged for 0.5 h at 100,000 *g* in an ultracentrifuge (TL 100; Beckman Instruments). The volumes of pellets and supernatants were first adjusted with 0.1 M MES, 0.5 mM $MgCl_2$, 1 mM EGTA, 0.02% NaN_3 (pH 6.5), to 1 ml, before 1 ml of 1 M Tris (pH 7.0), was added. Both samples were again ultracentrifuged as described above, and the supernatants, which contained soluble coat proteins were saved. Auxilin was quantitatively removed from the supernatants by a 2-h incubation with 50 μg of mAb 100/4, coupled to a Sepharose matrix. After extensively washing the Sepharose with 0.5 M Tris (pH 7.0), and PBS, the amount of auxilin bound to the antibody and was quantified by SDS-PAGE. By immunoblotting, it was ascertained that all the auxilin present in the Tris-extracts was adsorbed by the antibody.

Miscellaneous Techniques. Free flow electrophoresis of coated vesicles was performed essentially as described previously (18). In brief, 10–20-mg coated vesicles obtained by differential centrifugation according to Campbell et al. (4) were further purified by electrophoresis in a Hirschmann

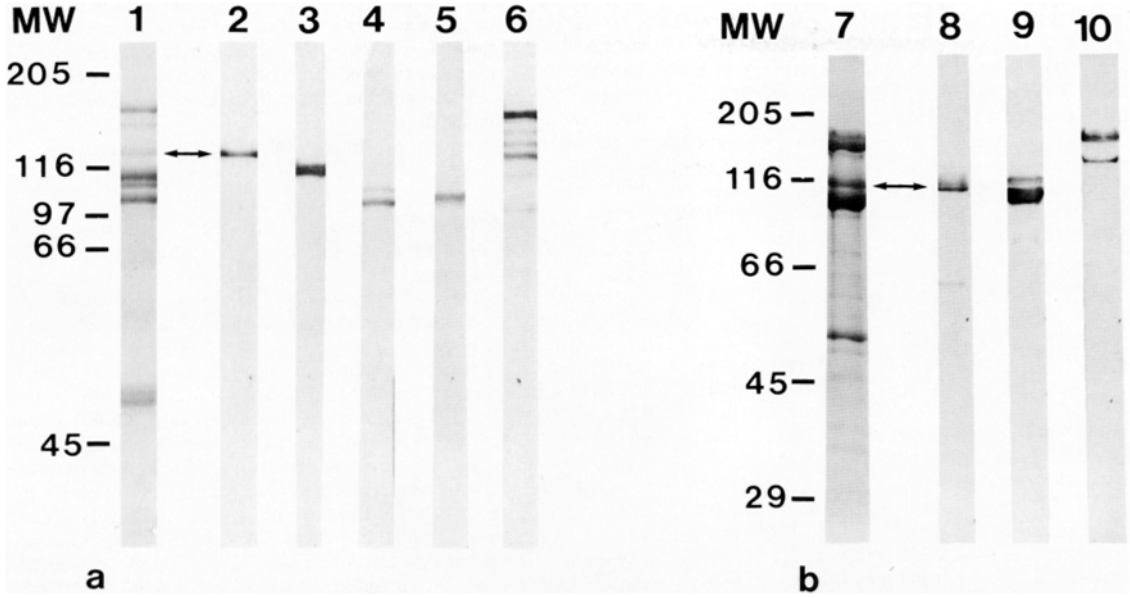

Figure 1. Identification of a new protein (auxilin) in the assembly protein fraction. Total assembly proteins obtained by gel filtration of Tris-solubilized coat proteins were analyzed by SDS-urea–PAGE (*a*) and Laemmli type (standard) SDS-PAGE (*b*). (Lanes *1* and *7*) Coomassie blue–stained protein zones; (lanes *2* and *8*) immunoblot stained with antiauxilin (mAb 100/4); (lanes *3* and *9*) immunoblot stained with anti–β type adaptor subunit (mAb 100/1); (lane *4*) immunoblot stained with anti–α type adaptor subunit (mAb 100/2); (lane *5*) immunoblot stained with anti–γ type adaptor subunit (mAb 100/3); (lanes *6* and *10*) immunoblot stained with anti–AP 180. The arrows denote auxilin.

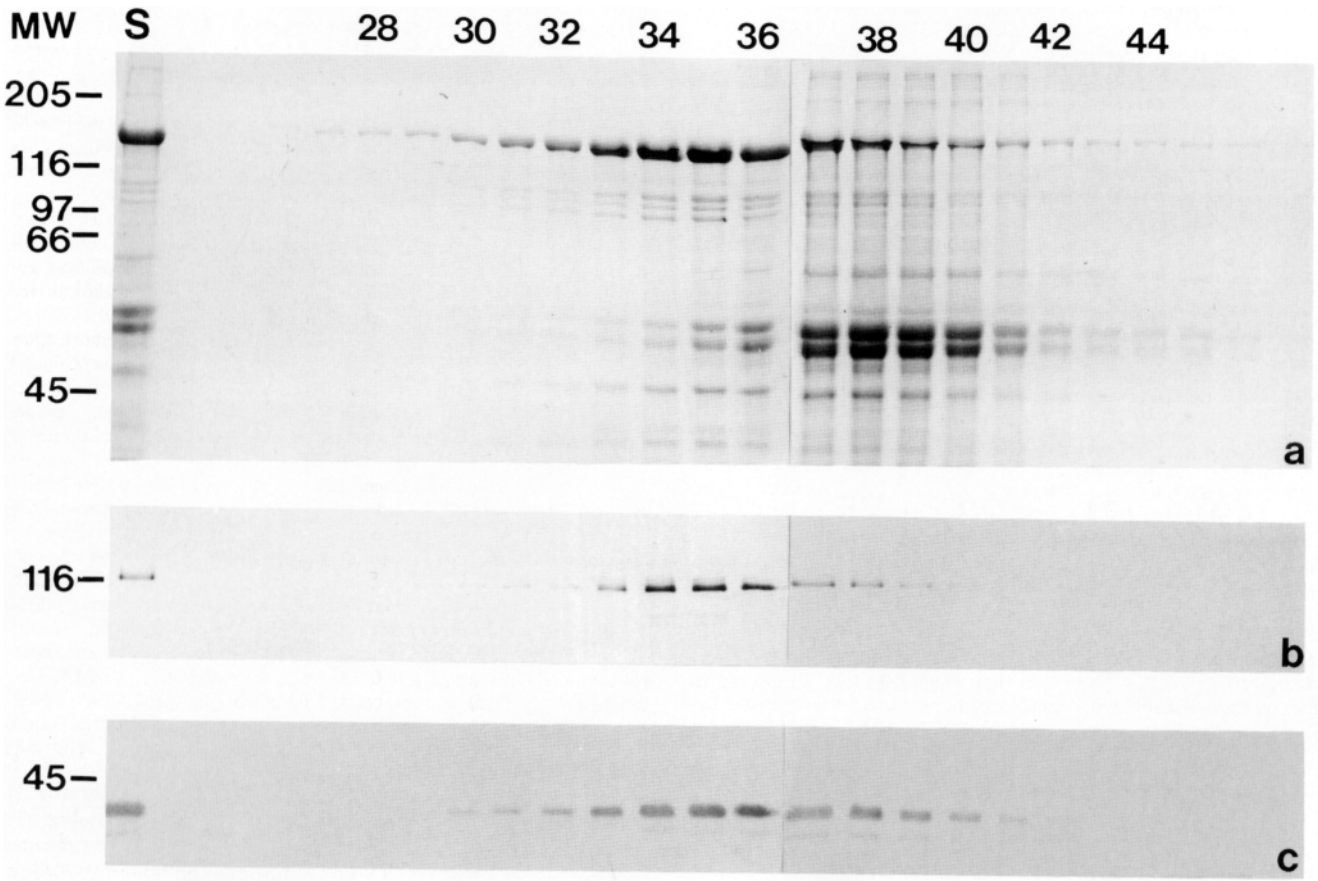

Figure 2. Co-migration of auxilin with clathrin-coated vesicles in free flow electrophoresis. Coated vesicles were purified by the method of Campbell et al. (4) and then subjected to free flow electrophoresis. Fractions were analyzed by SDS-urea PAGE and immunoblotting. The starting material (*S*) is in the lane on the far left. (*a*) Coomassie-stained gel of the fractions; (*b*) relevant part of an immunoblot stained with antiauxilin (mAb 100/4); (*c*) relevant part of an immunoblot stained with a monoclonal antibody (CVC.7) against a light chain (LC_A) of clathrin (13). Note that the distribution of auxilin follows that of clathrin and the light chain.

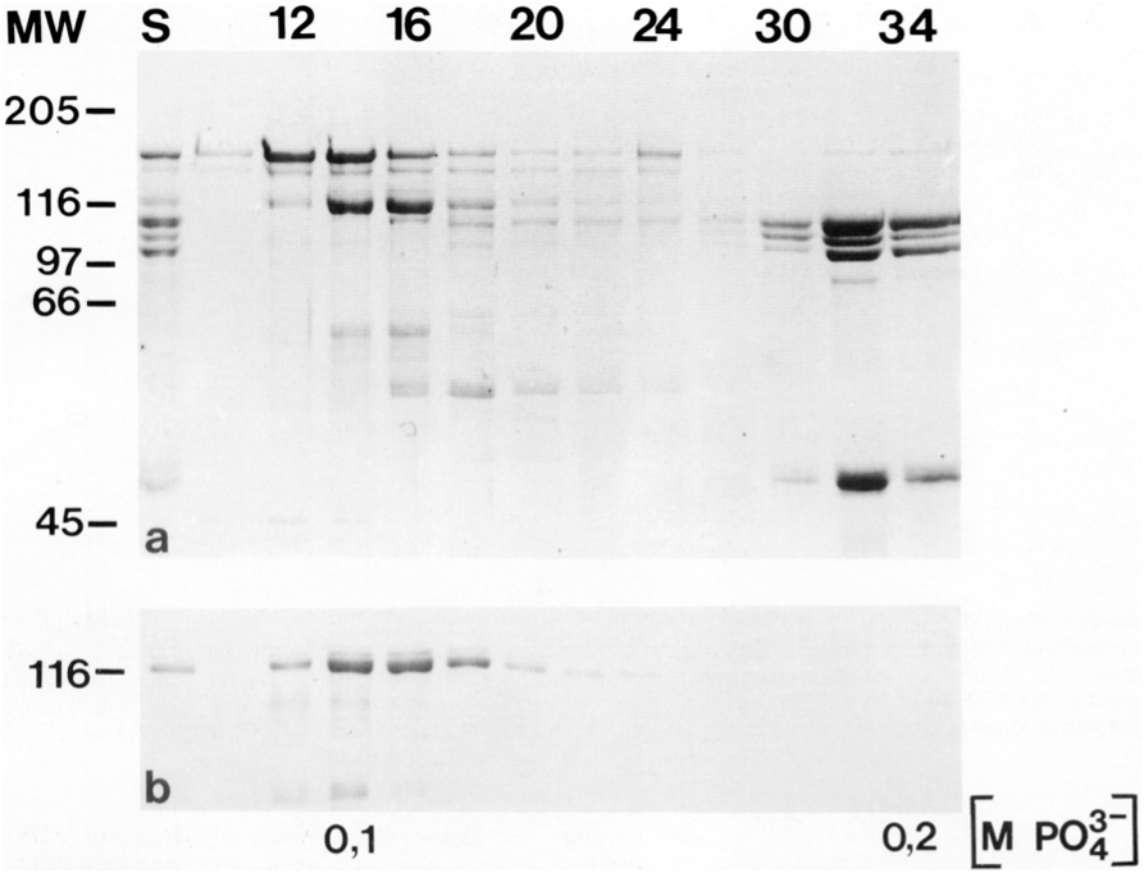

Figure 3. Hydroxyapatite chromatography of assembly proteins. Protein-containing column fractions were electrophoresed in urea-SDS polyacrylamide gels and either stained for protein with Coomassie blue (*a*) or transferred to nitrocellulose paper and probed with antibodies against auxilin (*b*). The lane on the far left shows the starting material (*S*). Fractions are indicated at the top of the gel and corresponding phosphate molarities beneath.

VAP 5 apparatus at 900 V (200 mA). The buffer was 19 mM MES, 0.5 mM EGTA, 0.25 mM $MgCl_2$, pH 6.4, with a conductivity of 1,000 μSiemens. The electrode buffer was 0.1 M MES (pH 6.4). SDS-PAGE was performed according to Laemmli (16). SDS-Urea PAGE was performed in 7.5% acrylamide minigel slabs (7.5 × 8.0 × 0.075 cm), containing 0.1% SDS, 6 M urea, and 2 mM EDTA in the separation gel. Electrophoresis was performed in a Hoefer Mighty Small II unit. The gels were either stained with Coomassie brilliant blue or electroblotted onto nitrocellulose paper for probing with monoclonal antibodies. Coomassie binding to auxilin and clathrin was quantified by applying protein loads of 1–5 μg in duplicate to 10% mini gel slabs. The gels were stained with Coomassie blue, destained, and densitometered in a Camag instrument. Areas under zone profiles were plotted against the amount of protein.

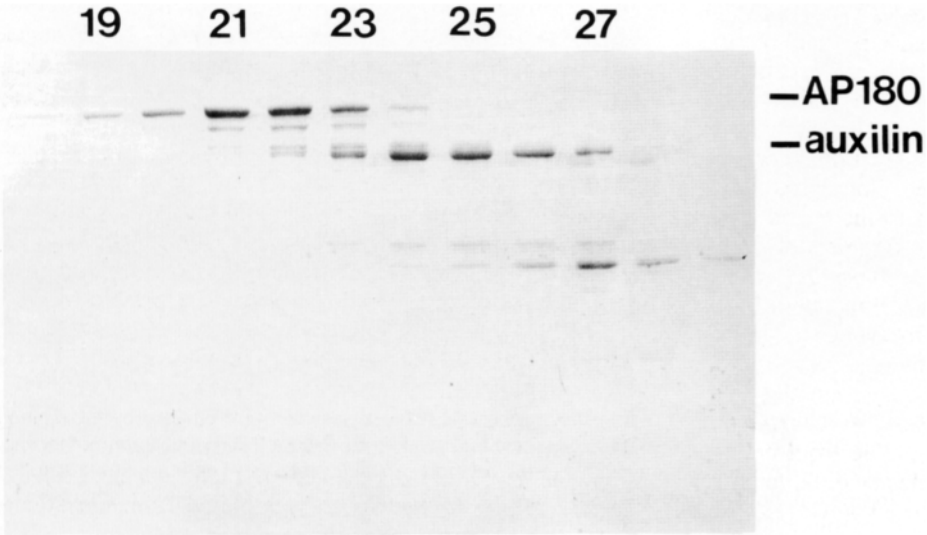

Figure 4. Final purification step of auxilin by gel filtration. Auxilin, obtained by hydroxyapatite chromatography (fractions 13–16 in Fig. 3), was concentrated and then subjected to gel filtration on Superose 6 to remove AP 180 and low molecular weight contaminants. Fractions were analyzed by standard SDS-PAGE. Fractions 24 and 25, which contain almost pure auxilin, were pooled.

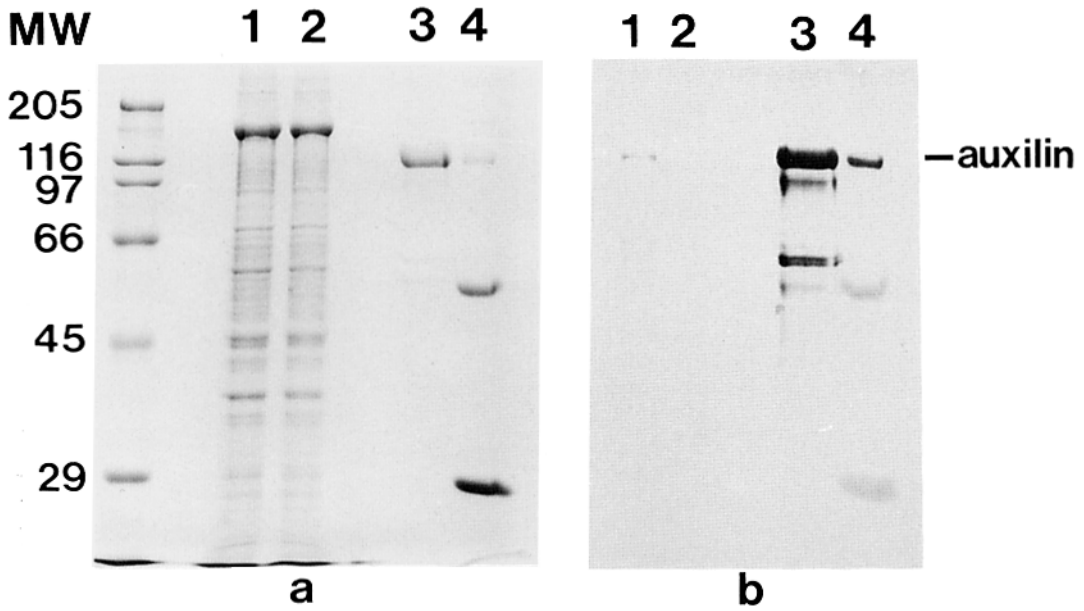

Figure 5. Purification of auxilin by affinity chromatography. A Tris extract of crude membranes from bovine brain was passed through an affinity column, containing immobilized monoclonal antibody against auxilin (mAb 100/4). (*a*) Coomassie-stained gel showing membrane extract before (track 1) and after passage (track 2) through the affinity column. (Track 3), Auxilin eluted with 0.35 M $MgCl_2$. (Track 4) Auxilin remaining bound to the antibody after elution with magnesium. (*b*) Corresponding immunoblots stained for auxilin with mAb 100/4. The dense protein zones at 50 and 25 kD in track 4 are antibody molecules, released from the Sepharose beads with residual auxilin by 0.1% SDS.

Results

Identification of a New Coated Vesicle Component

Coat proteins were extracted from purified clathrin-coated vesicles with 0.5 M Tris and then subjected to gel filtration to separate clathrin from the adaptors and the assembly protein AP 180. When a pool of fractions, containing the HA1 and HA2 adaptor complexes, protein AP 180 and other minor components (previously described as assembly protein fraction [11]) was analyzed by SDS-PAGE in the presence of 6 M urea, we noted a polypeptide of M_r ~126,000, which was not resolved from other components in the 100–116 kD molecular mass range in the standard system of Laemmli (16) (Fig. 1). This polypeptide was not stained on immunoblots by monoclonal antibodies directed against the previously characterized subunits of the two adaptor complexes or against AP 180 (2, 3). However, our repertoire of monoclonal antibodies that were elicited in mice by injection with total coat proteins, contained one antibody (mAb 100/4), which reacted exclusively with the new polypeptide (Fig. 1; lanes *2* and *8*). Immunoblotting showed that this species migrates on standard SDS-PAGE in a position corresponding to M_r ~110 kD, which is very close to the β and β'-type adaptor subunits. A major proteolytic fragment of AP 180, which migrates close to the new polypeptide in urea-SDS-PAGE (Fig. 1, lane *6*) does not accompany it in standard SDS-PAGE (Fig. 1, lane *4*). To avoid ambiguity in designating the new protein in terms of an apparent molecular mass, we shall henceforth refer to it as auxilin.

To show that auxilin is a component of clathrin-coated vesicles and not a contaminant originating from unrelated membranes, we purified coated vesicles by differential centrifugation followed by free flow electrophoresis (18). Fractions obtained from the latter were analyzed by urea-SDS-PAGE and immunoblotting with monoclonal antibodies against auxilin and against known constituents of clathrin-coated vesicles. The result shows that auxilin copurifies with clathrin and with a light chain of clathrin (LC_A) in free flow electrophoresis and is thus very likely to be associated with clathrin coated vesicles (Fig. 2).

Purification of Auxilin

We undertook the purification of auxilin, starting from the assembly protein fraction, initially by conventional chromatographic methods such as hydroxyapatite chromatography and gel filtration. The fractions from the hydroxyapatite column were analyzed by urea-SDS-PAGE and the presence of auxilin was confirmed by immunoblotting. Auxilin desorbed from the column between 0.1 and 0.15 M phosphate together with AP 180 and traces of the HA1 adaptor (Fig. 3). Fractions containing auxilin were then chromatographed on a Superose 6 gel filtration column to remove AP 180 and other minor contaminants (Fig. 4). Although it proved possible to remove AP 180 and other minor polypeptides from auxilin by gel filtration, the yield and purity of auxilin obtained in this way was generally less than satisfactory. Attempts to purify auxilin by Mono Q ion exchange chromatography instead of gel filtration were frustrated by high losses of the protein and were therefore abandoned. We therefore explored the possibility of immunoadsorbing auxilin from the 0.5 M Tris extract of a crude membrane fraction. In pilot experiments, it was established that the mAb 100/4 binds strongly to auxilin in 0.5 M Tris and that this interaction was effectively dissociated by high concentrations of $MgCl_2$. >70% of the auxilin was eluted from the affinity

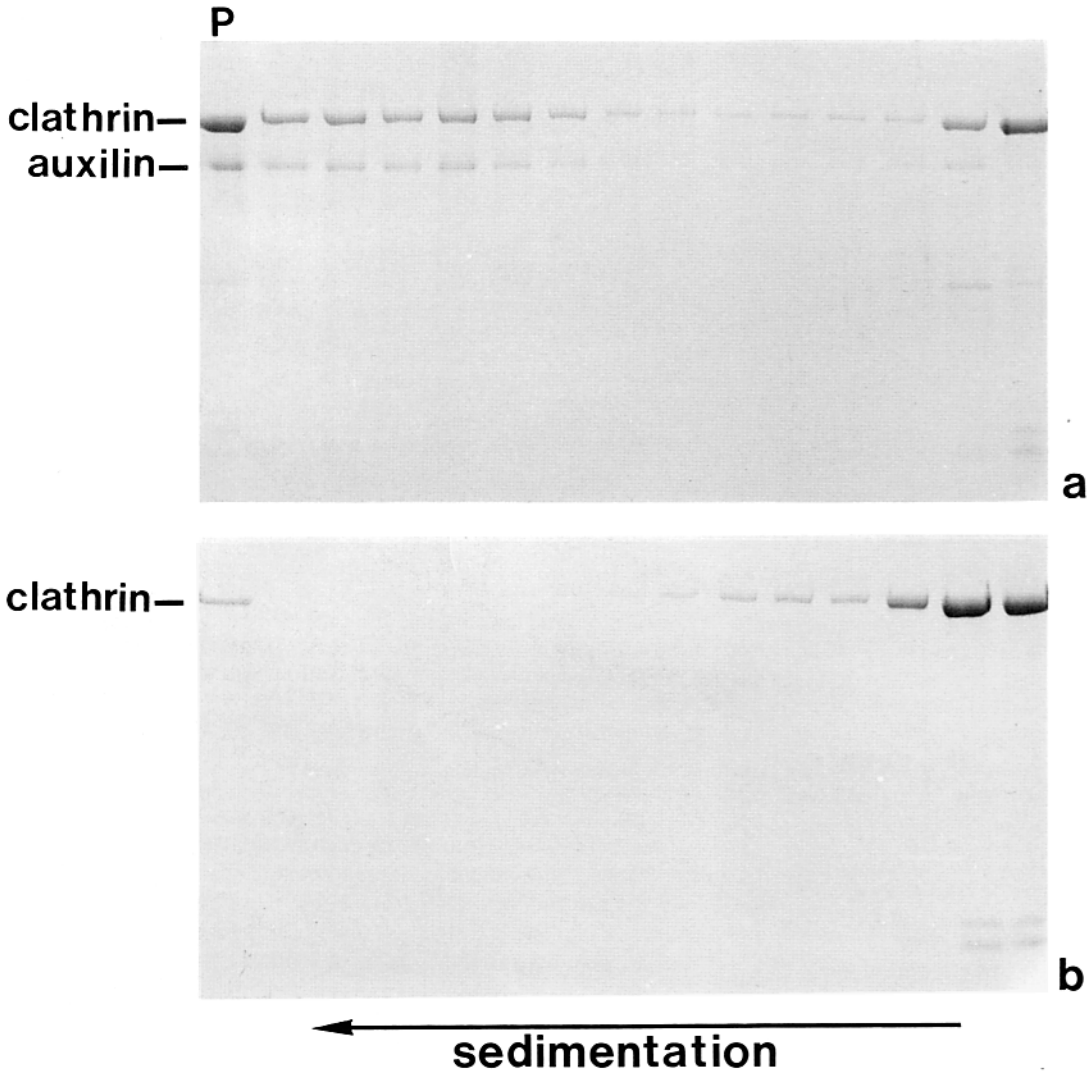

Figure 6. Auxilin-induced assembly of clathrin cages. 50 μg clathrin triskelia were dialyzed into 0.1 M MES, 0.5 mM $MgCl_2$, 1 mM EGTA, 0.02% NaN_3, pH 6.5, with 34 μg auxilin, and in a control experiment, 50 μg clathrin was dialyzed in the absence of auxilin. To assess assembly, aliquots of the samples were sedimented through 5–30% sucrose gradients. Fractions from the gradients were analyzed by electrophoresis in 10% Laemmli-type polyacrylamide gels, which were stained for protein with Coomassie. (*a*) Sucrose gradient fractions of clathrin-auxilin complexes; (*b*) sucrose gradient of unpolymerized clathrin triskelia; lanes on the far left (*P*) show aggregated material, which was pelleted by low speed centrifugation in a bench top centrifuge.

column by 3.5 M $MgCl_2$ (Fig. 5). By this means, we usually obtained ~1 mg auxilin from 40-ml pelleted crude membranes, containing some 24 mg total assembly protein. Compared to the biochemical procedure described above, immunoaffinity purification yielded ~2.5 times more auxilin and was therefore routinely employed. In electrophoresis in standard SDS gels, the purified protein appears as a relative broad zone, which may reflect some microheterogeneity (Fig. 5). The nature of this effect has not been further explored. It may arise from proteolysis and/or posttranslational modifications. In addition, immunoblots almost always showed two to three satellite bands. Proteolysis of auxilin could be inhibited, but never completely eliminated by addition of protease inhibitors to the Tris-extraction buffer. Upon elution of the auxilin from the affinity column, care was taken to reduce the high salt concentration rapidly by gel filtration. Auxilin could be stored in 50% glycerol at −20°C without any detectable loss of activity (see below).

Functional Characterization of Auxilin

The definitive identification of auxilin as a structural component of the coated vesicle must rest on a demonstration of saturable binding to clathrin or one of its associated proteins. Binding of AP 180 and of the HA2 adaptor complex to clathrin triskelia induces their assembly into cagelike structures. To investigate whether auxilin has similar properties, we dialyzed clathrin triskelia alone and in the presence of auxilin against a buffer known to support only assembly protein-dependent polymerization of clathrin. The dialyzed samples were briefly spun in a table top centrifuge to remove large aggregates and then fractionated by centrifugation on sucrose gradients. The distribution of clathrin and auxilin was determined by SDS-PAGE and densitometry. As expected, in the absence of auxilin all of the clathrin remained unassembled in the top fractions of the sucrose gradient (Fig. 6 *b*). However, upon addition of auxilin, up to 45% of the

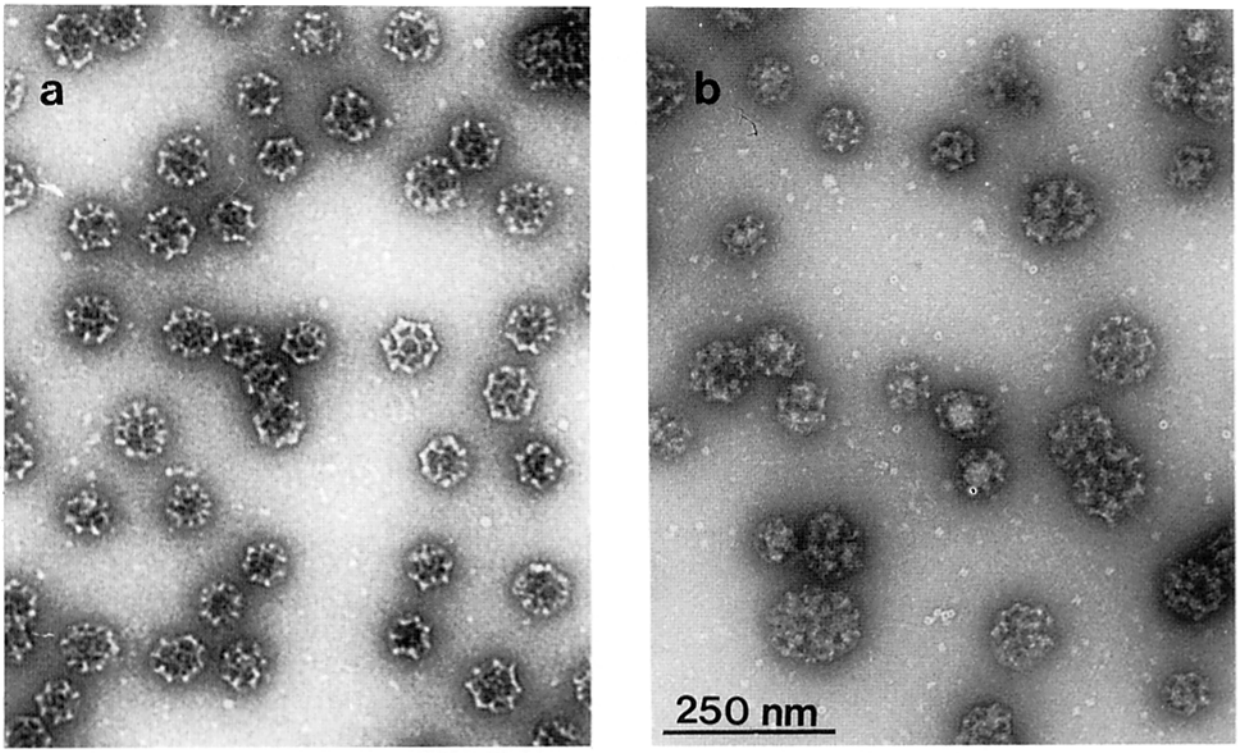

Figure 7. Electron microscopy of reassembled cages. Clathrin triskelia were assembled either in the presence of AP 180 (*a*) or of auxilin (*b*). Note that auxilin-induced cages are less uniform in diameter.

clathrin sedimented together with auxilin in the position expected for clathrin cages (Fig. 6 *a*). 31% of the clathrin remained unassembled, while 24% formed large aggregates that were pelleted by low speed centrifugation. 64% of auxilin sedimented with clathrin in the position of cages, and 28% were found in the low speed pellet. The remainder was recovered from the top of the gradient.

Electron microscopy of negatively stained aliquots of the dialysate confirmed that auxilin functions as a clathrin assembly protein (Fig. 7). Compared to the very homogenous population of AP 180-induced cages, 85% of which have a diameter in the range of 60–79 nm, the size distribution of auxilin induced cages was much broader (Fig. 8). Only 55% of the cages were within the 60–79-nm range. Auxilin-induced cages also appeared to be more obviously filled or decorated with protein. In this respect, they resemble the cages assembled in the presence of the HA2 adaptor (see Fig. 7 in reference 22). We also investigated the binding of auxilin to clathrin cages, preassembled in the presence of 2 mM calcium. Since auxilin proved to be exceptionally prone to aggregation below pH 7.0, the binding experiment was performed at pH 7.2 in a Hepes/Tartrate buffer. A constant amount of clathrin cages (9 μg) were incubated with increasing amounts of auxilin for 30 min on ice and then ultracentrifuged to separate bound from unbound auxilin. Pellets and supernatants were analyzed by SDS-PAGE (Fig. 9). In the absence of clathrin, auxilin does not sediment under the conditions employed for the binding assay (compare tracks 19 and 20 in Fig. 9). Densitometric analysis of the titration experiment shows that binding of auxilin to clathrin cages saturates at three auxilin molecules per clathrin triskelion, corresponding to one auxilin per clathrin heavy chain (Fig. 10).

Structural Characterization of Auxilin

To determine whether auxilin is structurally related to the two other known clathrin-binding constituents, AP 180 and the β-subunit of the HA2 adaptor complex, peptide maps (5) of the three were compared. Auxilin and AP 180 were purified by immunoprecipitation and SDS-PAGE. The β subunit was obtained by electrophoresis of the HA2 adaptor

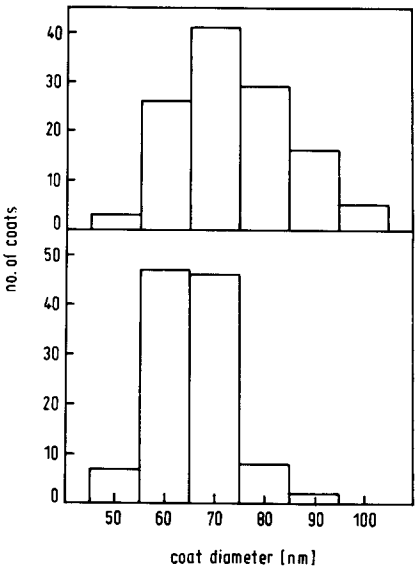

Figure 8. Histogram of coat diameters. Cages were reassembled either in the presence of auxilin (*top*) or of AP 180 (*bottom*).

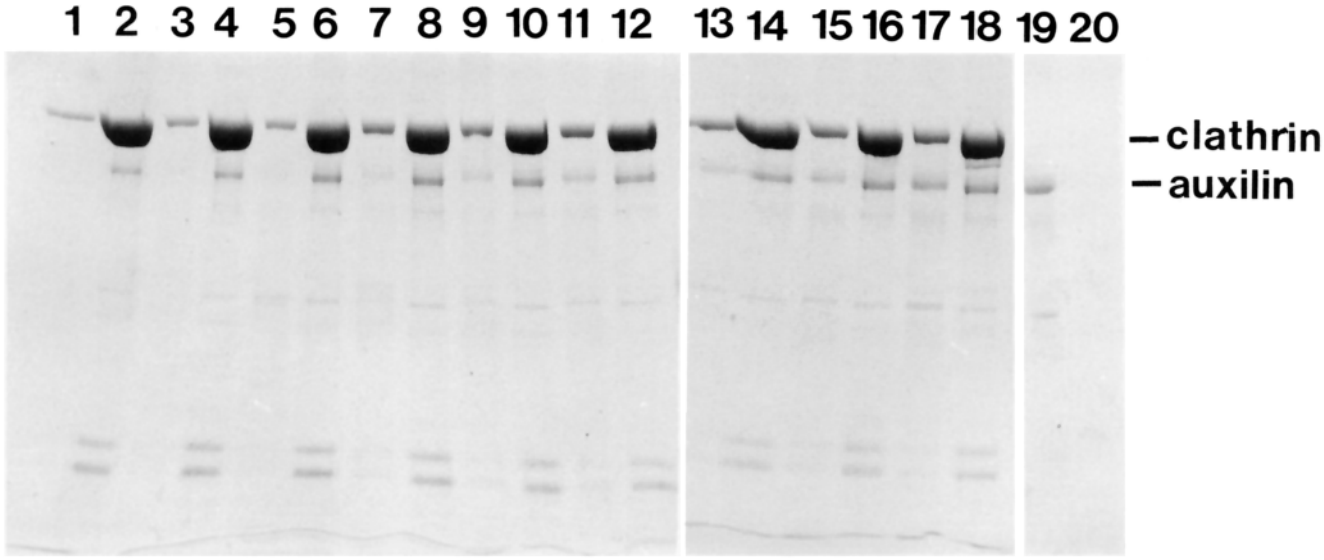

Figure 9. Interaction of auxilin with preformed clathrin cages. 1.6–7.6 μg of auxilin was incubated in binding buffer either alone or with 9 μg clathrin cages and then centrifuged for 15 min at 88,000 *g* in an ultracentrifuge (TL-100; Beckman Instruments). The protein content of pellets and supernatants was analyzed by SDS-PAGE. Odd numbers denote tracks of supernatants and even numbers denote the tracks of the corresponding pellets. Note that auxilin did not sediment when clathrin was omitted (lane *20*).

in urea-containing SDS gels. All proteins were iodinated in the gel and eluted after digestion with trypsin. The iodinated peptides were analyzed by two-dimensional peptide mapping on thin layer plates (Fig. 11). As with the immunological criterion, there is no extensive structural similarity between AP 180, auxilin and the β subunit of HA2, although we cannot totally exclude a limited homology of peptides in the part of the map bracketed in Fig. 11. We also mapped the 64 and 43 kD polypeptides, which copurified with auxilin. Both maps were very similar to that of auxilin, which confirms their identity as proteolytic fragments of auxilin (data not shown).

Molecular mass determinations of polypeptides by SDS-PAGE sometimes give erroneous results (31). This is true of AP 180, which migrates as a 180-kD polypeptide in SDS-PAGE (2, 34), but was shown by three other methods to have a molecular mass of ~120,000 (2, 25). We therefore determined the molecular mass of the reduced and unfolded auxilin by gel filtration on a calibrated Superose column in 6 M guanidinium hydrochloride (17). The elution volume corresponded to a protein of 86,000 mol wt (Fig. 12). We also deduced the molecular weight of native auxilin from its Stokes radius and sedimentation coefficient. For the determination of the Stokes radius, auxilin was chromatographed on a calibrated Superose 12 gel filtration column equilibrated in 0.5 M Tris. The Stokes radius was found to be 62 Å (Fig. 13), which is close to that of the HA2 adaptor, but much smaller than that of AP 180 (81 Å, reference 1). By centrifugation in calibrated sucrose gradients, we obtained a sedimentation coefficient of 3.2 S (not shown). Assuming a partial specific volume of 0.73 ml/g, we obtain a molecular weight of 84,000, in good agreement with the value from gel filtration of the unfolded chain. Thus, the value determined by SDS gel electrophoresis is wrong and, in 0.5 M Tris, auxilin is like AP 180 released as a monomer from the coated vesicle membrane.

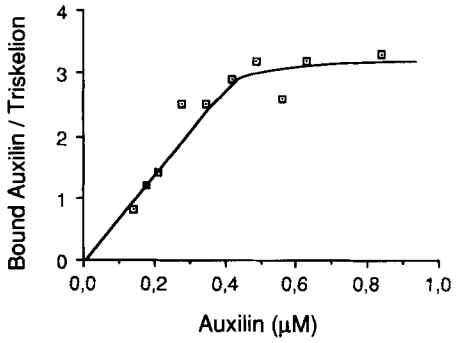

Figure 10. Binding of auxilin to preformed clathrin cages is saturable. Binding data were obtained from densitometry of SDS-PAGE lanes such as those shown in Fig. 9. The data from two independent titration experiments are shown.

Distribution of Auxilin

Bovine brain, liver, and adrenal gland were examined by immunoblotting for the presence of auxilin. Immunologically detectable amounts of auxilin were found only in brain homogenates (Fig. 14). Thus, it seems likely that auxilin like AP 180, is confined to neuronal tissue (3, 19). This conclusion is also supported by our failure to detect auxilin in coated vesicles from placenta and adrenal gland by biochemical means (data not shown). Quantitative immunoprecipitation of auxilin in brain homogenates showed that ~45% of auxilin is associated with membranes while 55% was found in the cytosolic fraction. This ratio is similar to that obtained for the adaptors in brain (data not shown).

Discussion

With the aid of electrophoresis methods and a panel of monoclonal antibodies against coated vesicle coat proteins, we dis-

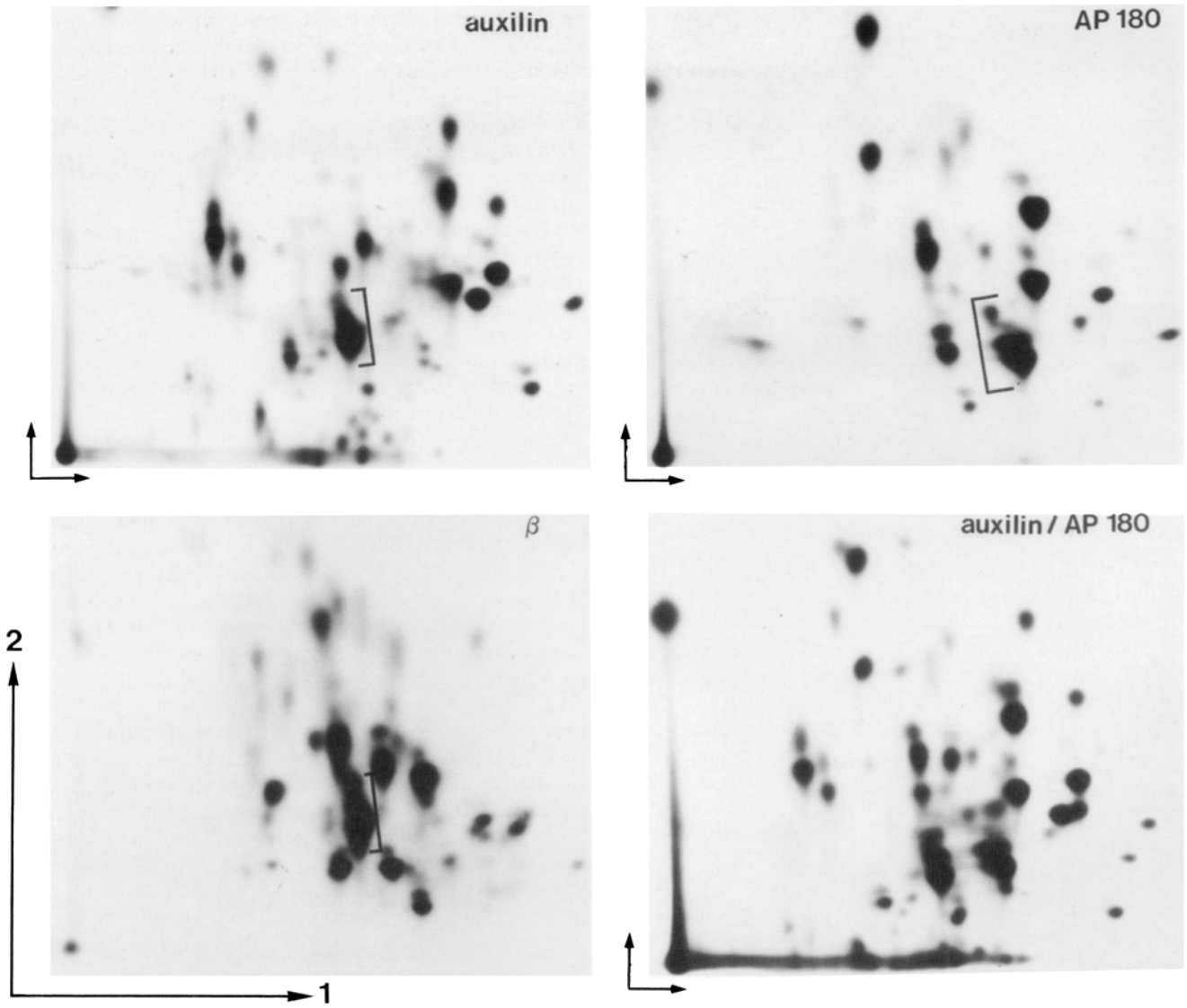

Figure 11. Autoradiographs of two-dimensional peptide maps of ^{125}I-labeled tryptic peptides from auxilin, AP 180, and the β subunit of the HA2 adaptor. Tryptic peptides were separated on cellulose thin layer plates by electrophoresis at pH 3.5 in the first dimension and ascending chromatography in the second. Brackets indicate peptides that could be common to all three proteins.

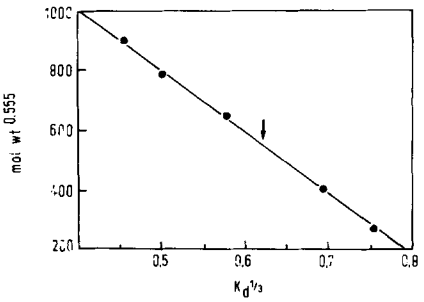

Figure 12. Molecular weight of auxilin. Total coat protein unfolded in 6 M guanidinium-HCl, 2 mM EDTA, and 1 mM DTT was applied to a calibrated Superose 6 gel filtration column. The elution volume of auxilin which was determined by SDS-PAGE and immunoblotting (see Materials and Methods for details) corresponded to a molecular weight of 86,000. The column was calibrated with myosin (210 kD), thyroglobulin (165 kD), β-galactosidase (116 kD), rabbit immunoglobulins heavy (55 kD), and light chains (25 kD). The arrow denotes the elution position of auxilin. K_d, Partition coefficient of the protein between the mobile and stationary phases.

covered a new 86,000 D coat protein, which we have named auxilin. We have identified it as a component of clathrin-coated vesicles on the grounds that it co-migrates with them in differential centrifugation and in free flow electrophoresis and moreover that it interacts stoichiometrically with clathrin in vitro. Auxilin is a peripheral membrane protein, which is extracted from the coated vesicle membrane with 0.5 M Tris together with clathrin, the adaptor complexes and AP 180. Auxilin represents in coated vesicle from brain tissue ~9% of the total 100–120-kD polypeptides. This corresponds to one auxilin per HA1 adaptor, to three molecules

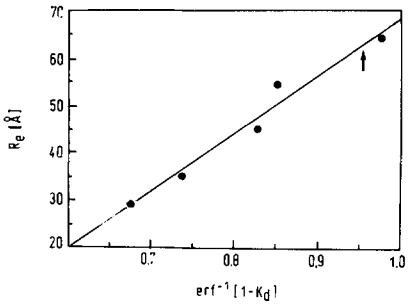

Figure 13. Stokes radius of auxilin in 0.5 M Tris. The apparent Stokes radius of auxilin (R_e) was determined by gel filtration on a calibrated Superose 12 column in 0.5 M Tris, 2 mM EDTA, pH 7.0. The column was calibrated with apoferritin (64 Å), rabbit immunoglobulin (55 Å), aldolase (45 Å), BSA (35 Å), and ovalbumin (29 Å). The elution volume of auxilin, which was determined by immunoblotting, corresponded to a Stokes radius of 62 Å. The arrow denotes the elution volume of auxilin. K_d, Partition coefficient for the protein between the mobile and stationary phases.

of AP 180, or to five molecules of the HA 2 adaptor. Thus, auxilin cannot be regarded as a particular minor component of coated vesicles. Auxilin supports assembly of clathrin into polygonal cages that are similar in size to those induced by AP 180. The mechanism by which auxilin induces clathrin assembly has not been investigated, but it could be related to its tendency to self-associate under conditions that also favor clathrin assembly. For example, if clathrin-bound auxilin were able to form dimers, cross-linking of adjacent triskelion legs would result. The ratio of one auxilin per clathrin heavy chain found in cages is compatible with a model of this kind.

Auxilin is immunologically unrelated to any of the other proteins in clathrin coated vesicles. Peptide mapping has excluded any major similarity to the β subunit of the HA2 adaptor and AP 180, which are both known to interact directly with clathrin (1, 2, 25), although we clearly cannot entirely rule out limited homologies between these proteins. Unless the three proteins bind to different sites on clathrin, local homologies would not be unexpected, but, apart from a set of two to three poorly resolved peptides, which may be common to all three proteins, we do not yet have any indications to this effect. Furthermore, peptide maps of α and γ subunits of the adaptors (see Figs. 5 and 6 in reference 3) show no significant homologies to auxilin.

The purification of two presumably different polypeptides with clathrin assembly promoting properties in the molecular mass range of 100–110 kD (as judged by their mobility on standard SDS-PAGE) has been described in reports by Edelhoch and his co-workers (8, 26). Based on the available information, however, it is difficult to relate them unambiguously either to the subunits of the adaptor complexes or to auxilin. Both proteins were exposed to 2 or 3 M urea during purification, conditions known to cause dissociation of adaptor subunits (1). However, on the basis of physical properties such as sedimentation constants and molecular weight, it appears very unlikely that the 114,000-D protein, which was studied by Prasad and colleagues in 3 M urea (26), is auxilin. A 110-kD polypeptide, extracted with 2 M urea from bovine brain–coated vesicles and then purified on lysine-Sepharose was not further characterized, and could thus have been a liberated adaptor subunit, auxilin, or an unrelated species (8).

Although distinct proteins, AP 180 and auxilin have more in common with each other than with the structurally more complex adaptors. Both appear to be restricted to neuronal tissue (3, 19); they are released from coated vesicles by 0.5 M Tris as monomers (2), behave anomalously in SDS gel electrophoresis (2, 25, 35), are very susceptible to proteolytic attack (2), and have very low sedimentation constants, suggestive of an extended structure for both. We therefore hesitate to equate auxilin and AP 180 with additional adaptors. The apparent restriction of both proteins to neuronal tissue implies that coated vesicles from other tissues function without them. This then affords another example of the adaptation of coated vesicles to specific requirements of neuronal

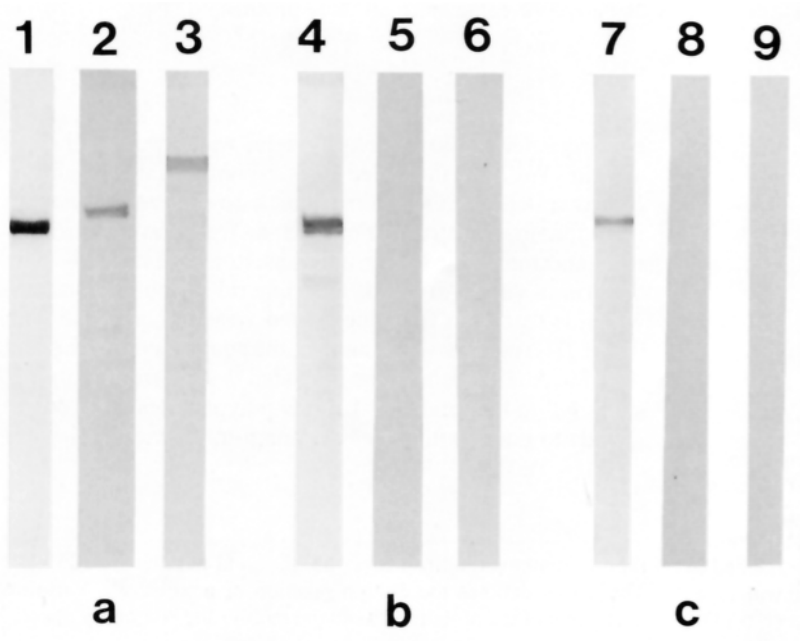

Figure 14. Tissue specificity of auxilin. Crude membranes from brain (*a*), liver (*b*), and adrenal gland (*c*) were extracted with 0.5 M Tris and then fractionated by SDS-PAGE. Nitrocellulose replicas of the gel were reacted with antibodies against the β and β' adaptor subunits (tracks 1, 4, and 7), antiauxilin (tracks 2, 5, and 8), and anti-AP 180 (tracks 3, 6, and 9). Note that auxilin and AP 180 immunoreactivity appears only in brain tissue.

cells. The light chains and the α subunit of the HA2 adaptor have already been shown to contain brain-specific inserts (9, 14, 15, 28). The functions of auxilin and AP 180 are unlikely to be restricted to promoting clathrin assembly. This property might be only a reflection of their preference for assembled over free clathrin. The substoichiometric amounts of both proteins in coated vesicle preparations (relative to clathrin) suggests a restriction to subpopulations of coated vesicles, in which either occurs in stoichiometric amounts. We hope to prove the existence of these with available monoclonal antibodies either by immunopurification of a subpopulation of coated vesicles or by immunoelectronmicroscopy.

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References

1. Ahle, S., and E. Ungewickell. 1989. Identification of a clathrin binding subunit in the HA2 adaptor protein complex. *J. Biol. Chem.* 264:20089–20093.
2. Ahle, S., and E. Ungewickell. 1986. Purification and properties of a new clathrin assembly protein. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:3143–3149.
3. Ahle, S., A. Mann, U. Eichelsbacher, and E. Ungewickell. 1988. Structural relationships between clathrin assembly proteins from the Golgi and the plasma membrane. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:919–929.
4. Campbell, C., J. Squicciarini, M. Shia, P. Pilch, and R. Fine. 1984. Identification of a protein kinase as an intrinsic component of rat liver coated vesicles. *Biochemistry.* 23:4420–4426.
5. Elder, J. H., R. A. Pickett, J. Hampton, and R. A. Lerner. 1977. Radioiodination of proteins in single polyacrylamide gel slices. *J. Biol. Chem.* 252:6510–6515.
6. Glickman, J. N., E. Conibear, and B. M. F. Pearse. 1989. Specificity of binding of clathrin adaptors to signals on the mannose 6-phosphate/insulin-like growth factor II receptor. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:1041–1047.
7. Goldstein, J. L., M. S. Brown, R. G. W. Anderson, D. W. Russel, and W. J. Schneider. 1985. Receptor-mediated endocytosis: concepts emerging from the LDL-receptor system. *Annu. Rev. Cell Biol.* 1:1–39.
8. Irace, G., R. E. Lippoldt, H. Edelhoch, and P. K. Nandi. 1982. Properties of clathrin coat structures. *Biochemistry.* 21:5764–5769.
9. Jackson, A. P., H. F. Seow, N. Holmes, K. Drickamer, and P. Parham. 1987. Clathrin light chains contain brain-specific insertion sequences and a region of homology with intermediate filaments. *Nature (Lond.).* 226:154–159.
10. Keen, J. H. 1987. Clathrin assembly proteins: affinity purification and a model for coat assembly. *J. Cell Biol.* 105:1989–1998.
11. Keen, J. H., M. C. Willingham, and I. Pastan. 1979. Clathrin coated vesicles: isolation, dissociation, and factor-dependent reassociation of clathrin baskets. *Cell.* 16:303–312.
12. Kirchhausen, T., and S. C. Harrison. 1981. Protein organization in clathrin trimers. *Cell.* 23:755–761.
13. Kirchhausen, T., S. C. Harrison, P. Parham, and F. M. Brodsky. 1983. Location and distribution of the light chains in clathrin trimers. *Proc. Natl. Acad. Sci. USA.* 80:2481–2485.
14. Kirchhausen T., P. Scarmato, S. C. Harrison, J. J. Monroe, E. P. Chow, R. J. Mattaliano, K. L. Ramachandran, J. E. Smart, A. H. Ahn, and J. Brosius. 1987. Clathrin light chains LCA and LCB are similar, polymorphic and share repeated heptad motifs. *Science (Wash. DC).* 230:320–324.
15. Kirchhausen, T., K. L. Nathanson, W. Matsui, A. Vaisberg, E. P. Chow, C. Burne, J. H. Keen, and A. E. Davis. 1989. Structural and functional division into two domains of the large (100–115 kDa) chains of the clathrin-associated protein complex AP-2. *Proc. Natl. Acad. Sci. USA.* 86:2672–2676.
16. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.
17. Mann, K. G., and W. W. Fish. 1972. Protein polypeptide chain molecular weights by gel chromatography in guanidinium chloride. *Methods Enzymol.* 26:28–41.
18. Morris, S. A., K. Hannig, and E. Ungewickell. 1988. Rapid purification of clathrin-coated vesicles by free flow electrophoresis. *Eur. J. Cell Biol.* 47:251–258.
19. Morris, S. A., S. Ahle, and E. Ungewickell. 1989. Clathrin coated vesicles. *Curr. Opinion Cell Biol.* 1:684–690.
20. Pearse, B. M. F. 1975. Coated vesicles from pig brain: purification and biochemical characterization. *J. Mol. Biol.* 97:93–98.
21. Pearse, B. M. F. 1987. The EMBO medal review: clathrin and coated vesicles. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:2507–2512.
22. Pearse, B. M. F. 1988. Receptors compete for adaptors found in plasma membrane coated pits. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:3331–3336.
23. Pearse, B. M. F., and M. S. Bretscher. 1981. Membrane recycling by coated vesicles. *Annu. Rev. Biochem.* 50:85–101.
24. Pearse, B. M. F., and M. S. Robinson. 1984. Purification and properties of 100kD proteins from coated vesicles and their reconstitution with clathrin. *EMBO (Eur. Mol. Biol. Organ). J.* 3:1951–1957.
25. Prasad, K., and R. E. Lippoldt. 1988. Molecular characterization of the AP180 coated vesicle assembly protein. *Biochemistry.* 27:6098–6104.
26. Prasad, K., T. Yora, O. Yano, R. E. Lippoldt, H. Edelhoch, and H. Saroff. 1986. Purification and characterization of a mol wt 100,000 coat protein from coated vesicles obtained from bovine brain. *Biochemistry.* 25:6942–6947.
27. Robinson, M. S. 1987. 100-kD coated vesicle proteins: molecular heterogeneity and intracellular distribution studied with monoclonal antibodies. *J. Cell Biol.* 104:887–895.
28. Robinson, M. S. 1989. Cloning of cDNAs encoding two related 100-kD coated vesicle proteins (α adaptins). *J. Cell Biol.* 108:833–842.
29. Schmidt, S. L., and J. E. Rothman. 1985. Enzymatic dissociation of clathrin cages in a two stage process. *J. Biol. Chem.* 260:10044–10049.
30. Scopes, R. K. 1974. Measurement of protein by spectrophotometry at 205nm. *Anal. Biochem.* 59:277–282.
31. See, Y. P., and G. Jackowski. 1989. Estimating molecular weights of polypeptides by SDS gel electrophoresis. *In* Protein Structure, A Practical Approach. T. E. Creighton, editor. IRL Press Limited, Oxford. 1–21.
32. Siegel, L. M., and K. J. Monty. 1966. Determination of molecular weights and frictional ratios of proteins in impure systems by use of gel filtration and density gradient centrifugation. Application to crude preparations of sulfite- and hydroxylamine reductase. *Biochim. Biophys. Acta.* 112:346–362.
33. Unanue, E. R., E. Ungewickell, and D. Branton. 1981. The binding of clathrin triskelions to membranes from coated vesicles. *Cell.* 26:439–446.
34. Ungewickell, E., and D. Branton. 1981. Assembly units of clathrin coats. *Nature (Lond.).* 289:420–422.
35. Ungewickell, E., and L. Oestergaard. 1989. Identification of the clathrin assembly protein AP180 in crude calf brain extracts by two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Anal. Biochem.* 179:352–356.
36. Virshup, D. M., and V. Bennett. 1988. Clathrin-coated vesicle assembly polypeptides: physical properties and reconstitution studies with brain membranes. *J. Cell Biol.* 106:39–50.