Clathrin-coated Vesicle Assembly Polypeptides: Physical Properties and Reconstitution Studies with Brain Membranes

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Abstract. The assembly polypeptides are an integral component of coated vesicles and may mediate the linkage of clathrin to the vesicle membrane. We have purified assembly polypeptides in milligram quantities from bovine brain by an improved procedure. Hydrodynamic and chemical crosslinking studies indicate that the protein is an asymmetric heterotetramer with a molecular weight of 252,000, containing two subunits of M_r 98,000-115,000, one subunit of 52,000, and one subunit of 16,000. Two-dimensional peptide maps of the subunits show that the 16- and 52-kD polypeptides are not derived from the higher molecular weight species, and that the group of bands at 98-115 kD are related. Electron microscopic visualization shows an essentially globular protein with one or two knob-like tails.

We demonstrate a specific membrane protein binding site for ^{125}I-labeled assembly polypeptides in 0.1 N sodium hydroxide–extracted bovine brain membranes based on the following criteria: (a) binding is displaceable by unlabeled ligand, (b) the binding site is destroyed by protease treatment of the membranes, and (c) the distribution of binding between vesicle-depleted membranes and coated vesicle membranes parallels the in vivo localization of assembly polypeptides and clathrin. This binding site is likely to be an integral membrane protein because (a) it is enriched in the sodium hyroxide–extracted membranes stripped of most of their peripheral membrane proteins, and (b) the binding site is partially extracted by 0.5% Triton X-100. A similar binding site appears to be present in coated vesicles. Clathrin binds to the hydroxide-stripped membranes in an assembly polypeptides dependent manner, and this binding is diminished by Triton extraction of the membranes. This assay may aid in identification of the membrane receptor for the assembly polypeptides.

THE nature of the linkage between membrane receptors and the intracellular structures responsible for their sorting, localization, movement, and processing is a central question in cell biology. Receptor-mediated endocytosis is one of the best studied examples of this process. Morphologic studies have shown that membrane receptors cluster in the plane of the membrane, acquire a clathrin-containing coat, and invaginate to form a clathrin-coated vesicle. These vesicles fuse in the cytoplasm, and the contents are sorted for delivery to a variety of intracellular destinations. The intermediates in this pathway, the clathrin-coated vesicle and its constituent proteins, have been extensively studied (3, 18, 22, 35, 38).

The assembly polypeptides are an integral component of the clathrin-coated vesicles, which were originally identified on the basis of their ability to stimulate clathrin cage formation in vitro (24, 47). Several lines of evidence suggest that the assembly polypeptides may serve as the linkage between membrane receptors and the clathrin coat in coated pits and vesicles, and, as such, may be important both in regulation of endocytosis and the sorting of some membrane proteins. First, clathrin can be released from coated vesicles in vitro under conditions that do not release the assembly polypeptides (2, 44). Second, clathrin can then rebind to these vesicles unless (a) the assembly polypeptides are also removed with buffers such as 1 M Tris (29), (b) the vesicles are treated with low concentrations of elastase which selectively cleave the assembly polypeptides subunits at 98-115 kD (hereafter referred to as the 100-kD subunit) (44), or (c) anti–assembly polypeptide antibody is present (32). Third, the assembly polypeptides have also been demonstrated in three-dimensional reconstructions of clathrin cages and coated vesicles in vitreous ice to lie between the clathrin shell and the receptor-containing vesicle membrane (45, 46). Last, several groups have demonstrated that the isolated 100-kD subunits

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can bind directly to clathrin and stimulate clathrin cage formation (1, 40, 48).

Clathrin is found in multiple sites within the cell and is thought to participate in many different transport processes. In fibroblasts in tissue culture, coated pits cover 2% of the plasma membrane (31). Immunofluorescence studies by Robinson (41) and Robinson and Pearse (42) have demonstrated colocalization of assembly polypeptides with clathrin in perinuclear Golgi complex and in coated pits on cell membranes in addition to their presence in coated vesicles from which they were originally purified. These studies also suggest that distinct subsets of assembly polypeptides are found in different parts of the cell. A recent study by Anderson and co-workers (31) also found that assembly of coated pits on stripped fibroblast membranes was coincident with the selective adherence of clathrin and a 110-kD protein from cytosol. Thus, it appears likely that assembly polypeptides are important in clathrin coat formation throughout the cell in addition to their well-recognized role in endocytic vesicles.

As an approach to understanding the link between clathrin and membrane receptors, we have purified the assembly polypeptides from a high speed pellet of bovine brain cytosol. Assembly polypeptides thus purified have a molecular weight of 252,000, can be chemically crosslinked to give a complex of M_r 260,000, and have a subunit composition of two 100-, one 52-, and one 16-kD polypeptides. These assembly polypeptides bind to a site on sodium hydroxide-extracted brain membranes that is protease sensitive and can be partially extracted with 0.5% Triton X-100. Clathrin binding to these membranes is dependent on the presence of assembly polypeptides.

Materials and Methods

Carrier-free Na^{125}I was from Amersham Corp. (Arlington Heights, IL). ^{125}I-Bolton Hunter reagent was from ICN Radiochemicals (Irvine, CA). Plastic-backed 0.1-mm cellulose TLC sheets were from Merck & Co., Inc. (Rahway, NJ). α-Chymotrypsin (54 U/mg) was from Worthington Biochemical Corp. (Freehold, NJ). Diisopropylfluorophosphate (DFP), leupeptin, pepstatin A, phenylmethylsulfonyl fluoride (PMSF), dithiothreitol (DTT), EGTA, Tris base, and Tween 20 were from Sigma Chemical Co. (St. Louis, MO). Triton X-100 and hydroxylapatite were from Boehringer Mannheim Diagnostics (Houston, TX). Cyanogen bromide-activated Separose 4B, Sephacryl S-400, and protein A were from Pharmacia Fine Chemicals (Piscataway, NJ). Gel filtration and sedimentation standards were from Boehringer Mannheim Diagnostics and Pharmacia Fine Chemicals. BSA was from Miles Laboratories Inc. (Naperville, IL). Sucrose and urea were from Schwarz/Mann (Spring Valley, NY). Polyethylene glycol 8000 was from Fisher Scientific Co. (Pittsburgh, PA). Electron microscopy supplies were from Ernest F. Fullam, Inc. (Schenectady, NY), Ted Pella, Inc. (Irvine, CA), and Eastman Kodak Co. (Rochester, NY). Crosslinking agents ethylene glycol succinimidyl succinate (EGS) and dithiosuccinimidyl propionate (Lomant's reagent) (27) were from Pierce Chemical Co. (Rockford, IL). Bradford reagent, nitrocellulose, molecular weight standards, and other electrophoresis reagents were from Bio-Rad Laboratories (Richmond, CA). Bovine brains were obtained from a local slaughterhouse within 30 min of death, stripped of meninges, and rinsed in 0.32 M sucrose; the cerebral cortex was then frozen in liquid nitrogen and stored at −90°C. Frozen brain was used within 6 wk.

Methods

Protein determinations were performed by the method of Lowry (28) or Bradford (7) with BSA as a standard. Phospholipid determinations were by

1. *Abbreviations used in this paper*: DFP, diisopropylfluorophosphate; EGS, ethylene glycol succinimidyl succinate; HA, hydroxylapatite; ^{125}I-assembly polypeptides and ^{125}I-AP, ^{125}I-Bolton-Hunter–labeled assembly polypeptides.

the method of Duck-Chong (14). SDS-polyacrylamide discontinuous gel electrophoresis was generally performed on 5–15% linear gradient gels (25) using Bio-Rad Laboratories and Bethesda Research Laboratories (Gaithersburg, MD) molecular weight standards. For the separation of crosslinked proteins, 3.5–17% exponential gradient continuous gels with the buffers of Fairbanks (16) were used, with human erythrocyte ghosts as molecular weight standards.

Purified assembly polypeptides were iodinated with Bolton-Hunter reagent (6). Autoradiography was performed at −80°C on Kodak X-Omat AR film using Cronex intensifier screens (DuPont Co., Wilmington, DE).

Electron microscopy was performed as previously described (17). Assembly polypeptides at 10 μg/ml were applied to glow-discharged Formvar-coated grids and negatively stained with 0.75% uranyl formate. For shadowed specimens, protein was suspended in 0.1 M NH$_4$COOH, 30% glycerol, sprayed onto freshly cleaved mica, and platinum/carbon shadowed in a P650A vacuum coater (Polaron Instruments Inc., Hatfield, PA) at pressures <5 × 10^{-7} torr. Unidirectional shadowing was performed from 10° and rotary shadowing from 6°. Horse spleen ferritin was included in some fields as an internal standard.

Physical Properties

Sedimentation coefficients were estimated by rate-zonal sedimentation of ^{125}I assembly polypeptides on 5–20% sucrose gradients in 10 mM sodium phosphate, 50 mM NaCl, 1 mM EGTA, 1 mM DTT, 1 mM NaN$_3$, pH 7.3, in an SW 50.1 rotor (Beckman Instruments, Inc., Palo Alto, Ca) (30), with beef liver catalase (11.3 S$_{20,w}$), rabbit muscle aldolase (7.3 S$_{20,w}$), BSA (4.6 S$_{20,w}$), and horse heart cytochrome c (1.75 S$_{20,w}$) as standards. The Stokes radius (R_s) was estimated by gel filtration of ^{125}I assembly polypeptides on a Pharmacia Superose 6B FPLC column in the same buffer with 0.02% Tween 20, calibrated with aldolase (48 Å), catalase (52 Å), horse spleen ferritin (61 Å), and *Escherichia coli* β-galactosidase (68 Å), using a standard curve of (-log K_{av})$^{1/2}$ versus R_s (26, 43). Amino acid analysis was performed on a Picotag system (Waters Instruments, Inc., Rochester, MN) by Dr. Richard Nakashima, Department of Biological Chemistry, Johns Hopkins University School of Medicine, Baltimore, MD.

Bovine brain clathrin was prepared by a modification of published methods (20). Antibody to the 100-kD band of the assembly polypeptides was produced in rabbits by standard methods (10) and affinity purified on an assembly polypeptide-Sepharose column.

Preparation of Coated Vesicles and Brain Membranes for Binding Assays

Coated vesicles were prepared by the method of Nandi et al. (34), and stripped by incubation in 1 M Tris, pH 7.0, for 30 min at room temperature. Stripped vesicles were collected by centrifugation at 100,000 g for 60 min at 3°C and washed twice in assay buffer without BSA and Tween. Brain membranes were prepared by homogenization of 20 g frozen bovine brain in 80 ml 0.32 M sucrose, 2 mM EGTA, 1 mM NaN$_3$, with 5 μg/ml leupeptin and pepstatin, 0.1% DFP, and 200 μg/ml PMSF. After pelleting nuclei and large debris at 900 g for 10 min, the membranes were collected by centrifugation at 30,000 g for 15 min. The membranes were then demyelinated by washing in 1 M sucrose and pelleting at 30,000 g for 30 min; washed once in 10 mM sodium phosphate buffer, pH 7.2, with 1 mM EGTA, 1 mM DTT, 1 mM NaN$_3$, 1 mM MgCl$_2$; and stripped of peripheral membrane proteins by a 30-min exposure to 0.1 N NaOH as previously described (12). Extracted membranes were then collected by centrifugation at 30,000 g for 30 min through a 10% sucrose barrier in the 10 mM phosphate buffer followed by washing of the pellet with the phosphate buffer until the pH was at or below 7.4, and resuspension in one-half the starting volume. Only the membranes that pelleted through the 10% sucrose barrier were used. Both stripped vesicles and NaOH-extracted membranes were used only the day they were made. All membrane preparations and assays were performed at 4°C unless otherwise noted.

Binding Assays

Stripped vesicles or NaOH-extracted membranes (2–3 μg/assay) were incubated with ^{125}I-assembly polypeptides with constant gentle shaking for 3 h or as indicated in a final volume of 200 μl in assay buffer (10 mM sodium phosphate, pH 7.2, 50 mM NaCl, 1 mM EGTA, 1 mM DTT, 1 mM NaN$_3$, 0.012% Tween 20, 2 mg/ml BSA). 80-μl samples were then layered over a 200-μl 10% sucrose barrier in assay buffer in 400-μl Eppendorf polyethylene tubes and centrifuged at 5,000 g for 30 min in a swinging bucket rotor. The tubes were frozen in dry ice, the tips cut off, and membrane-bound
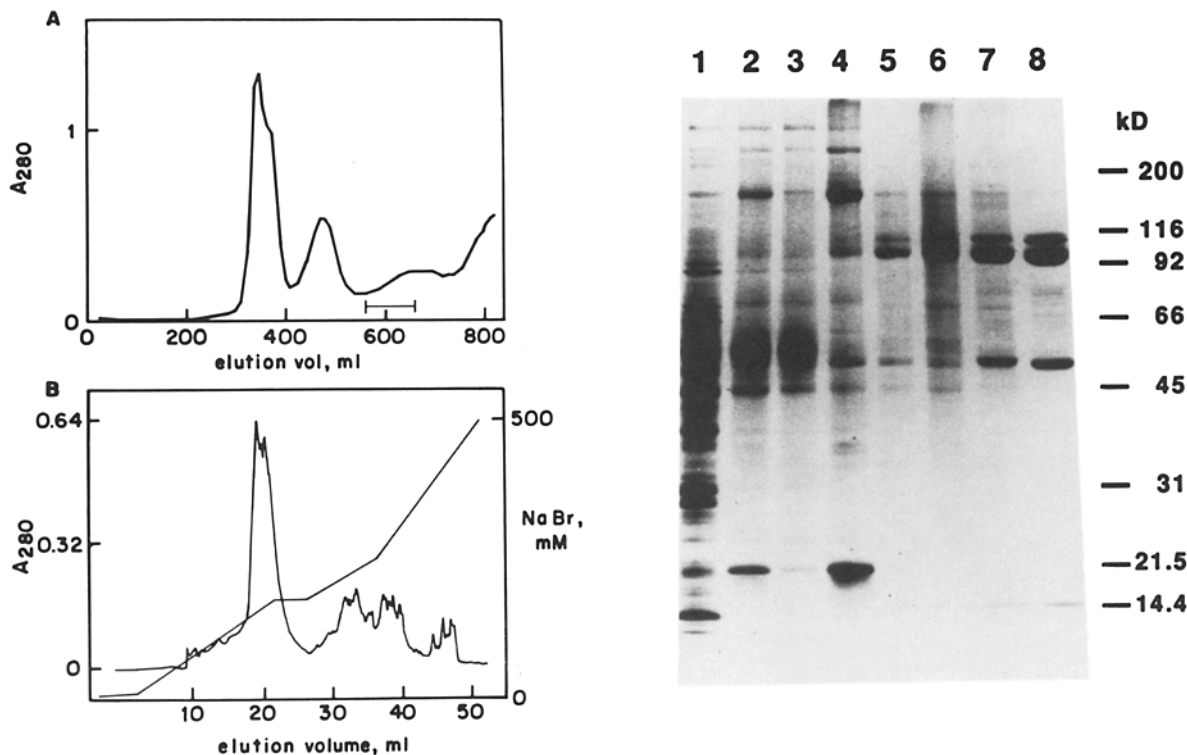
Figure 1. Purification of assembly polypeptides from bovine brain. 1 kg of previously frozen (−90°C) bovine brain cortex was thawed and homogenized in a total volume of 4 liters 50 mM sodium phosphate buffer, 1 mM EGTA, 1 mM DTT, 1 mM NaN₃, pH 6.8, with 2 μg/ml leupeptin and pepstatin A, 200 μg/ml PMSF, and 1:10,000 DFP. A 100,000 g pellet of cytosol was collected and washed three times in homogenization buffer. The second wash included 0.5% Triton X-100 and 1:10,000 DFP. Clathrin and associated proteins were solubilized by resuspension of the pellets in 1 M Tris-HCl, pH 7.0, with a 30-min incubation at room temperature. The stripped vesicles were removed by centrifugation at 100,000 g for 60 min, and the extracted proteins precipitated by addition of ammonium sulfate to 40% saturation after dilution of the supernatant with an equal volume of 1 mM DTT. The precipitated proteins were resuspended in 40 ml 0.5 M Tris, 0.5 M NaBr, 0.05% Tween 20, 1 mM EDTA, 1 mM DTT, 1 mM NaN₃, pH 7.0, applied to a 5 × 55-cm Sephacryl S-400 column pre-equilibrated with the same buffer, and eluted at 40 ml/h for 24 h (*A*). The assembly polypeptide-containing fractions (*A, brackets*) as assessed by SDS-PAGE, were pooled, made 2 mM in MgCl₂, and loaded onto a 1 g hydroxylapatite (HA) column. The HA was washed with 10 mM potassium phosphate, 0.5 M NaBr, 0.05% Tween 20, 1 mM MgCl₂, 1 mM DTT, 1 mM NaN₃, pH 6.7; contaminating proteins and the HA-I assembly polypeptides eluted with 75 mM potassium phosphate, and the HA-II fraction eluted with 150 mM potassium phosphate, in the same buffer. The 150 mM phosphate HA (HAP) peak was centrifuged at 130,000 g for 45 min and the supernatant diluted 11-fold in 1 mM EGTA, 10% sucrose, 0.5% Tween 20. This material (usually ∼120 ml) was loaded on a Pharmacia Mono-Q FPLC column and eluted with a 0–500 mM NaBr gradient in 10 mM sodium phosphate, pH 7.4, 0.5% Tween 20, 1 mM EGTA, 1 mM DTT, 1 mM NaN₃ (*B*). The assembly polypeptides were eluted at 120 mM NaBr and were generally better than 90–95% pure as judged by scanning of Coomassie Blue–stained SDS-polyacrylamide gels. All operations were carried out at 4°C unless otherwise noted. (*Right*) Lane *1*, crude cytosol; lane *2*, crude vesicle pellet; lane *3*, Tris-extracted crude vesicles; lane *4*, 1 M Tris extract from crude coated vesicles; lane *5*, assembly polypeptides fractions from S-400 column; lane *6*, 75 mM phosphate cut from HAP column; lane *7*, 150 mM phosphate cut from HAP column; lane *8*, assembly polypeptides peak from Mono-Q column.

Results

The assembly polypeptides were purified as described (Fig. 1) by selective extraction of a Triton-extracted crude vesicle fraction, gel filtration, hydroxylapatite chromatography, and finally, anion-exchange chromatography on a Mono-Q HPLC column. 1 kg of starting bovine brain cortex, from approximately four cows, gave 2–3 mg of assembly polypeptides in 90–95% purity as assessed by densitometric scanning of Coomassie Blue–stained gels. The complex consisted of sub-

units of M_r 98–115,000, 52,000, and 16,000, corresponding to the previously described HA-2 fraction (39). We found that dilution of the assembly polypeptides rather than dialysis into low salt buffers before loading onto the Mono-Q anion exchange column was essential for a good yield; otherwise, assembly polypeptide-containing aggregates formed.

The solution molecular weight and subunit stoichiometry of the assembly polypeptides were determined by three approaches: hydrodynamic properties, chemical crosslinking, and two-dimensional gel analysis of subunit ratios in reversibly crosslinked species. In contrast to previous studies (39, 48), we found that all three methods indicate the complex has a molecular mass of 250–260 kD and is composed of subunits in the ratio of two 100-kD, one 52-kD, and one 16-kD polypeptide. First, crosslinking studies in the presence (Fig. 2 *a, i*) and absence (Fig. 2 *a, ii*) of urea showed
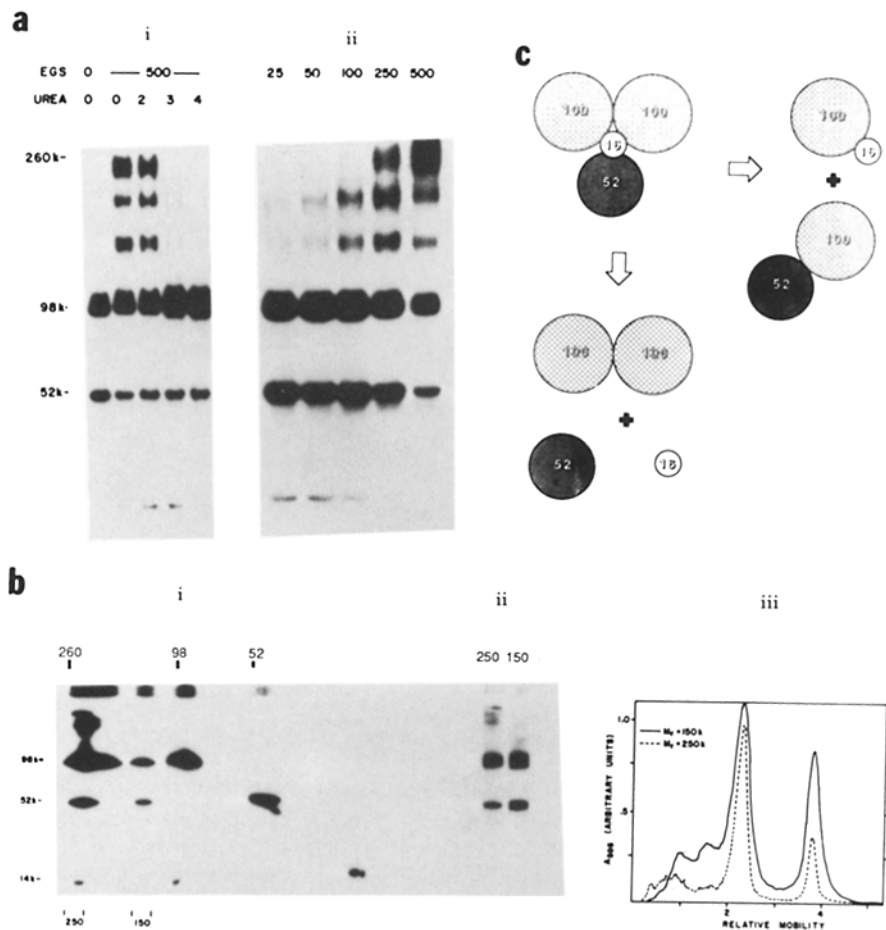
Figure 2. Crosslinking of ^{125}I-assembly polypeptides (*a*) Autoradiographs of 10 nM ^{125}I-assembly polypeptides cross-linked with EGS. In both cases, cross-linked protein was separated on a 3.5–17% exponential gradient SDS-polyacrylamide gel which was then dried and autoradiography performed. Human erythrocyte ghosts were used as molecular weight standards. (*i*) 10 nM ^{125}I-assembly polypeptides were incubated with 0, 2, 3, and 4 M urea for 30 min, followed by the addition of EGS to a final concentration of 500 µg/ml. The reaction was allowed to proceed for 30 min at room temperature, then quenched with the addition of glycine to 1 mg/ml. (*ii*) 10 nM ^{125}I-assembly polypeptides were incubated with from 25 to 500 µg/ml EGS for 30 min at room temperature, followed by the addition of glycine to 1 mg/ml. (*b*) Second dimension reducing gel of reversibly crosslinked assembly polypeptides demonstrates subunit stoichiometry. (*i*) ^{125}I-Assembly polypeptides were crosslinked with 500 µg/ml Lomant's reagent and separated on a 3.5–17% exponential gradient nonreducing continuous gel (16). The lane was excised, equilibrated with electrophoresis sample buffer with 50 mM DTT to reverse the crosslinking, and electrophoresed in the second dimension on a 5–15% linear gradient reducing gel (25). (*ii*) The autoradiograph lanes derived from the 260- and 150-kD crosslinked products were excised and (*iii*) the relative amounts of the 100- and 52-kD polypeptides derived from each estimated by densitometric scanning. (*c*) Proposed subunit structure. The hydrodynamic properties and crosslinking data suggest that the assembly polypeptides exist in solution as an ∼260,000 complex composed of two 100-kD, one 52-kD, and one 16-kD subunits. The formation of crosslinks between individual polypeptides is illustrated.

formation of species that migrated with apparent M_r of 150,000, 200,000, and 260,000 on a 3.5–17% exponential gradient gel that can resolve polypeptides up to M_r 1,000,000. No crosslinked species of M_r >260,000 were seen even at the highest crosslinker concentration. Similar results were seen both when a different chemical crosslinker, dithio-*bis*-succinimidyl propionate (Lomant's reagent) (27), was used as crosslinking agent and when unlabeled, as opposed to Bolton-Hunter labeled, protein was crosslinked. The crosslinking was blocked when the ^{125}I-assembly polypeptides were preincubated with urea at concentrations of 3 M and higher (Fig. 2 *a*, *i*), suggesting dissociation of the oligomer, as has been seen by others (40).

The crosslinked molecular weight of 260,000 is in excellent agreement with the solution molecular weight of 252,000 calculated from the Stokes radius of 66 Å, the sedimentation coefficient of 8.6 S, and the partial specific volume from amino acid analysis of 0.74 cm³/g (Table I). These figures are in good agreement with previously published values (39, 48). Similar hydrodynamic properties were obtained for the crosslinked protein as well, suggesting that no major distortion of configuration occurred upon crosslink-

ing. The subunit composition of the 100:52:16-kD polypeptides that best fits this molecular weight is 2:1:1.

Because crosslinked proteins may migrate anomalously, and the hydrodynamic M_r is subject to error, we further investigated the subunit composition of the crosslinked species using a cleavable chemical crosslinker. ^{125}I-Assembly polypeptides crosslinked with 500 µg/ml Lomant's reagent were subjected to two-dimensional SDS-PAGE (Fig. 2 *b*, *i*); the crosslinker's disulfide bond was reduced by an excess of DTT before the second dimension electrophoresis. The 260-kD species is composed of 100-, 52-, and 16-kD subunits, the 200-kD species is composed of 100-kD subunits only, and the 150-kD species is composed of 100- and 52-kD subunits. The 16-kD band crosslinked to a 100-kD band since migrating slightly slower than the 100-kD band in the first dimension is a species that in the second dimension contains both 100- and 16-kD bands.

The second dimension lanes were cut out of on-scale autoradiographs (Fig. 2 *b*, *ii*) and the ratio of 100- to 52-kD bands quantitated by scanning on a densitometry attachment of a spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, OH) (Fig. 2 *b*, *iii*). The 260-kD species con-

tains two 100-kD subunits per 52-kD subunit, and the 150-kD species contains one 100-kD per 52-kD band.

Since previous studies (39, 48) had determined subunit ratios by determination of relative dye binding, an attempt was made to quantitate polypeptide ratios in the purified protein by densitometric scanning of gels stained with various protein dyes. Results of this technique were variable and appeared to depend on length of time the gels stained and destained, as well as the stain used, suggesting differential dye binding by the subunits. Ratios of the 100:52-kD polypeptides stained in a polyacrylamide gel with amido black, ponceau S, fast green, and Coomassie Blue ranged from 1:1 to 2:1. The 16-kD subunit was consistently present in a 1:1 ratio with the 52-kD subunit (data not shown).

Peptide Mapping

To assess the relationship between the multiple subunits of the assembly polypeptides, two-dimensional chymotryptic peptide maps of ^{125}I-labeled protein were prepared. As Fig. 3 demonstrates, the 16- and 52-kD subunits contain only unique spots and are not derived from each other or from the higher molecular weight bands. The 98- and 110–115-kD bands produced maps that contain a large number of identical fragments (Fig. 3, c–f), strongly suggesting that the major peptides in each of these groups are highly related. One-dimensional peptide maps of three subsets of the 100-kD polypeptides have previously shown some homologies between two of the bands (42). Higher resolution separation of the 100-kD polypeptides may reveal groups of closely related polypeptides.

Table I. Physical Properties

Property	Native	Crosslinked
Stokes radius* ($\mathring{A}$)	66	64
Sedimentation coefficient‡ ($S_{20,w}$)	8.6	8.9
Partial specific volume§ (v)	0.74	0.74
M_r, calculated¶	252,000	252,900
Frictional ratio¶ (f/f_0)	1.36	1.32
M_r, SDS-PAGE	110,000	260,000
	98,000	200,000
	52,000	150,000
	16,000	

Stokes radius, sedimentation coefficient, and partial specific volume were determined as described (Materials and Methods). To obtain hydrodynamic data on chemically crosslinked protein, 10 nM ^{125}I-assembly polypeptides were first treated with 500 µg/ml EGS for 30 min as described in Results. M_r for crosslinked protein was estimated from SDS-PAGE on 3.5–17% exponential gradient gels with the buffers of Fairbanks et al. (16) using human erythrocyte ghost proteins as standards. The slowest migrating crosslinked product comigrated with spectrin α-chain.
* From gel filtration on Superose 6B.
‡ From sedimentation on 5–20% sucrose gradient.
§ Estimated from amino acid composition.
¶ Calculated from equations:

$$M_r = \frac{6\pi N R_s S_{20,w}}{1 - v\, \rho_{20,w}}$$

and

$$f/f_0 = R_s \left[\frac{4\pi N}{3 M_r (v + \delta/\rho)} \right]^{1/3},$$

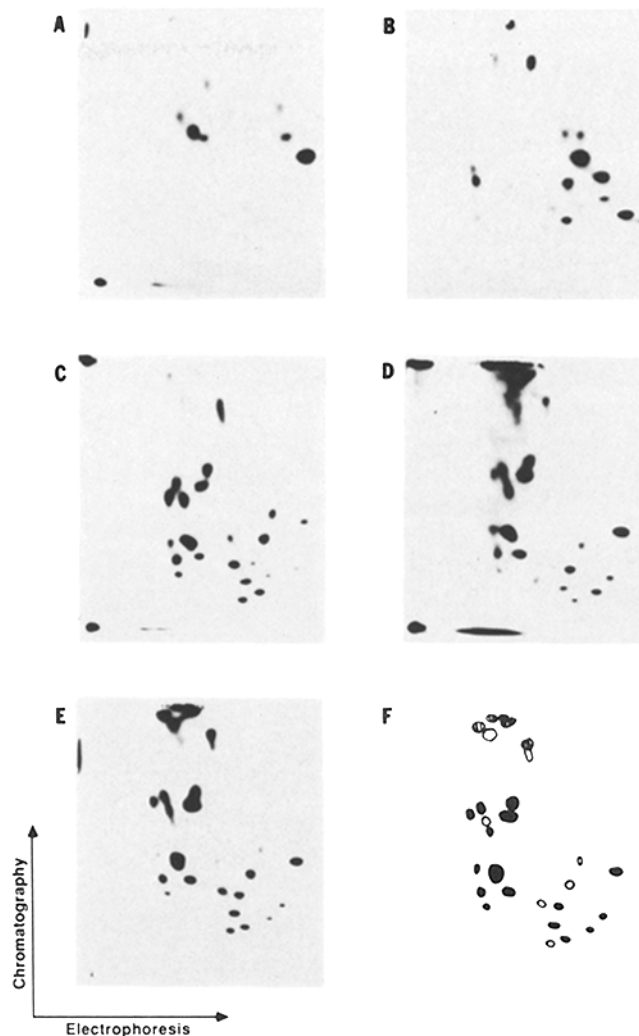where δ was assumed to be 0.4 g of solvent/g of protein.

Figure 3. Two-dimensional chymotryptic peptide maps of assembly polypeptides subunits. (A) 16-kD subunit; (B) 52-kD subunit; (C) 98-kD subunit; (D) 110–115-kD subunits; (E) mixture of 98 and 110–115 kD; (F) diagram indicating shared and unique spots in E, filled spots represent common spots, open and stripped circles indicate spots unique to the 98- and 110–115-kD subunits, respectively. Chymotryptic peptide maps of ^{125}I-labeled assembly polypeptides were prepared by chloramine-T iodination of assembly polypeptides in vitro followed by gel electrophoresis and chymotryptic digestion of the excised gel slices as previously described (11). C and D were also prepared by iodination of protein in excised gel slices by the method of Elder et al. (15), with equivalent results.

Electron Microscopy

Purified protein was visualized in the electron microscope by negative staining, rotary shadowing, and unidirectional shadowing (Fig. 4, Materials and Methods). The molecule appears to have a globular body ~70 Å in diameter with at least one knob-like tail (Fig. 4 D). In several views, two of these tails may be visible. The complex also appears capable of unfolding to form a more elongate structure. Assembly polypeptides with two tails have been seen by deep etching of the protein adsorbed to mica (Heuser, J., Washington University, and J. Keen, Temple University, personal communication); the difference may be due to increased sensitivity of the deep etch technique or a change in the structure of the protein
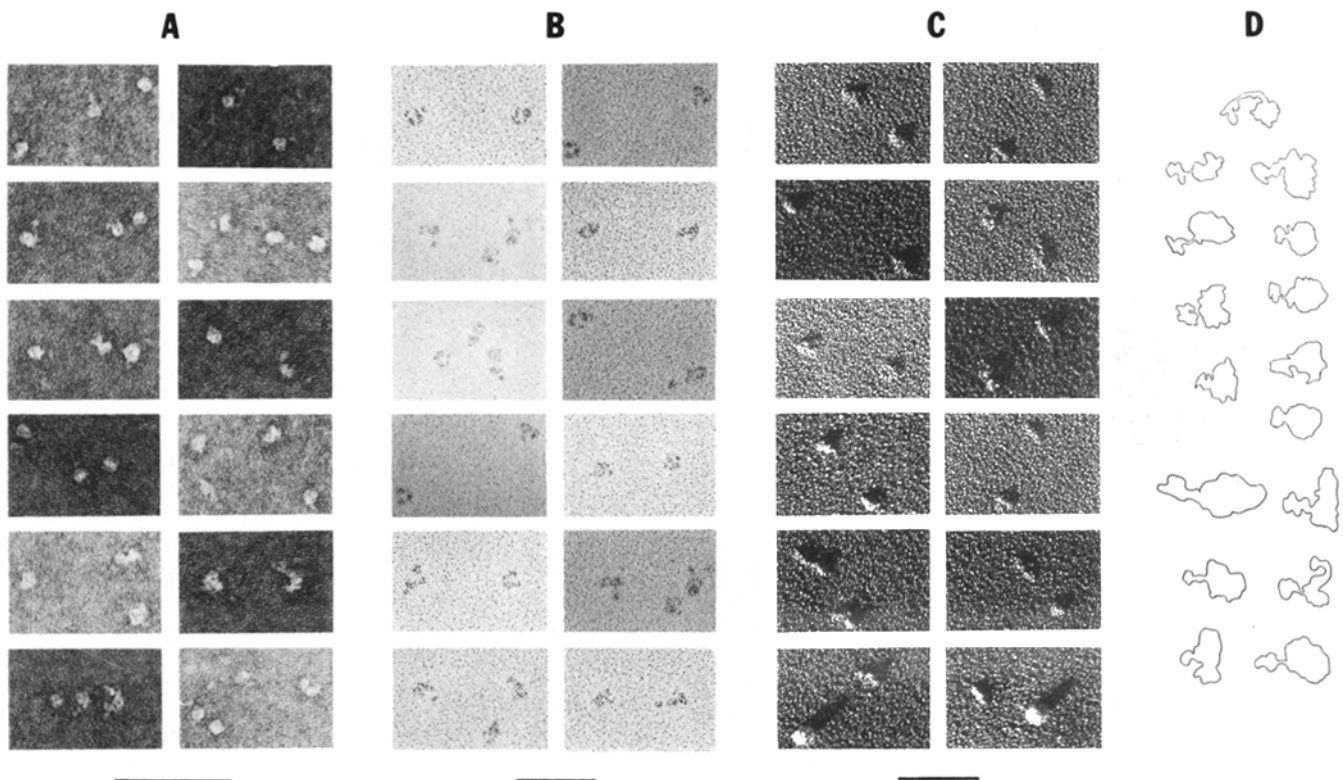
Figure 4. Electron microscopic visualization of purified assembly polypeptides. Negative staining (*A*), rotary shadowing (*B*), and unidirectional shadowing (*C*) of purified assembly polypeptides. Bar, 1,000 Å. (*D*) Tracings of selected images from negative staining and rotary shadowing.

upon adsorption to mica in the absence of glycerol. Both the presence of a tail or tails, and the ability to fold and unfold would produce an asymmetric frictional ratio and explain why the Stokes radius taken by itself predicts a much larger protein. Ferritin (61 Å) is included in the bottom picture of the unidirectional shadowing column as an internal standard (Fig. 4 *C*).

Assembly Polypeptides Promote Clathrin Cage Reassembly

To determine if the assembly polypeptides purified as a complex of 252 kD could stimulate clathrin cage formation, purified bovine brain clathrin and assembly polypeptides were mixed and dialyzed overnight into 100 mM 2-(*N*-morpholino)ethane sulfonic acid (MES), pH 6.5 (Fig. 5). Under these conditions, small amounts of assembly polypeptides and clathrin sedimented when dialyzed individually; when assembly polypeptides and clathrin were dialyzed together under conditions of clathrin excess (39), virtually all the assembly polypeptides sedimented, and electron microscopy showed the formation of uniformly sized cages. This demonstrates that the assembly polypeptides purified from the crude vesicle fraction quantitatively retain the ability to stimulate clathrin cage formation.

Subcellular Localization Studies

A major goal in the study of the assembly polypeptides is to characterize and identify their membrane binding site or sites. One measure of physiologic relevance in such an en-

deavor is that the location of binding sites parallel the in vivo distribution of the ligand. We therefore assessed the quantitative distribution of clathrin and assembly polypeptides in crude subcellular fractions of bovine brain (Fig. 6). About 75% of the 100-kD assembly polypeptide band was associated with the demyelinated membrane fraction (Fig. 6, lane *I*) and 25% with the vesicle fraction (Fig. 6, lane *J*), with no detectable 100-kD polypeptide in the cytosol (Fig. 6, lane *H*). Clathrin distributed evenly between membranes (Fig. 6, lane *F*) and vesicles (Fig. 6, lane *G*), with ∼10% free in cytosol (Fig. 6, lane *E*). This value is in good agreement with the results of both Goud et al. (19), and Bruder and Wiedenmann (8). These findings are also in agreement with immunofluorescence studies, which suggest that a significant fraction of the assembly polypeptides reside in perinuclear Golgi complex and in coated pits rather than primarily in coated vesicles (41, 42), and electron microscopic studies, which show the presence of large amounts of clathrin on the plasma membrane. Anderson and co-workers (31) have recently demonstrated that cytosolic proteins of 110 and 180 kD bind to the cytoplasmic face of fibroblast membranes during the re-formation of clathrin-coated pits. Thus, it is reasonable to expect the existence of assembly polypeptide binding sites on brain membranes as well as on coated vesicles.

Assembly Polypeptides Have a Protein Binding Site on Sodium Hydroxide–stripped Brain Membranes

[125]I-Assembly polypeptide binding to coated vesicles stripped of the majority of clathrin and assembly polypeptides by ex-
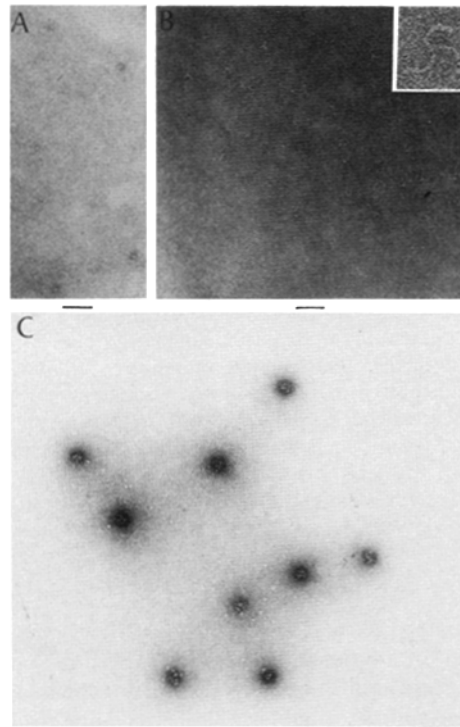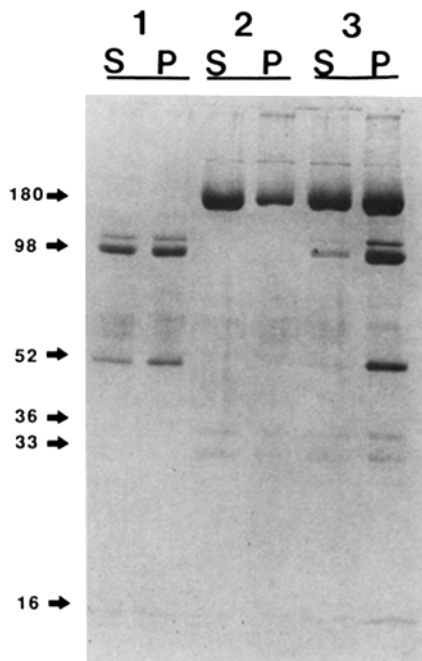
Figure 5. Assembly polypeptides stimulate clathrin cage formation. Bovine brain clathrin (27 µg) and/or assembly polypeptides (12 µg) in a final volume of 320 µl were dialyzed overnight into 100 mM MES pH 6.5, 1 mM EGTA, 1 mM MgCl$_2$, 1 mM DTT, 1 mM NaN$_3$. 100 ml of the mixture was then centrifuged through a 6% sucrose barrier at 160,000 g for 35 min at 4°C in a Beckman TLA 100 rotor. (*Left*) Aliquots of supernatant (*S*) and pellet (*P*) were examined by SDS-PAGE. Column *1*, assembly polypeptides alone; *2*, clathrin alone; *3*, assembly polypeptides plus clathrin. (*Right*) Uncentrifuged aliquots dialyzed into pH 6.7 buffer were examined by electron microscopy after negative staining with 0.75% uranyl formate. (*A*) Assembly polypeptides alone; (*B*) clathrin alone; (*C*) assembly polypeptides plus clathrin. Bar, 1,000 Å.
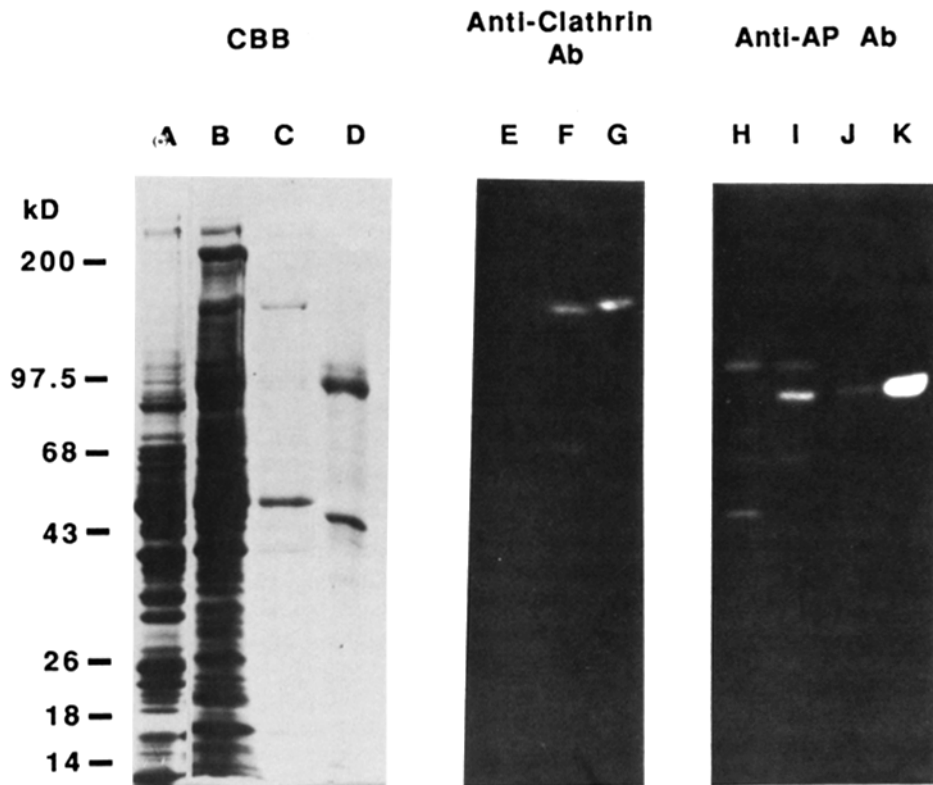

Figure 6. Subcellular distribution of clathrin and assembly polypeptides. 25 g of frozen bovine cerebral cortex was thawed and homogenized in a total volume of 100 ml 0.32 M sucrose, 2 mM EGTA, 1 mM DTT, 1 mM NaN$_3$, with 5 µg/ml leupeptin and pepstatin, 0.1% DFP, and 200 µg/ml PMSF. Nuclei and large debris were removed by centrifugation at 2,000 g for 10 min. A crude membrane fraction was obtained by centrifugation of the supernatant at 30,000 g for 20 min. The resulting membrane pellet was demyelinated by washing once in 1 M sucrose, then once with buffer A, pH 6.8, before resuspending the pellet in a final volume of 100 ml. The 30,000 g supernatant was centrifuged at 100,000 g for 60 min to give a crude vesicle pellet which was washed once in buffer A and resuspended to a final volume of 100 ml. The 100,000 g supernatant (cytosol) was made up to 100 ml. Equal aliquots of each crude subcellular fraction were subject to SDS-PAGE and stained with Coomassie Brilliant Blue (*A–D*); or electrophoretically transferred to nitrocellulose and immunoblotted with anti–clathrin antibody (*E–G*) or anti–assembly polypeptide 98-kD antibody (*H–K*). Lanes *A, E, H*, cytosol; lanes *B, F, I*, demyelinated membranes; lanes *C, G, J*, crude vesicles; lanes *D, K*, partially purified assembly polypeptides.
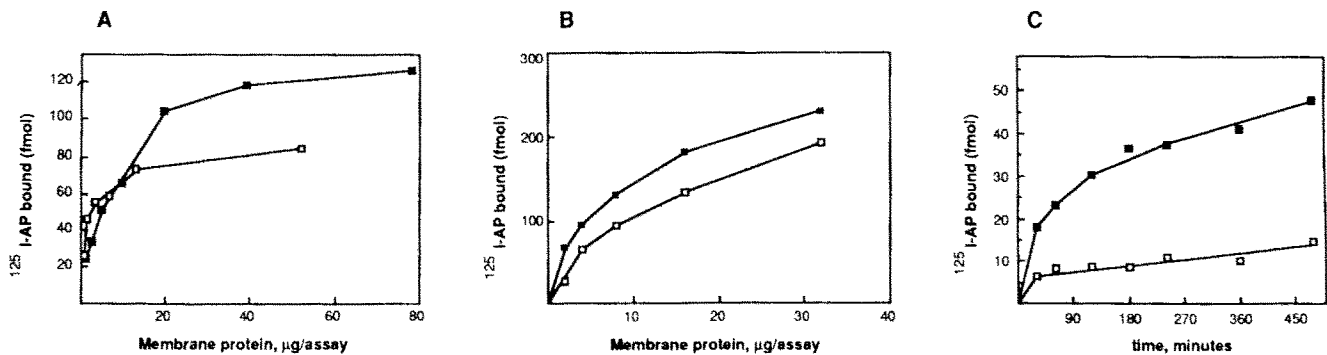
Figure 7. Assembly polypeptides bind to Tris-extracted coated vesicles and NaOH-extracted brain membranes. (*A*) Binding of ^{125}I-assembly polypeptides (10 nM, 72,833 cpm/pmol) to increasing amounts of NaOH-extracted brain membranes (■) and 1 M Tris-extracted coated vesicles (□) was measured, subtracting binding in the presence of 400 mM NaCl as an estimate of nonspecific binding. Binding to Tris-extracted coated vesicles was measured by filtration of 80-μl aliquots through prewet 0.2-μm hydrophilic Durapore membranes (Millipore Corp., Bedford, MA) by the method of Hanspal (20). (*B*) Binding of ^{125}I-assembly polypeptides (10 nM, 86,055 cpm/pmol) to increasing amounts of demyelinated brain membranes (□) and NaOH-extracted brain membranes (■) was measured (Materials and Methods). (*C*) Time course of binding. ^{125}I-Assembly polypeptides (10 nM, 80,347 cpm/pmol) was incubated with 2 μg NaOH-extracted membranes for various times and specific membrane binding determined (■). Nonspecific binding, estimated by the amount of membrane-associated assembly polypeptides after incubation in the presence of 0.4 M NaCl, is shown as well (□).

posure to 1 M Tris, pH 7.0 was compared with binding to 0.1 N NaOH-stripped demyelinated brain membranes (Fig. 7 *A*). Stripped vesicle binding was measured using the filtration assay developed by Hanspal et al. (20). Binding to hydroxide-extracted brain membrane was performed using an assay developed to measure association of membrane skeleton components with integral membrane proteins (12). Any binding sites remaining on these membranes is most likely due to an integral membrane protein since virtually all peripheral proteins are removed by the 30-min exposure to hydroxide. The ideal control in this assay would be the use of a vast excess of unlabeled ligand to swamp the limited number of specific sites. However, to use this control routinely requires the expenditure of large amounts of purified ligand. Therefore, in all assays except where indicated nonspecific binding was estimated by binding of ligand in the presence of 0.4 M NaCl. This value was in good agreement with results obtained using the amount of binding in the presence of 30-fold excess unlabeled assembly polypeptide.

Fig. 7 *A* shows that the ^{125}I-assembly polypeptides bound to both the Tris-stripped vesicles and NaOH-extracted brain membranes. Binding in the linear part of the assay (<5–10 μg membrane protein) shows a similar capacity of the membranes; however, the Tris-stripped vesicles contain a large amount of peripheral membrane proteins as well as residual clathrin and assembly polypeptides, whereas the hydroxide-extracted membranes are largely devoid of peripheral membrane proteins, so that a direct comparison of capacity cannot be made.

[125]I-Assembly polypeptides binding to NaOH-extracted membranes was compared with binding to unextracted demyelinated membranes (Fig. 7 *B*). On a pmol/mg membrane protein basis, binding was enriched in the NaOH-extracted membranes from which the peripheral membrane proteins had been removed. This indicates that residual clathrin on the membrane is not the primary binding site being measured in these assays, since the membrane-associated clathrin and assembly polypeptides are completely removed by the hydroxide extraction as assessed by immunoblotting.

Membrane-bound and free ^{125}I-assembly polypeptides were examined by electrophoresis and autoradiography and were identical, confirming that the binding is not due to a minor contaminant of the purified protein (data not shown). Binding was a linear function of membrane protein up to 5 μg of NaOH-extracted membrane protein, so subsequent assays were performed with 2–3 μg per assay. At most, only 20–25% of the added ligand bound to the membranes at the highest membrane concentration, suggesting that only a subset of the ligand was competent to bind to these membranes. The time course of binding (Fig. 7 *C*) displayed a rapid initial phase followed by a slower phase which did not plateau at 8-h incubation. In contrast, the binding in the presence of 0.4 M NaCl occurred rapidly as would be expected for nonspecific interactions.

Binding of ^{125}I-assembly polypeptides to the NaOH-extracted membranes was displaced by unlabeled assembly polypeptides with 50% inhibition at ∼80 nM (Fig. 8 *A*). When these data are expressed as a saturation curve by calculating specific activity for each concentration of ligand (Fig. 8 *B*), at the highest concentration of unlabeled protein ∼250 pmol ^{125}I-assembly polypeptides were bound per milligram membrane protein.

The assembly polypeptides are specifically associating with a protein component of the membranes since digestion of the membranes with α-chymotrypsin destroys 80% of the binding (Fig. 8 *C*). Loss of binding occurred in parallel with loss of protein from the membranes, and was not due to decreased ability of the membranes to sediment in the assay since the phospholipid content of the sedimentable material remained constant. Inhibition of binding was not due to residual membrane-associated protease degrading the ligand since electrophoresis and autoradiography of samples after the incubation period showed no breakdown of ligand even at the highest protease concentrations.

Integral membrane proteins can often be solubilized from the membrane with mild nonionic detergents. Treatment of the NaOH-extracted membranes with 0.5% Triton X-100 eliminated ∼40% of the assembly polypeptide binding sites
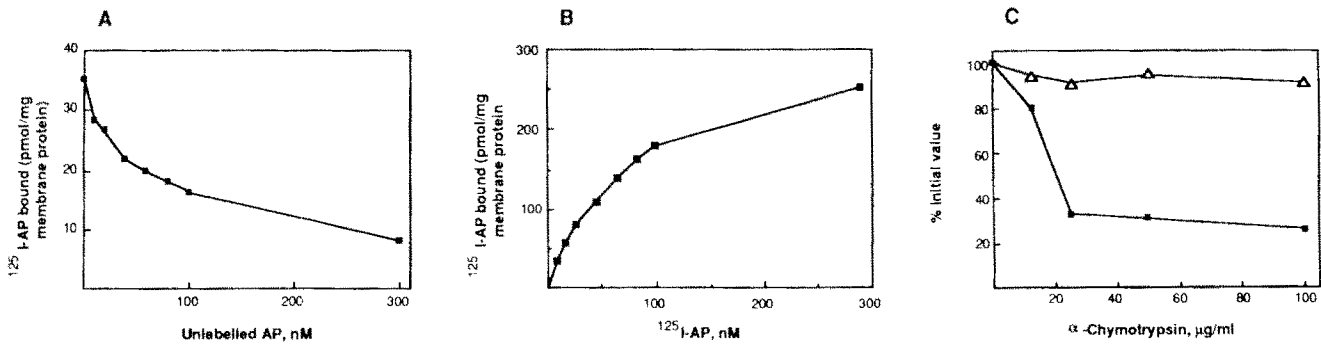
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Figure 8. Binding of ¹²⁵I-assembly polypeptides to NaOH brain membranes is saturable and protease sensitive. (*A*) 10 nM ¹²⁵I-assembly polypeptides (94,164 cpm/pmol) were incubated with 2.78 µg NaOH extracted brain membranes in the presence of increasing concentrations of unlabeled assembly polypeptides for 135 min, and values for nonspecific binding in the presence of 0.4 M NaCl were subtracted. (*B*) The specific activity was calculated for each point in *A* and the data expressed as a saturation curve. (*C*) NaOH-extracted brain membranes were incubated for 60 min at 22°C in the presence of increasing concentrations of α-chymotrypsin. At the end of the digestion, DFP was added to a final dilution of 1:10,000; after an additional 10 min, the membranes were re-extracted with 0.1 N NaOH to remove any residual protease and centrifuged through a 10% sucrose barrier, then washed twice in the presence of PMSF. Control membranes were treated identically except for the addition of protease. Membranes were incubated for 4 h with ¹²⁵I-assembly polypeptides (10 nM, 59,020 cpm/pmol) in the presence or absence of 340 nM unlabeled assembly polypeptides as a control for nonspecific adsorption to the membranes, and specific binding determined (■, Materials and Methods). Phospholipid (△) was also determined (Materials and Methods). 100% values are: ¹²⁵I-assembly polypeptides binding, 63 fmol, 36.5 pmol/mg membrane protein; membrane protein, 1.72 µg per assay; phospholipid, 53 µg P/ml (994 nmol P/mg undigested membrane protein).

while removing only 5% of the membrane protein (Fig. 9 *a*). Loss of binding was not due to denaturation of sites since Scatchard analysis of saturation binding to both sets of membranes showed equal affinity interactions but a loss of sites from the Triton-treated membranes. The polypeptide composition of unextracted and Triton-extracted NaOH-stripped membranes were analyzed as shown (Fig. 9 *b*; see also Fig. 10, lanes *C* and *D*). There was no significant difference in protein composition between the membrane samples as visualized by Coomassie Brilliant Blue staining. However, the ¹²⁵I–wheat germ agglutinin blot, which visualizes mem-

brane glycoproteins that may be poorly seen by Coomassie staining, shows depletion of several bands and elimination of one band altogether (Fig. 9 *b*, lanes *C* and *D*, *arrowhead*).

If the binding of assembly polypeptides to the NaOH-extracted brain membranes is a reasonable in vitro model for the in vivo mechanism of the attachment of clathrin to membrane proteins, then the addition of assembly polypeptides to these membranes ought to reconstitute clathrin binding. As seen in Fig. 10, lane *B*, clathrin alone does not cosediment with these membranes. Assembly polypeptides and clathrin together do not sediment without the addition of membranes
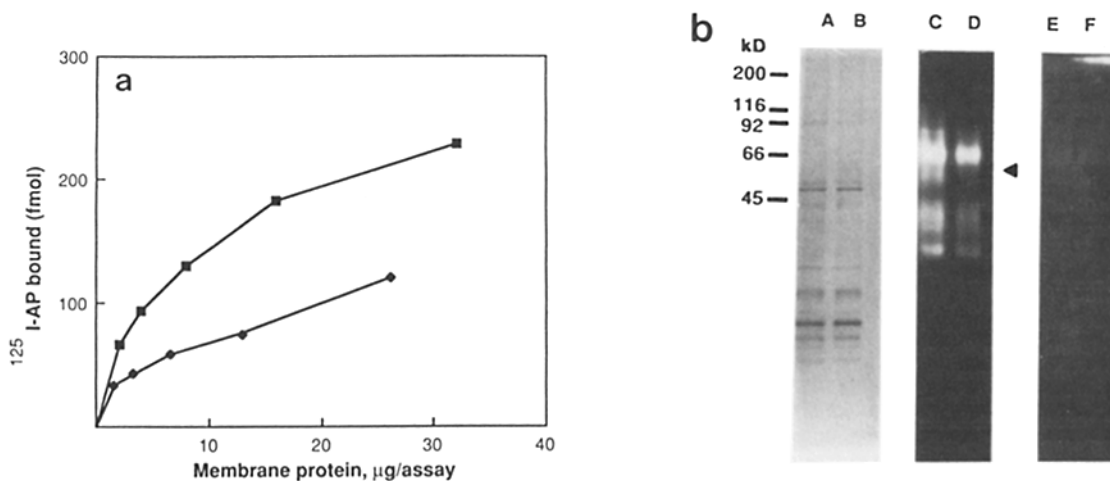

Figure 9. ¹²⁵I-Assembly polypeptides binding is depleted in Triton X-100–extracted membranes. NaOH-extracted brain membranes were incubated in 0.5% Triton X-100, 200 µg/ml PMSF for 15 min on ice and then washed twice (◆). Control membranes (■) were treated identically except for the addition of detergent. (*A*) Binding of ¹²⁵I-assembly polypeptides (10 nM, 86,065 cpm/pmol) to increasing concentrations of membranes was measured (Materials and Methods). Binding in the presence of 0.4 M NaCl was used as an estimate of nonspecific adsorption and subtracted from total binding. (*B*) NaOH-extracted brain membranes before (lanes *A*, *C*, *E*) and after 0.5% Triton X-100 extraction (lanes *B*, *D*, *F*) were subject to SDS-PAGE and stained with Coomassie Brilliant Blue (lanes *A*, *B*). Duplicate samples were transferred to nitrocellulose and incubated with ¹²⁵I-wheat germ agglutinin in the absence (lanes *C*, *D*) or presence (lanes *E*, *F*) of 0.2 M *N*-acetyl glucosamine according to the method of Bartles and Hubbard (4).
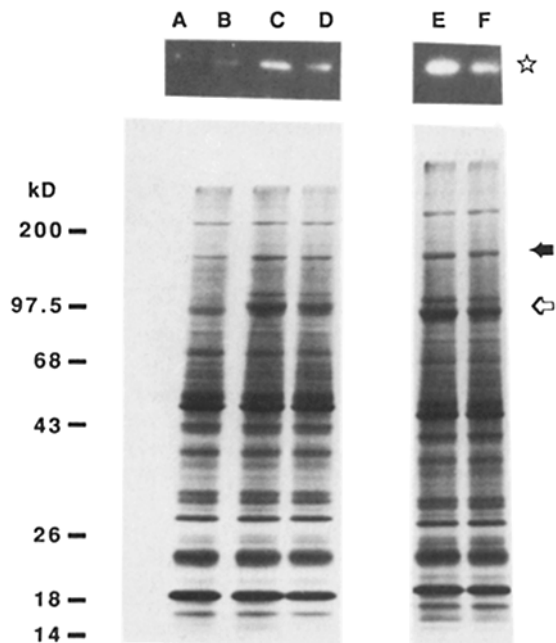
Figure 10. Assembly polypeptides dependent association of clathrin with NaOH-extracted brain membranes. 85 μg NaOH brain membranes or Triton-extracted NaOH brain membranes were incubated for 90 min on ice with 6 or 12 μg assembly polypeptides, followed by the addition of 12 μg bovine brain clathrin, in a final volume of 200 μl in 100 mM Hepes, pH 6.8, 10 mM NaCl, 1 mM EGTA, 1 mM DTT, 1 mM NaN₃, 0.5 mM MgCl₃, 0.01% Tween 20. After an additional 90-min incubation, 180 μl was layered over a 10% sucrose barrier and centrifuged at 5,000 *g* for 30 min in a swinging bucket rotor. Supernatants were aspirated, and pellets were solubilized in electrophoresis sample buffer and analyzed by SDS-PAGE followed by staining with Coomassie Brilliant Blue or transfer to nitrocellulose and immunoblotting with anti–clathrin antibody (☆). Lane *A*, clathrin and 6 μg assembly polypeptides, no membranes; *B*, clathrin and NaOH membranes, no assembly polypeptides; *C*, clathrin, 6 μg assembly polypeptides, and NaOH membranes; *D*, clathrin, 6 μg assembly polypeptides, Triton-extracted membranes; *E*, clathrin, 12 μg assembly polypeptides, NaOH membranes; *F*, clathrin, 12 μg assembly polypeptides, Triton-extracted membranes. → indicates clathrin; ⇨ indicates assembly polypeptides 98-kD subunit.

(lane *A*). However, clathrin added to membranes preincubated with assembly polypeptides pellet with the membranes (lane *C*), and this effect is increased with increased assembly polypeptide concentration (lane *E*). Triton X-100–extracted NaOH-extracted brain membranes concurrently lose assembly polypeptide and clathrin binding sites (lanes *D* and *F*). The reduction of clathrin binding to Triton-extracted membranes demonstrates that the sedimentation of clathrin in the presence of assembly polypeptides and membranes is not due to nonspecific trapping of clathrin cages by membranes. These results are consistent with the hypothesis that the assembly polypeptides attach clathrin to an integral membrane protein in brain membranes.

Discussion

We have demonstrated that the assembly polypeptides can be purified from a crude vesicle fraction with a yield of 2–3

mg/kg of bovine brain cortex. Electron microscopy shows a flexible globular protein with at least one tail, and reconstitution studies indicate the presence on brain membranes of a protease-sensitive specific binding site(s) that is partially Triton X-100 extractable.

This purification is a modification of previous procedures (39) with the major improvement being the extraction of clathrin and assembly polypeptides from a Triton-treated crude vesicle pellet of bovine brain cytosol rather than a more highly purified coated vesicle fraction. Use of a crude vesicle fraction has the advantages of both speed, and an increase in the quantity of protein extracted, which can then be further purified through conventional means. Assembly polypeptides are located on many organelles other than coated vesicles, especially Golgi complex and plasma membrane, as discussed above. Use of a 100,000 *g* pellet of cytosol may include some amount of assembly polypeptides from these sources as well. This procedure also provides a large quantity of assembly polypeptides corresponding to the HA-1 fraction of Pearse, which could be further purified. Finally, we confirm the finding of Zaremba and Keen (48) that a 16-kD polypeptide copurifies with the HA-2 fraction of assembly polypeptides.

Hydrodynamic and crosslinking studies show that the assembly polypeptides as purified here are an asymmetric heterotetramer with a solution molecular weight of ~252,000 and a subunit composition of two 100-kD, one 52-kD, and one 16-kD polypeptide. Earlier estimates of the molecular weight of the assembly polypeptides in solution have centered on 300,000, with a subunit structure of two 100- and two 52-kD chains. These estimates were based on Stokes radius alone, which is inaccurate in predicting the M_r of asymmetric proteins, or on the M_r of the crosslinked protein run on single percentage SDS-PAGE gels. Crosslinking studies using an exponential gradient gel with a very low initial acrylamide concentration, which can clearly separate 260,000 from 300,000 M_r bands, show that even at the highest crosslinker concentration no species migrates slower than M_r 260,000. The M_r of the crosslinked protein is consistent with both the solution molecular weight and with the calculated molecular weight derived from the subunit stoichiometry of two 100,000, one 52,000, and one 16,000 polypeptide. These crosslinking studies also indicate that the 16- and 100-kD polypeptides are closely associated. It cannot be excluded that this purification yields assembly polypeptides from which a loosely associated 52-kD subunit has been removed under dissociating conditions such as 0.5 M NaBr, that a second 52-kD subunit is in equilibrium with a 2:1:1 complex, or that the crude vesicle fraction contains a subset of protein different from purified coated vesicles. However, not even a small subset of higher molecular weight assembly polypeptides was seen in crosslinking and hydrodynamic studies.

Despite the apparent differences in subunit composition from those previously published, these assembly polypeptides quantitatively retain the ability to promote clathrin cage formation. Others have shown that this activity is attributable to the 100-kD chains both in the complex and alone in solution (1, 40, 48).

The details of the molecular linkage between clathrin and membrane receptors in the endocytic pathway have been approached from both directions. Several groups have demon-

strated that specific amino acid sequences in the cytoplasmic tail of receptors are necessary to target receptors in the plasma membrane to clathrin-coated pits and the endocytic pathway (10, 18, 33). Others have shown that the assembly polypeptides are required for clathrin to associate with the coated vesicle membrane. Since the assembly polypeptides are located between clathrin and the membrane in coated vesicles, and the assembly polypeptides colocalize with clathrin in the plasma membrane, it is probable, although not proven, that they have a similar relationship in coated pits on the plasma membrane. It is not known if assembly polypeptides exist in vivo associated with membranes independent of clathrin before receptor clustering and coated pit formation, or if they bind to the membranes during coat formation, and what role, if any, the kinase activities found in the coated vesicles (5, 23, 36) play in regulating these associations.

The availability of purified protein allows the development of in vitro assays that measure its association with cellular structures. Such an assay must be validated before conclusions regarding in vivo relevance can be drawn. We have demonstrated that the ^{125}I-assembly polypeptides can bind to brain membranes stripped of their peripheral membrane proteins by brief exposure to 0.1 N NaOH, and that the binding is enriched 30–50% in NaOH-stripped versus unstripped membranes despite the removal of ~60% of the membrane protein, indicating at least one class of binding sites are integral membrane proteins. The quantity of binding sites per milligram remaining membrane protein did not increase as dramatically as might be expected if integral membrane proteins were the only assembly polypeptide binding sites, suggesting that a significant fraction of the demyelinated membrane binding is due to membrane-associated clathrin, and/or that NaOH extraction of the membranes denatures a significant subset of the remaining integral membrane sites. This binding is progressively reduced in the presence of unlabeled assembly polypeptides, demonstrating the specificity of the association. The binding site can be destroyed by α-chymotrypsin, indicating it is a protein and can be partially extracted by 0.5% Triton X-100, which supports the hypothesis that it is an integral membrane protein. The availability of binding sites in vitro parallels the in vivo distribution of clathrin and assembly polypeptides. Lastly, clathrin binding to NaOH-stripped brain membranes is dependent on the presence of the assembly polypeptides. These findings taken together strongly suggest that this assay measures a physiologically relevant assembly polypeptide receptor or receptors in these membranes.

Several features of the data support the hypothesis that the assembly polypeptides are binding to more than one site on the membrane and suggest the possibility that the assembly polypeptides may self-associate on the membrane. First, the time course of binding appears biphasic. The second, slower phase of binding may indicate a second site with a slow on rate, or the slow formation of a second class of sites. Second, even at the highest membrane concentration only 20–25% of the ligand binds to the membranes. Although it may be that 80% of the ligand has had its membrane-binding domain denatured during purification, an alternative hypothesis is that 80% of the assembly polypeptides bind to other sites within the cell such as in the Golgi complex, or that some classes of binding sites are selectively denatured by the NaOH extraction of the membranes. Third, Triton extracts

only 40% of the binding sites. The remainder may be a different protein(s) that is less easily extractable, or the same protein(s) more firmly linked to membrane-associated structures. Last, the capacity of the NaOH extracted brain membranes seen in the saturation binding curve, ~300 pmol/mg membrane protein, is quite high. To bind this quantity of assembly polypeptides to the membrane requires many sites. NaOH-extracted membranes are highly enriched in integral membrane proteins, and a large fraction of these may be capable of binding assembly polypeptides. There is no one single protein visible on SDS-PAGE gels that appears likely to account for this quantity of sites. Alternatively, nucleation of assembly polypeptides on a single class of membrane sites may lead to assembly polypeptide polymerization, similar to the polymerization of mannose-6-phosphate receptors and assembly polypeptides reported by Pearse (37).

The binding of assembly polypeptides to brain membranes involves several layers of heterogeneity, both in terms of the presence of multiple forms of assembly polypeptides, and in the probable existence of multiple classes of binding sites within the cell. One system that may be more homogeneous is the reticulocyte membrane. Transferrin receptors constitute the vast majority of membrane receptors in these cells, and are well known to enter the cell via a clathrin-coated vesicle (9, 11, 21). It is reasonable to speculate that these cells might contain a single class of assembly polypeptides binding to a single class of membrane sites. In that case, it could prove an especially powerful system to further our understanding of assembly polypeptide–membrane interactions.

In summary, we have described an improved purification of bovine brain assembly polypeptides and investigated their subunit structure. Reconstitution assays provide evidence that the assembly polypeptides mediate clathrin attachment to brain membranes by binding to integral membrane proteins. The assay used may prove useful in monitoring the purification of the assembly polypeptide membrane binding site and in testing the hypothesis that the assembly polypeptides bind directly to the cytoplasmic domains of membrane receptors.

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