centers, and which influence interactions between soluble proteins.

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Stabilization of Clathrin Coats by the Core of the Clathrin-Associated Protein Complex AP-2†

William Matsui and Tomas Kirchhausen*
Department of Anatomy and Cellular Biology, Harvard Medical School, 220 Longwood Avenue, Boston, Massachusetts 02115
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ABSTRACT: AP-2 is the class of clathrin-associated protein complex found in coated vesicles derived from the plasma membrane of eukaryotic cells. We demonstrate here, using a chemical method, that an AP-2 complex is an asymmetric structure consisting of one large α chain, one large β chain, one medium AP50 chain, and one small AP17 chain. The complex has been shown to contain a core and two appendages. The AP core includes the small AP17 and the medium AP50 chains together with the amino-terminal domains of the large α and β chains. One appendage corresponds to the carboxy-terminal domain of the β chain. We find that as in the case of the β chains, the carboxy-terminal portion of the α chains is an independently folded domain corresponding to the second appendage. We use limited tryptic proteolysis of clathrin/AP-2 coats to show the release of the appendages from the interior of the coats and the retention of the AP core by the remaining clathrin lattice. In addition, we find that the AP core stabilizes the coat and prevents its depolymerization. These results are consistent with the proposal that the AP core contains the binding site(s) for clathrin, while the α- and β-chain appendages interact with membrane components of coated pits and coated vesicles.

Clathrin-coated pits and coated vesicles are organelles that serve to concentrate membrane proteins and ligands destined for vesicular membrane traffic [reviewed in Goldstein et al. (1985) and Pfeffer and Rothman (1987)]. The major constituents of the coat are clathrin and its associated protein complexes (APs), which together represent about 90% of the protein content. It seems likely that these components act as a molecular trap or filter, to recruit and retain proteins selectively in the coated pit (Pearse & Bretsher, 1981). In addition, it is thought that assembly of the clathrin lattice actually drives the vesiculation step, transforming a coated pit into a coated vesicle (Harrison & Kirchhausen, 1983). It is not yet known which of the components of the coat allow the uptake to be selective but the properties of the AP complexes

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Table I: Composition and Molecular Weight of the Polypeptide Chains from Clathrin and Its Associated Protein Complexes, AP-1 and AP-2

	subunit	molec mass[a]	
clathrin	heavy chain HC	192	Kirchhausen et al., 1987b
	light chain LCA1	27	Kirchhausen et al., 1987a
	light chain LCB2	25.1	Kirchhausen et al., 1987a
AP-1	large chain γ	~100	SDS–PAGE
	large chain β'	104.7	Kirchhausen et al., 1989
	medium chain AP47	47	SDS–PAGE
	small chain AP19	19	SDS–PAGE
AP-2	large chain αa	107.6	Robinson, 1989
	large chain αc	104	Robinson, 1989
	large chain β	104	Kirchhausen et al., 1989
	medium chain AP50	49.6	Thurieau et al., 1988
	small chain AP17	17.1	Kirchhausen, Frucht, and Davis, manuscript in preparation

[a] The molecular masses were calculated from deduced cDNA sequences, unless otherwise indicated.

suggest that they play a central role in this process. Two different classes of APs have so far been recognized (Robinson & Pearse, 1986; Keen, 1987; Robinson, 1987; Ahle et al., 1988): AP-1 complexes, found in association with clathrin-coated structures localized at the Golgi apparatus, and AP-2 complexes, associated with similar structures at the plasma membrane. Under physiological ionic conditions in which pure clathrin will not ordinarily assemble, APs interact and coassemble with clathrin to form a stable coat whose structure resembles the cytoplasmic lattices surrounding coated pits and coated vesicles (Zaremba & Keen, 1983; Pearse & Robinson, 1984). The APs lie between the clathrin lattice and the membrane of coated vesicles (Heuser & Kirchhausen, 1985; Vigers et al., 1986). In addition, APs allow the binding of clathrin to membranes (Unanue et al., 1981; Virshup & Bennett, 1988), and they appear to interact in vitro with the cytoplasmic domains of several transmembrane proteins destined for endocytosis or export (Pearse, 1988; Glickman et al., 1989). The cellular localization of each class of AP complex also suggests interactions with membrane-specific docking proteins. Therefore, AP complexes may be viewed as bifunctional structures that interact simultaneously with the relatively homogeneous population of clathrin trimers and a heterogeneous set of transported membrane receptors and docking proteins.

Each class of AP complex contains a different but related set of polypeptide chains, ranging in size from 17 to 110 kDa and classified as small, medium, and large (Table I and Figure 1). However, their precise quaternary structure is still a matter of controversy, and two models have been proposed. In the first model, AP-2 complexes are dimeric structures containing two copies each of the small, medium, and large chains (Pearse & Robinson, 1984; Manfredi & Bazari, 1987; Keen, 1987). In an alternative model more recently presented by Virshup and Bennett (1988), an AP complex is an asymmetric structure containing two large chains, but only one copy each of the small and medium chains. Moreover, immunoprecipitation experiments show that the two large chains in a complex are different (Ahle et al., 1988).

In the first part of this report we resolve the discrepancy between the two structural models using a chemical method first used to establish the stoichiometry of polypeptide chains in the H^+ pump of clathrin-coated vesicles (Arai et al., 1988). This approach allows one to establish the minimal quaternary structure independently of the physical state of the complex. Our results confirm the model of Virshup and Bennett and conclusively demonstrate that the AP-2 complexes are asymmetrical structures consisting of one copy each of the large α and β chains, one medium chain, and one small chain.

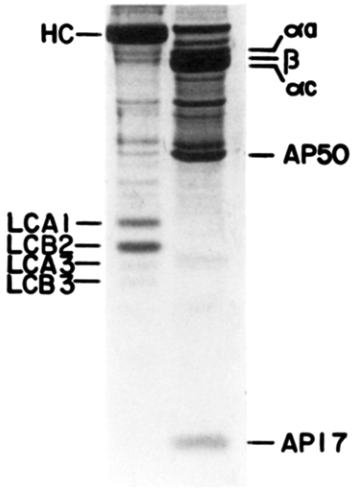

FIGURE 1: SDS–PAGE analysis of clathrin and its associated protein complex. Clathrin and its associated protein complex AP-2 were fractionated by SDS–13% PAGE and stained with Coomassie Blue. Lanes 1 and 2 are aliquots of the clathrin and AP-2 samples used for the quantitative amino acid analysis described under Methods.

AP-2 complexes are divided into a core and two appendages, as shown by proteolytic digestion experiments (Zaremba & Keen, 1985; Kirchhausen et al., 1989) and electron microscopy of AP-2 complexes (Heuser & Keen, 1988). The AP core includes the invariant proteins of the AP complex, the medium AP50 chain and the small AP17 chain, as well as the amino-terminal domains of the large α and β chains (Zaremba & Keen, 1985; Kirchhausen et al., 1989). One of the appendages corresponds to the carboxy-terminal domain of the β chain. The proteolytic cleavage patterns of the α and β chains are similar, and we proposed that the other appendage corresponds to the carboxy-terminal domain of the α chains (Kirchhausen et al., 1989).

On the basis of the bipartite organization of the AP-2 complexes, we also proposed a model in which the core contains the binding site(s) necessary for interaction with clathrin and the appendages participate in the selection of membrane receptors for inclusion in coated pits and coated vesicles. A direct test for the ability of these domains to promote the assembly of coats has not been successful, since cleavage of AP complexes prevents them from directing the in vitro assembly of clathrin/AP coats (Zaremba & Keen, 1985). Recently, Keen and Beck (1988) performed a binding experiment in which AP-2 cores were shown to cosediment with preformed clathrin cages and to bind clathrin trimers immobilized on a Sepharose matrix.

In the second part of this report we confirm that as in the case of the β chains, the carboxy-terminal portions of the α chains from AP-2 complexes are independently folded domains. We then show that the carboxy-terminal domains of the α and β large chains from AP-2 are released from the interior of the coat after proteolytic cleavage and that they do not associate with clathrin. We also show that after cleavage the remaining components of the AP complex copurify with coats and provide stability to the assembled lattice. Taken together, these results support the proposal that while the AP core is responsible for binding clathrin, the α- and β-chain appendages are available for interaction with docking proteins and selective retention of receptors in the coated pit.

Methods

Purification of Clathrin and Its Associated Proteins (APs). Calf brain clathrin-coated vesicles were obtained by differential

centrifugation (Kirchhausen & Harrison, 1984) in the presence (Pearse & Robinson, 1984) or absence (Campbell et al., 1984) of Triton X-100. Triton X-100, which substantially decreases the relative yield of AP-1 with respect to AP-2 complexes (our observation), was normally used for the isolation of coated vesicles used in proteolysis and binding experiments. Clathrin and its associated proteins (APs) were solubilized from coated vesicles by overnight incubation at room temperature with 0.8 M Tris, pH 7.4, 1 mM EGTA, 0.5 mM DTT, 0.5 mM phenylmethanesulphonyl fluoride (PMSF, Sigma Co.), and 0.02% NaN_3 (Keen et al., 1979; Kirchhausen & Harrison, 1984) followed by high-speed centrifugation at 100000*g* for 90 min at 4 °C to clear the sample of vesicles and large aggregates. The supernatant was directly applied to a Sepharose CL-4B (Pharmacia) sizing column equilibrated with 0.5 M Tris, pH 7.4, 1 mM EGTA, 0.5 mM DTT, and 0.02% NaN_3 and eluted at room temperature. Clathrin-containing fractions were pooled and used directly for cage or coat assembly. Fractions containing APs were pooled and either used directly for coat assembly or subjected to further purification by ionic exchange chromatography (Ahle et al., 1988) using the following modifications. The pool of crude APs was diluted with four volumes of a solution containing 50 mM Tris, pH 7.4, 2 mM EGTA, 0.5 mM DTT, and 0.02% NaN_3 and directly applied to a 1-mL Mono-Q or Sepharose-Q fast-flow column (HR5/5; Pharmacia). AP complexes were eluted with a 30-mL linear gradient from 0 to 0.45 M NaCl in the same solution at a flow rate of 0.5 mL/min. Samples containing AP complexes were pooled and when necessary concentrated by centrifugation (Centricon-30, Amicon).

Stoichiometry of Polypeptide Chains. Subunit stoichiometry determinations of clathrin and AP-2 complexes were performed essentially according to the method of Arai (1988). Briefly, polypeptide subunits were separated by 13% SDS–PAGE (Laemmli, 1970), electroblotted onto Immobilon membranes (Millipore), and stained with Coomassie Blue (Matsudaira, 1987). Individual polypeptides were excised from the membrane and subjected to quantitative amino acid analysis (Harvard Biological Laboratories Microchemistry Facility). Samples were subjected to acid hydrolysis at 110 °C for 24 h in a Waters Pico-tag workstation followed by amino acid analysis using a Hewlett-Packard Model 1084B HPLC apparatus (Ebert, 1986).

The efficiency of blotting for a given polypeptide chain was determined in duplicate samples, containing cold and ^{125}I-labeled clathrin or AP-2 complexes, by comparing the radioactivity present in the Immobilon membrane after the electrotransfer to that present in the polyacrylamide gel before transfer. Clathrin and AP-2 complexes (0.2 mL at 0.2–0.4 mg/mL in 50 mM Tris, pH 7.4, and 200 mM NaCl) were iodinated with $Na^{125}I$ (0.002 mL at 10 μCi/mL); New England Nuclear) for 15 min at room temperature in glass tubes coated with Iodogen (Pierce Chemical Co.) and separated from free ^{125}I by gel filtration on a Sephadex G-25 column (1 × 4 cm). The samples were stored at −20 °C and used within two weeks of iodination.

Assembly and Disassembly of Clathrin Cages and Clathrin/AP Coats. Cages were assembled from purified clathrin (0.8 mg/mL) by overnight dialysis at 4 °C against cage buffer (20 mM NaMES, pH 6.2, 2 mM $CaCl_2$, 0.5 mM DTT, 0.5 mM PMSF, 0.02% NaN_3) (Kirchhausen & Harrison, 1981). Clathrin/AP-2 coats were obtained from a 4:1 w/w mixture of clathrin with either size-purified APs or with ionic/exchange purified AP-2 complexes (final protein concentration of 0.1 mg/mL) by overnight dialysis at 4 °C against coat buffer (100 mM NaMES, pH 6.5, 0.5 mM DTT, 0.5 mM PMSF, 0.02% NaN_3). Aggregates were removed by low-speed centrifugation at 10000*g* for 10 min at 4 °C, then cages or coats were separated from nonassembled proteins by high-speed centrifugation in the airfuge (Beckman) at 30 psig for 15 min at room temperature. The pellets containing intact or proteolyzed cages or coats were resuspended at room temperature in cage or in depolymerization buffer (50 mM Tris, pH 7.4) to a concentration of ~0.5 mg/mL. After 20 min, the mixtures were processed for electron microscopy of negatively stained samples or for SDS–PAGE analysis and Coomassie Blue staining of supernatants and pellets obtained after a high-speed centrifugation in the airfuge.

Proteolytic Cleavage of AP-2, Cages, and Coats. Prior to digestion, aggregates were removed by centrifugation at 10000*g* for 10 min at 4 °C. Proteolytic digestion was performed at room temperature with DPCC–trypsin (Sigma Co.) and reactions stopped by addition of 0.5 mM PMSF. Retained or released fragments from the digested samples were identified by SDS–PAGE analysis of Coomassie Blue stained pellets and supernatants obtained by high-speed centrifugation in the airfuge.

Amino-Terminal Sequencing. About 130 μg of AP-2 complexes was subjected to mild tryptic digestion (0.7% trypsin by weight) for 15 min at room temperature and cleaved fragments were separated by preparative SDS–13% PAGE. After electrophoretic transfer onto an Immobilon membrane (Millipore), amino-terminal sequences of selected fragments were obtained by automated Edman degradation in a gas-phase sequencer (Applied Biosystems; Matsudaira, 1987).

Electron Microscopy. Samples were adsorbed to freshly glow-discharged carbon-coated grids, negatively stained with 1.2% uranyl acetate and observed with a JEOL 100-CXII electron microscope at 80 kV (Kirchhausen & Harrison, 1984).

Results

Quaternary Structure of AP-2 Complexes. The reliability of the procedure used to determine the stoichiometric ratio of polypeptide chains in the AP-2 complex was first tested by using a sample of clathrin of known composition. Clathrin isolated from calf brain coated vesicles contains three copies of a single species of heavy chain and three copies of different classes and types of light chains (Kirchhausen & Harrison, 1981; Ungewickell & Branton, 1981). The relative intensity of Coomassie Blue staining of the light chains from a sample of clathrin subjected to SDS–PAGE confirms that the light chains LCA1 and LCB2 are present in a molar ratio of 1:2 and that together they represent about 90–95% of the total amount of light chains (Figure 1, lane 1). The direct determination of the molar ratio of heavy and light chains was established following the procedure of Arai et al. described under Methods. Table II demonstrates that the amino acid composition of the chains obtained by chemical analysis of the polypeptides transferred to the Immobilon membrane is in close agreement with those calculated from their corresponding cDNA sequences (Kirchhausen et al., 1987a,b). The molar content of polypeptide chains in each band of the acrylamide gel was then determined from the amounts of amino acids in the membrane after correcting for the efficiency of transfer and the corresponding molecular weights (Table III). The molar ratio of heavy chains with respect to the light chains LCA1 and LCB2 defined by these calculations is 0.9 and is consistent with the ratio deduced from the Coomassie Blue staining described above.

Using the same method we have found that the molar ratio of the large, medium, and small chains in AP-2 complexes is

Table II: Amino Acid Composition of the Polypeptide Chains of Clathrin and of the Associated Protein Complex AP-2[a]

	clathrin				AP-2					
	HC[b]		LC[b]		$(\alpha + \beta)$[c]		AP50[c]		AP17[c]	
amino acid	aa	cDNA	aa[d]	cDNA[e]	aa[f]	cDNA[g]	aa	cDNA	aa	cDNA
Asp/Asn	9.7	10.5	10.6	10.1	11.2	9.7	9.1	8.5	8.0	12.6
Glu/Gln	14.7	13.7	17.9	19.3	12.1	10.6	11.8	11.0	13.2	12.6
Lys	5.9	5.8	5.5	6.1	5.8	5.6	8.3	8.7	5.6	6.3
Arg	6.6	4.8	5.3	4.4	4.0	4.7	6.4	6.2	5.5	5.6
His	2.2	2.1	0.6	0.6	1.2	1.7	1.2	1.7	1.4	2.8
Ser	5.2	5.0	7.9	7.8	7.2	7.4	7.3	7.4	7.6	1.4
Thr	4.2	3.9	2.9	2.8	4.0	5.2	3.1	4.4	4.1	4.2
Tyr	3.4	4.1	1.6	2.1	2.2	2.7	2.6	3.2	3.3	5.6
Gly	6.2	4.1	13.7	7.4	6.7	5.0	15.5	6.2	15.2	2.1
Ala	8.8	8.1	12.5	13.4	9.8	8.7	6.6	5.1	8.7	5.6
Pro	4.3	4.2	5.1	5.5	5.8	5.6	3.3	3.9	4.5	0.0
Val	6.6	6.9	4.5	4.8	7.7	7.4	7.2	8.3	7.4	10.5
Leu	11.6	11.0	5.4	5.2	11.8	11.9	6.6	6.7	7.0	11.0
Ile	5.8	5.8	3.4	3.4	5.2	5.3	6.1	8.5	4.1	6.3
Phe	4.5	4.4	3.2	3.4	3.6	4.0	4.0	4.8	3.6	7.7
Met	0.2	2.4	0.1	1.3	1.6	1.8	1.4	3.0	0.9	2.8

[a] The amino acid composition analyses (aa) of the polypeptide chains of calf brain clathrin and AP-2 complexes were performed on excised bands after their separation by SDS-PAGE and electrotransfer onto Immobilon membranes. These values are expressed in mole percent and are compared to the amino acid composition of each class of chain calculated from their respective cDNA sequences (cDNA). [b] One amino acid analysis. [c] Three amino acid analysis. [d] Pool of LCA1 and LCB2. [e] Average composition for the deduced sequences of LCA1 and LCB2 using a molar ratio of 1:2 (Kirchhausen & Harrison, 1981; Ungewickell & Branton, 1981). [f] Pool of the large chains of AP-2 including the electrophoretic variants αa1, αa2, αc1, αc2, and β. [g] Average composition for the deduced sequences of αa, αc, and β chains using a molar ratio of 1:1:2 (based on the relative intensity of Coomassie Blue stained bands).

Table III: Polypeptide Chain Stoichiometry of Clathrin and AP-2 Complexes[a]

trial	polypeptide chain	total amino acids in membrane (nmol)	blotting efficiency (%)	corr amino acids in gel (nmol)	total protein in gel (pmol)	molar ratio
1	HC (190 kDa)	47.2	46	102.6	57.2	1.0
	LC (26 kDa)	5.8	47	12.3	52.1	0.9
2	$\alpha + \beta$ (105 kDa)	30.0	70	42.3	45.8	2.0
	AP50 (49.6 kDa)	0.9	10	9.1	20.6	0.9
	AP17 (17.1 kDa)	1.3	nd			
3	$\alpha + \beta$	26.2	80	32.0	34.4	2.0
	AP50	3.3	49	6.8	15.3	0.9
	AP17	1.5	61	2.6	16.0	0.9
4	$\alpha + \beta$	56.6	75	75.5	79.0	2.0
	AP50	1.8	11	16.4	36.8	0.9
	AP17	2.5	41	6.1	38.0	1.0

[a] The total molar amount of each polypeptide was determined by correcting the quantity of amino acids obtained from amino acid analysis with the blotting efficiency of each peptide. The molecular weight of each subunit used in the conversion of nanomoles of amino acids to picomoles of protein was determined from primary structures derived from the corresponding cDNA clones (see text). The electrotransfers of the polypeptides from the gel to the Immobilon membrane were performed at 4 °C in 10 mM CAPS, pH 11.0, in the absence of methanol (trials 1, 2, and 4) or in the presence of 10% methanol (trial 3) for 8 h at 0.5 A (Transblot apparatus, Biorad). The molar ratios of the polypeptide chains in clathrin and AP-2 complexes were determined from the total protein in each band of the gel normalized to a value of 1 for the clathrin heavy chain and a value of 2 for the pool of AP-2 large chains.

2:1:1 (Table III). These experiments were performed in three independent trials using samples enriched in AP-2 complexes isolated from calf brain coated vesicles. As indicated in Table II, the presence of other proteins in the preparation has not affected our ability to select a single band, since a very close correspondence exists between the amino acid compositions of the polypeptide chains determined by this method and the compositions calculated from the cDNA sequences (Thurieau et al., 1988; Kirchhausen et al., 1989; Robinson, 1989; T. Kirchhausen, S. Frucht, and A. Davis, manuscript in preparation). We note the experimental error in the measurement of proline and serine for the small chain AP17 is probably due to the small amounts of AP17 transferred to the membrane. Likewise, the slightly elevated amounts of glycine determined in all samples probably reflects a carry-over from the electrophoresis buffer. Nevertheless, the highly redundant nature of the amino acid analysis for each polypeptide (16 different amino acids measured) prevents a significant bias on the estimate of the total amount of amino acids in the Immobilon membrane.

Molecular weights previously reported for intact AP-2 complexes range from 290 to 440 kDa (Manfredi & Bazari, 1987; Keen, 1987; Virshup & Bennett, 1988). This information, taken together with the molar ratio of chains in the complex (Table III) and the molecular weights for each of the polypeptides deduced from their cDNA sequences (Thurieau et al., 1988; Kirchhausen et al., 1989; Robinson, 1989; T. Kirchhausen, S. Frucht, and A. Davis, manuscript in preparation), indicates that an AP-2 complex contains one copy of each type of large chain in association with one copy of the medium chain and one copy of the small chain (see model in Figure 2).

Cleavage Pattern of AP-2 Complexes in Solution. We have previously shown, using limited proteolysis, that the α and β chains have a similar domain organization. On incubation of calf brain AP-2 complexes with trypsin, the large α and β chains are rapidly degraded into two distinct groups of relatively stable fragments, while the small AP17 and medium AP50 chains are comparatively resistant to proteolysis (Kirchhausen et al., 1989; confirmed here in the digestion

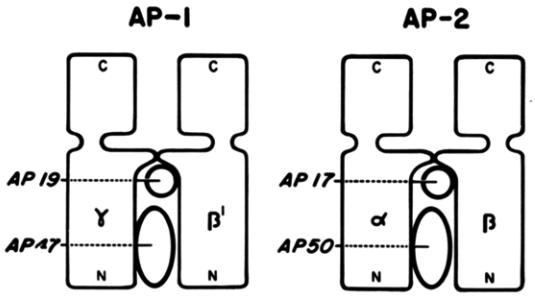

FIGURE 2: Proposed quaternary structure for AP-1 and AP-2 complexes. The assignment of specific contacts of the medium and small chains with the large chains is tentative and is based on the reversible cross-linking of AP-2 complexes performed by Virshum and Bennett (1988).

shown in Figure 3, lanes 1–6). The slower migrating group of fragments are species of 58–63 kDa and correspond to the amino-terminal end of the α and β chains (Kirchhausen et al., 1989). The faster migrating group of fragments includes species of about 39 and 35 kDa. A comparison of amino-terminal sequences from these smaller fragments with the protein sequence deduced from cDNAs for two rat brain β chains demonstrated that the 39-kDa species actually consists of equimolar amounts of two distinct species, one of which has been shown to correspond to the carboxy-terminal domain of the β chain from AP-2 complexes (Kirchhausen et al., 1989; Figure 4). Since the amino-terminal sequences of the other two fragments in this group were not present in the deduced primary structure of the β chains, we postulated that they derive from an analogous carboxy-terminal domain of the α chains (Kirchhausen et al., 1989). This prediction can now be confirmed by using the deduced primary structures of mouse brain αa and αc chains recently published by Robinson (1989). The amino-terminal sequences of the remaining 39- and 35-kDa species (shown in Figure 4) align with the protein sequences at the carboxyl side of Arg-664 and Arg-634 of αa and αc chains, respectively. These sites define carboxy-terminal domains of 313 and 304 residues, measured from the positions of cleavage toward the carboxyl ends of the complete αa and αc chains, whose predicted sizes (MW = 34 000 and 33 000) are slightly smaller than the apparent sizes of the corresponding tryptic fragments (39 and 35 kDa).

Of the two rat brain β chain cDNAs cloned, one of them codes for the large β chain of AP-2 complexes while the other corresponds to either a minor β chain variant or the related β' chain found in AP-1 complexes. The observation that the carboxy-terminal domain of the β chain, as defined by limited proteolysis, contains the sequence that differs most between the two cloned examples (Kirchhausen et al., 1989) can be extended to the α chains, since inspection of their deduced sequences (Robinson, 1989) shows 60.9% sequence identity between the carboxy-terminal domains of the αa and αc chains as opposed to 90.7% sequence identity between their corresponding amino-terminal domains.

From the analysis of the cleavage pattern just described, we conclude that the α and β chains have a strikingly similar domain organization despite the lack of sequence identity and the weakness of the only detectable sequence relationship [see Figure 4B for details of the scores obtained by using the alignment program Align (Dayhoff et al., 1983)].

Cleavage Patterns of AP-2 Complexes Free in Solution or Coassembled with Clathrin into Coats Are Related. Limited tryptic digestion of coats containing clathrin and AP-2 complexes favors the cleavage of the large α and β chains and the medium AP50 chain, while proteolysis of the small AP17 chain and of clathrin heavy and light chains is in general negligible (Figure 3, lanes 7–12). As an example, densitometric analysis of the bands remaining after the digestion in lane 11 shows a decrease of about 60% in the integrated intensities of the α and β chains and about 80% in the intensity of the AP50 chain, while the reduction in the amount of intact AP17 and clathrin light chain LCB2 is less than 10%. Incubation of the coats with a higher concentration of trypsin (lane 12) cleaves 80% of the α and β chains, more than 90% of the AP50 chain, and 40% of the clathrin light chain LCB2. In contrast, the cleavage of the clathrin heavy chain is relatively minor, judging

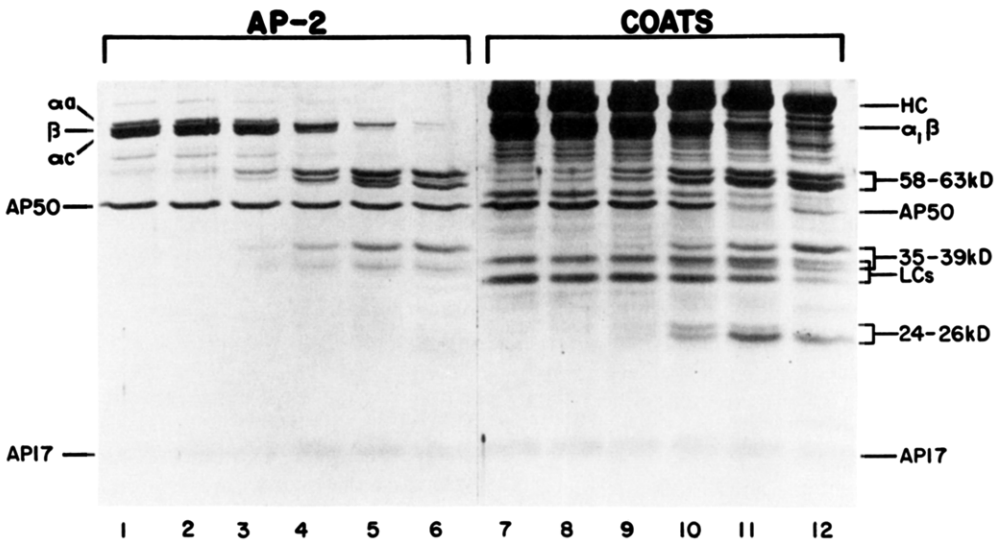

FIGURE 3: Electrophoretic analysis of the tryptic digestion of AP-2 complexes free in solution or coassembled with clathrin. AP-2 complexes (0.4 mg/mL; lanes 1–6) or clathrin/AP-2 coats (2.0 mg/mL; lanes 7–12) were treated at room temperature with trypsin for 15 min in 50 mM Tris, pH 7.4. Cleavage was stopped with 1.0 mM PMSF and the products analyzed by SDS–13% PAGE: before addition of protease (lanes 1 and 7) and after addition of trypsin by using the following enzyme/AP-2 ratios (w/w): 1:450 (lanes 2 and 8); 1:150 (lanes 3 and 9): 1:50 (lanes 4 and 10); 1:25 (lanes 5 and 11); 1:12.5 (lanes 6 and 12). HC and LC indicate clathrin heavy and light chains. αa, αc and β, and AP50 and AP17 are the large, medium, and small chains of the AP-2 complex, respectively. High molecular mass fragments (58, 60, 63 kDa) correspond to the amino-terminal domains of the large chains (Kirchhausen et al., 1989). One set of low molecular mass fragments (35 and 39 kDa) correspond to the carboxy-terminal domains of the large chains (Kirchhausen et al., 1989; this study). The other set (24 and 26 kDa) correspond to the amino terminus of AP50 (this study). Molecular weight markers were myosin, β-galactosidase, phosphorylase B, bovine serum albumin, chicken egg albumin, glyceraldehyde-3-phosphate dehydrogenase, carbonic anhydrase, trypsinogen, and α-lactalbumin.

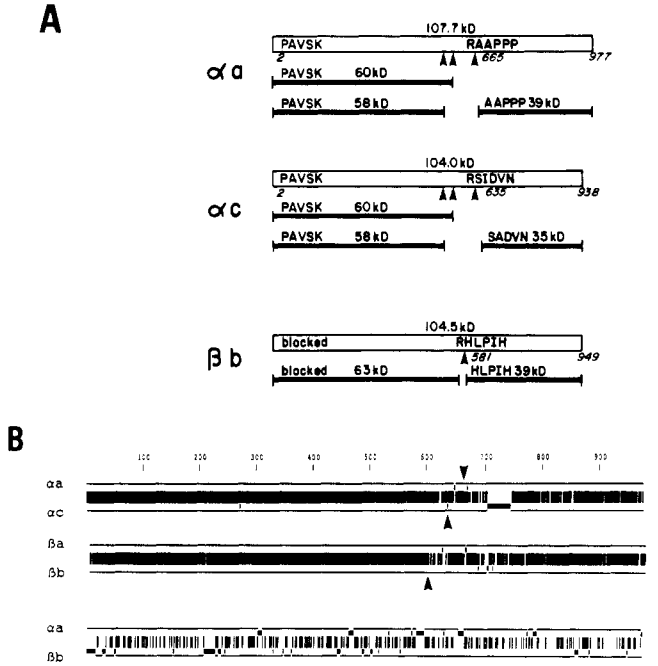

FIGURE 4: Domain organization and sequence comparison of the large α and β chains of AP-2 complexes. (A) Domain organization. The amino-terminal amino acid sequences from the intact large α and β chains of calf brain AP-2 complexes and their main tryptic fragments were located in the deduced primary structures of mouse αa and αc chains (Robinson, 1989) and of rat βa and βb chains (Kirchhausen et al., 1989). The size of the fragments was estimated from their electrophoretic mobilities. (B) Sequence comparisons. Optimal alignments of the deduced amino acid sequences of mouse αa and αc and of rat βb chains were obtained with the program Gapshow (Devcraux et al., 1984). Sequence identities are displayed by the vertical bars, while sequence gaps are indicated by thick horizontal lines. The level of sequence relatedness between the α and β chains was determined with the alignment program Align (Dayhoff et al., 1983). The calculations were performed by using the evolutionary matrix of homology and 100 independent randomizations. Alignment scores greater than 4.26 suggest that the sequence relationship is statistically significant with a $p > 10^{-5}$. The amino acid positions along the primary structures of the α and β chains tested for sequence relationship and the corresponding alignment scores were as follows: $\alpha(1-977)/\beta(1-949)$, 9.48; $\alpha(1-200)/\beta(1-200)$, 0.92; $\alpha(200-400)/\beta(200-400)$, 0.71; $\alpha(400-600)/\beta(400-600)$, −0.91; $\alpha(600-800)/\beta(600-800)$, 0.75; $\alpha(800-977)/\beta(800-949)$, 4.32.

by the appearance of two faint bands, one slightly smaller than AP50 (the terminal domain) and the other slightly larger than the α and β chains (the remaining leg). In addition, the small AP17 chain is not cleaved, and its amount remains as in the undigested sample.

The electrophoretic pattern reveals that the main cleavage products of coats (suspended in 50 mM Tris, pH 7.4) have mobilities and relative yields similar to those obtained by the digestion of pure AP-2 complexes (also suspended in 50 mM Tris). Therefore, it appears as if the interactions of AP-2 complexes with the clathrin lattice or with other AP-2 complexes do not limit the accessibility of the protease to the hinge regions located between the amino- and carboxy-terminal domains of the large α and β chains. However, the rapid cleavage of AP50 detected in coats (Figure 3, lanes 11 and 12) is in sharp contrast with its resistance to proteolysis when pure AP-2 complexes are digested under similar conditions (Figure 3, lanes 5 and 6). The tryptic cleavage of AP50 generates a fragment of 26 kDa, which is subsequently chased into a 24-kDa species and whose amino-terminal sequences align with the amino terminus of the intact AP50 (Thurieau et al., 1988). In addition, the 26-kDa fragment contains a minor product whose sequence aligns with the carboxyl side of Lys-281 of AP50. These results suggest that the AP-2 complex undergoes a conformational change upon coassembly with clathrin, leading to a more exposed AP50 near the center of its polypeptide chain.

The Carboxy-Terminal Domains of the Large Chains of AP-2 Complexes Are Released and the AP Cores Are Retained in Cleaved Coats. Clathrin/AP-2 coats were subjected to limited tryptic proteolysis followed by high-speed centrifugation, and the components that are released from coats and stay in the supernatant were distinguished from those that cosediment with the remaining clathrin. As shown in Figure 5, the proteolytic fragments of 35 and 39 kDa, corresponding to the carboxy-terminal domains of the large chains α and β, are exclusively found in the supernatant (Figure 5, lane 13). In contrast, the pellet contains most of the clathrin heavy and light chains, small amounts of uncleaved large chains (<10%), and the protein components of the AP core (the 58–63-kDa fragments, the remaining medium AP50 chain, and the small AP17 chain; Figure 5, lane 14). The efficient sedimentation of intact and cleaved coats is in marked contrast with the failure of pure clathrin cages to sediment in the same depolymerization solution (Figure 5, lanes 7 and 8). The possibility that AP cores are released from coats but sediment due to the formation of large aggregates is unlikely, since AP-2 complexes and all their cleaved components are soluble in the same solution as coats (50 mM Tris, pH 7.4) and remain in the supernatant fraction of a high-speed centrifugation (Figure 5, lanes 1 and 3). The concentration of APs inside the coats is relatively high, and we cannot rule out the formation of aggregates that may remain trapped within the lattices. Nevertheless, such hypothetical aggregates must still interact effectively with clathrin in order to maintain the observed structural integrity of the cleaved coats described below. These results suggest that the AP core remains specifically bound to clathrin, stabilizing the lattice and preventing its spontaneous dissociation into clathrin trimers. Similar stabilization of clathrin coats by intact AP-2 complexes was described by Zaremba and Keen (1983).

These observations were complemented by using electron microscopic visualization of negatively stained samples to demonstrate that cleaved and intact coats are indistinguishable in their appearance and structural stability. As a control we used cages assembled from pure clathrin trimers. These assemblies are hollow structures, generally 70–100 nm in diameter, which spontaneously depolymerize within 1 min of incubation in the depolymerization buffer containing 50 mM Tris (see electron micrographs in Figure 6A,B). In contrast, clathrin coats coassembled with AP-2 complexes generate smaller structures, ~70 nm in diameter, and do not depolymerize for at least 20 min of incubation in the same solution (Figure 6C,D). The same stabilization effect is found with coats subjected to mild tryptic proteolysis, to the extent that more than 90% of the AP large α and β chains and more than 50% of the medium AP50 chain have been cleaved (compare the micrographs in Figure 6E,F). The possibility that the greater stability of coats is due to stronger clathrin interactions in the small 70-nm lattice rather than to the presence of interacting AP-2 components can be ruled out by experiments such as the ones in Figure 6A,B. Samples of depolymerized clathrin cages completely lack recognizable structures, even though in the intact sample about 15–20% of the structures are of the smaller type.

Taken together, these results show that in spite of the complete release of the carboxy-terminal domains of the large chains of AP-2 complexes from proteolyzed coats, the re-

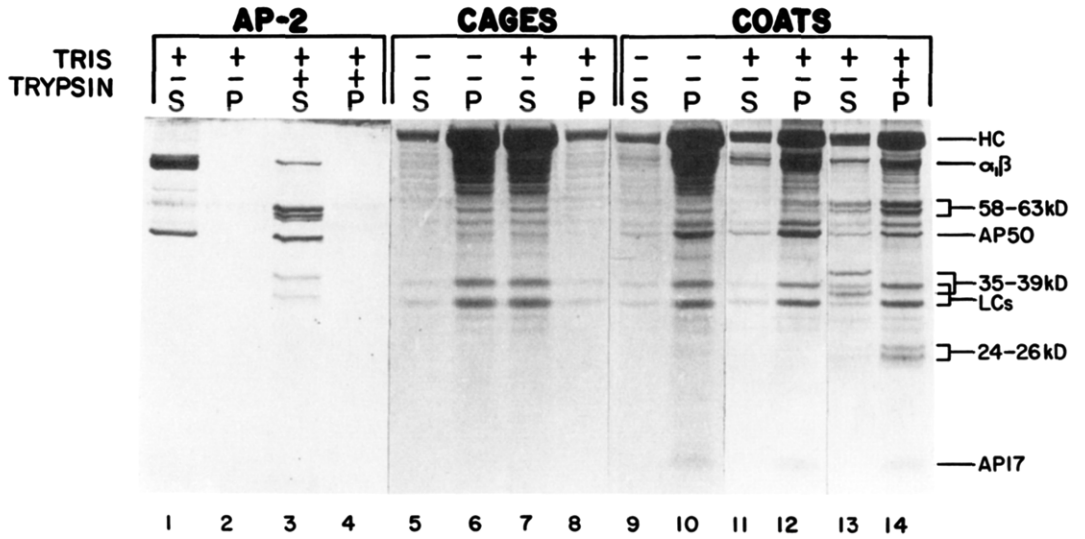

FIGURE 5: Proteolytic release of the carboxy-terminal domains of the large chains and retention of the AP cores in digested coats. Comparison of the sedimentation properties of AP-2 complexes (0.2 mg/mL; lanes 1–4), clathrin cages (0.8 mg/mL; lanes 5–8), and clathrin/AP-2 coats (1.0 mg/mL; lanes 9–14). Cages were suspended in cage buffer (lanes 5 and 6) or in 50 mM Tris, pH 7.4 (lanes 7 and 8). AP-2 and coats were suspended in 50 mM Tris, pH 7.4, and digested with trypsin at an enyme/AP-2 weight ratio of 1:200 for 15 min at room temperature. The reactions were stopped with PMSF and SDS–13% PAGE analysis was performed on supernatants (odd lanes) and pellets (even lanes) after centrifugation in the airfuge for 15 min at room temperature.

maining components of the AP complex retain enough binding site(s) to stabilize the interactions within the clathrin/AP-2 lattice, preventing its dissociation under otherwise unfavorable ionic conditions.

Discussion

Our results show that an AP-2 complex contains two large chains, one medium chain, and one small chain. Moreover, Ahle et al. (1988) used an immunoprecipitation approach to demonstrate that AP-1 complexes contain heterodimers of the unrelated γ and β' large chains while AP-2 complexes contain heterodimers of the corresponding α and β large chains. This relationship, together with sequence similarities found between the corresponding smaller chains of AP-1 and AP-2 complexes (Y. Nakayama, C. Hsu, and T. Kirchhausen, unpublished observation) and the same relative intensity of their Coomassie Blue stained bands (Ahle et al., 1988), leads us to propose the model shown in Figure 2. This representation is consistent with electron microscopic views of intact and proteolyzed AP-2 complexes (Heuser & Keen, 1988) and with the bipartite domain organization of AP-2 complexes (Kirchhausen et al., 1989). An important feature of the model is the lack of 2-fold symmetry. Indeed, an alignment of the protein sequences deduced from the cloned α- and β-chain cDNAs (Kirchhausen et al., 1989; Robinson, 1989) demonstrates a virtual absence of sequence identity and presence of only a weak relationship between the primary structures toward their carboxy-terminal ends. Additional asymmetry is given by the smaller AP17 and AP50 chains since a sequence comparison of their deduced primary structures (Thurieau et al., 1988; T. Kirchhausen, S. Frucht, and A. Davis, manuscript in preparation) indicates that they are not related to each other.

The results obtained from all of the chemical cross-linking experiments reported for AP-2 complexes (Pauloin et al., 1984; Pearse & Robinson, 1984; Virshup & Bennet, 1988) demonstrate that two large chains are in direct contact with each other. The smaller chains do not seem to touch each other, although they cross-link to either one of the large chains (Virshup & Bennet, 1988). These interchain contacts are located in the core of the AP complex and are mediated by noncovalent interactions between the amino terminal domains of the large chains, the medium chain, and the small chains (Zaremba & Keen, 1985; Kirchhausen et al., 1989).

Isolated AP-2 cores bind to preformed clathrin cages or to clathrin immobilized on a Sepharose column (Keen & Beck, 1988). Our binding results using cleaved coats confirm these observations and demonstrate that the AP cores not only remain attached to preformed clathrin lattices but also prevent dissociation under otherwise unfavorable ionic conditions. These results suggest that one or more components of the AP core contain the interacting site(s) responsible for clathrin/AP-2 coat stability. Likely candidates for such a component are the relatively invariant amino-terminal domains of the large chains. Direct contacts between clathrin and the large chains of AP-2 complexes were first shown by Prasad and colleagues (1986), who found that the large chains, isolated from AP-2 complexes by using chaotropic agents, favor clathrin coat formation. In a more recent study, Ahle and Ungewickell (1989) showed that purified β chains bind to preformed clathrin cages, thereby preventing the subsequent binding of AP-2. They therefore suggested that the β chain may mediate the interaction of AP-2 complexes with clathrin.

The AP complexes are located on the inner side of the clathrin lattice (Heuser & Kirchhausen, 1985; Vigers et al., 1986), but their exact position is unknown. The results described here provide some constraints on possible spatial arrangements. We observe that the composition of the AP-2 complex makes it unlikely that it binds to a position of local symmetry, since a single complex would not make identical contacts with more than one clathrin molecule. Whatever the precise location of AP complexes in the lattice, our results lead to the following picture for the organization of coated pits and vesicles. AP complexes are arranged with their cores contacting the membrane side of the clathrin lattice and with their appendages, containing the carboxy-terminal domains of the large chains, projecting toward the membrane. The appendages could interact with the cytoplasmic tails of membrane receptors or with docking proteins. Such a model can be extended to the related AP-1 complex, in view of the extensive similarities between the corresponding polypeptide chains of AP-1 and AP-2 complexes (Ahle et al., 1988; S. Frucht, Y. Nakayama, and T. Kirchhausen, manuscript in preparation)

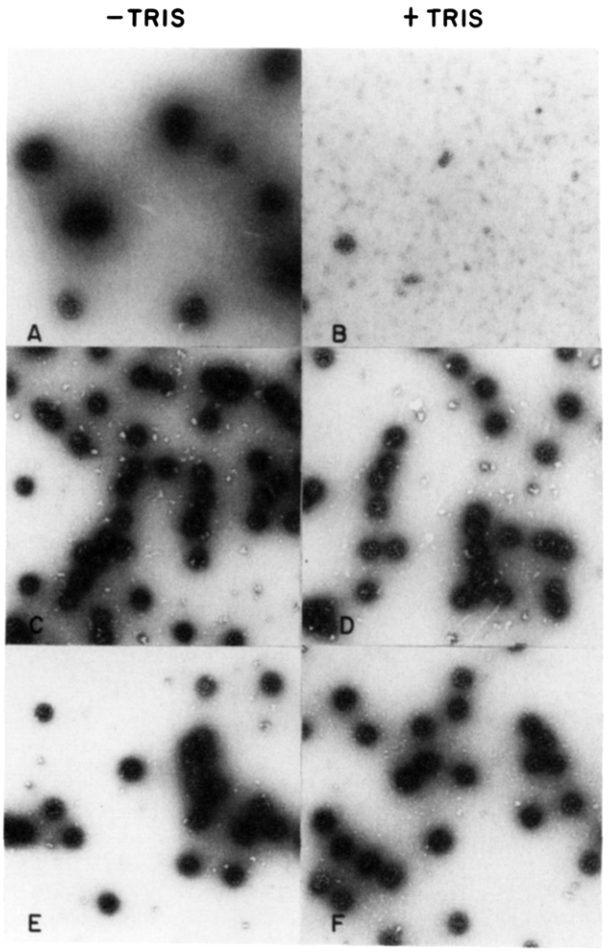

FIGURE 6: Structural stability of cages, coats, and digested coats. Aliquots of the samples described in Figure 5 (prior to centrifugation) were processed after 30 min at room temperature for negative staining and electron microscopic visualization. Clathrin cages suspended in cage buffer display closed lattices of 70–100 nm in diameter. Of 270 cages counted, 20% were ~70 nm in diameter and were classified as "small type" (A). Clathrin cages suspended in 50 mM Tris, pH 7.4, spontaneously depolymerize into clathrin trimers ($n = 0$) (B). Clathrin/AP-2 coats assembled in coat buffer display mostly the barrel-type structure ~70 nm in diameter ($n = 143$) (C) and are stable in 50 mM Tris, pH 7.4 ($n = 90$) (D). Digested coats display a similar number of barrel-type structures in coat buffer ($n = 167$) (E) and in 50 mM Tris, pH 7.4 ($n = 180$) (F). n is the number of assembled lattices counted in two micrographs taken at a primary magnification of 17400×.

and of the similar abilities of the two classes of complex to coassemble with clathrin and to promote the formation of coats (Robinson & Pearse, 1984; Keen, 1987).

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