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Structural and functional division into two domains of the large (100- to 115-kDa) chains of the clathrin-associated protein complex AP-2

(cDNA cloning/coated vesicles/vesicular-membrane traffic/protein structure)

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ABSTRACT **The clathrin-associated protein complex 2 (AP-2 complex) is a group of proteins associated with clathrin-coated vesicles and believed to interact with cytoplasmic domains of receptors found in the plasma membrane. AP-2 was purified as an assembly of several polypeptide chains (α, β, AP50, and AP17), of which only the α and β chains (100–115 kDa) show significant heterogeneity. We have obtained cDNA clones for two distinct rat brain β chains. We have also studied the domain organization of bovine brain AP-2 complexes by selective proteolysis. Results of these studies show that the α and β chains have a similar two-domain organization. Their amino-terminal domains are relatively invariant whereas their carboxyl-terminal domains are variable in both sequence and length. We propose that the variable domains select receptors for inclusion in coated vesicles.**

Clathrin-coated pits and -coated vesicles are associated at the plasma membrane with the early stages of receptor-mediated endocytosis (for review, see ref. 1) and in the Golgi region with organization of the nonconstitutive pathway of exocytosis (for review, see ref. 2). The major structural proteins of the coat are the heavy and light chains of clathrin (3–5) and the clathrin assembly proteins (6–8), referred to herein as associated proteins (APs). Together, these species represent ≈90% of the protein content of the coat. The APs are complexes made up of several kinds of polypeptide chains, large (100–115 kDa), medium (45–50 kDa), and small (17–20 kDa). Two major families of AP complexes, designated AP-1 and AP-2 (9), have been found in close association with the trans-Golgi and plasma membrane, respectively (7, 8). In the AP-2 family, which represents ≈80% of the APs isolated from bovine brain clathrin-coated vesicles, the medium and small chains are known as AP50 and AP17, and their sequences have been deduced from cDNA clones (ref. 10; S. Frucht and T.K., unpublished data). The large chains vary in apparent size, and at least six members can be resolved by $NaDodSO_4$/PAGE. One group, designated α chains (8), includes the immunologically related a1, a2, c1, and c2 species of apparent sizes 100, 102, 112, and 115 kDa, respectively. The other, designated β chains (8), includes the major b and minor b* species of 104 and 115 kDa, respectively. Cross-linking and immunoprecipitation experiments have shown that each AP-2 complex contains one α chain, one β chain, and either one or two each of the medium and small chains (8, 11, 12). A similar picture also appears to characterize complexes of the AP-1 family, which contain γ and β′ large chains associated with medium and small chains, known, respectively, as AP47 and AP19 (8). Thus, the AP complexes display a heterogeneity that suggests a role in interaction with membrane proteins destined for endocytosis or export. Their location, toward the membrane side of the hollow clathrin lattice (13, 14), is also consistent with such a function.

We report here the molecular cloning and primary structure of two distinct rat brain AP-2 β chains.** We also describe the proteolytic dissection of bovine brain AP-2 complexes. We combine these results to establish a bipartite structure for the α and β chains in the AP complex. We show that the core of an AP-2 complex contains, in addition to AP50 and AP17, the amino-terminal domains of the α and β chains. The carboxyl-terminal domains of the two β chains we have cloned differ more in sequence than do their amino-terminal domains. Moreover, the proteolytic dissection experiments show that in the α chains, it is the carboxyl-terminal domains that are responsible for significant size heterogeneity. On the basis of these results, we present a model for the organization of the AP complexes and for the functions of each of their two major parts.

METHODS

Clathrin and AP-2 Complexes. Clathrin and its APs were obtained from calf brain coated vesicles (6, 11, 15). AP-2 complexes were isolated by ion-exchange chromatography using Q Sepharose (Pharmacia) (8) or, in one case, by clathrin affinity column separation (9).

Proteolytic Cleavage and Amino-Terminal Sequencing of α and β Chains. Intact α and β chains were separated by preparative $NaDodSO_4$/PAGE (16) and directly sequenced either after elution (species a1, a2, and pool of b and c1) or transfer onto an Immobilon (Millipore) membrane (species b, c1, and c2). Internal sequences were obtained as follows. In one case, pure species b was separated on a urea/$NaDodSO_4$/8% polyacrylamide gel (8), transferred on to nitrocellulose, and treated *in situ* with L-1-tosylamide-2-phenylethyl choloromethyl ketone-treated trypsin (17). Cleaved fragments were fractionated by HPLC (17) and the amino-terminal sequence was determined for a selected fraction (peptides T5a, b, c, and d). In a second case, pure species b was separated on a urea/$NaDodSO_4$/polyacrylamide gel. The gel slice was treated with CNBr and cleaved products were resolved on a $NaDodSO_4$/12% polyacrylamide gel, and transferred on to Immobilon, and one fragment (CN2) was subjected to amino-terminal sequencing. In a third experiment a pool made of species b, c1, and c2 was obtained by separation on a $NaDodSO_4$/8% polyacrylamide gel. After

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Abbreviation: AP, associated protein.

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**The sequences reported in this paper are being deposited in the EMBL/GenBank data base (accession no. J04527 and J04528).

electroelution, an aliquot was treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin and cleaved fragments were separated by HPLC (18). Amino-terminal sequences were determined for peptides T57a,b, T61, T62, T63, T69, T71, T76, T96, and T97. A second aliquot was treated with CNBr (16) and fragments were resolved by $NaDodSO_4$/PAGE. After transfer onto Immobilon, the amino-terminal sequence was determined for peptides CN1, CN3, CN4a,b, CN5, and CN6a,b. Sequence determinations were performed by automated Edman degradation in a gas-phase sequencer (Applied Biosystems).

Proteolytic Cleavage of AP-2 Complexes. Limited enzymatic proteolysis of native AP-2 complexes was used to generate fragments of α and β chains. Prior to digestion, aggregates were removed by centrifugation at 10,000 × *g* for 10 min (4°C). Digestion was performed at room temperature with L-1-tosylamide-2-phenylethyl chloromethyl ketone-treated trypsin. The reaction was stopped with 1 mM phenylmethylsulphonyl fluoride and when necessary also with soybean trypsin inhibitor (Sigma) at 10 μg/ml. Cleaved products (T0, T1, T2, T3, and T4a,b) were sequenced after isolation on a $NaDodSO_4$/10% polyacrylamide gel and transfer on to Immobilon.

DNA Manipulations. A λgt10 rat brain cytoplasmic poly(A)$^+$ cDNA library (19) was screened at low stringency with a pool of four sets of 17-base synthetic oligonucleotides designed according to the amino acid sequence of peptide T-61. The sequence used was 5′-GGN TA(T,C) AT(T,C,A) TA(T,C) TGG (C,A)G, where N was G, A, T, or C, in each of the four oligonucleotide sets respectively. Subsequent screens were performed with restriction endonuclease fragments on a plasmid cDNA library from rat brain cortex poly(A)$^+$ mRNA (gift of M. Browstein, National Institute of Mental Health). DNA sequences were obtained by the enzymatic method (20).

RESULTS

Partial Amino Acid Sequences of α and β Chains and cDNA Clones. Amino-terminal sequences of the intact 100- to 115-kDa chains confirmed that the amino termini of the β chains appear to be blocked, whereas those of the α chains (species a1, a2, c1, and c2; Fig. 1) start with the common sequence Pro-Ala-Val-Ser-Lys-Gly-Asp-Gly-Met-Xaa-Gly-Leu-Ala-Val-Phe-Ile (where Xaa is an amino acid of uncertain determination; see also ref. 21). We chose peptide T61 (from a pool of species b, c1, and c2) to design oligonucleotide probes to screen a rat-brain λgt10 cDNA library. The hybridizing recombinant λTK44 was used to obtain additional recombinants isolated from the plasmid cDNA library (Fig. 2*A*). The clones derive from two classes of cDNA molecules, which are the products of two related genes encoding for ≈105-kDa β chains (see below). We designate these chains as AP105a and AP105b.

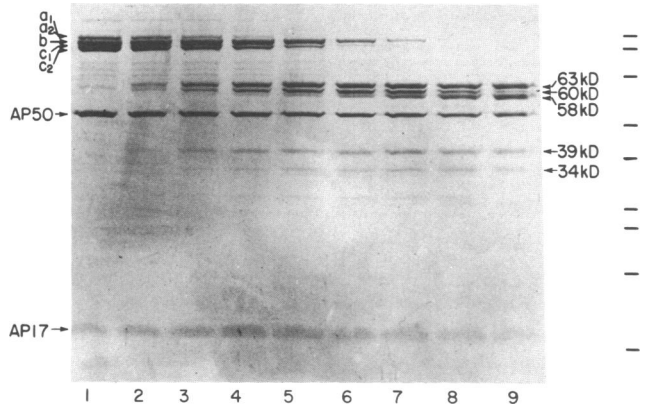


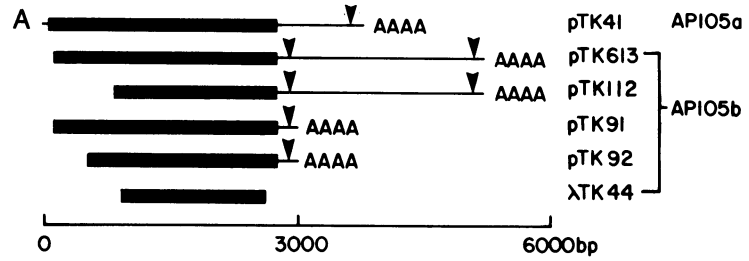
FIG. 1. Time course of proteolysis of AP-2 complexes. A sample of bovine brain AP-2 (0.25 mg/ml) was treated with trypsin at a protein/enzyme ratio of 1000:1 (wt/wt) and aliquots (10 μg) were subject to analysis on $NaDodSO_4$/13% polyacrylamide gels. Lanes: 1, before addition of protease; 2–9, after 1, 5, 10, 20, 40, 80, 160, or 320 min of digestion. a1, a2, c1, and c2 denote the main species of α chains. b denotes the main component of β chains. AP50 and AP17 correspond to the medium and small chains. The apparent size of the principal cleavage products of α and β chains is indicated. Size markers are 116, 97.4, 66, 45, 36, 29, 24, 20, and 14 kDa (kD).

AP105a and AP105b Are Two Members of the 100- to 115-kDa β Chains. Clone pTK41 (Fig. 2*A*) contains the complete open reading frame for a protein of 949 amino acids and of predicted molecular mass 104.7 kDa. We refer to this protein as AP105a. It is a β chain, because it contains the amino-terminal sequences of peptides T5a–d and CN2, obtained by digestion of purified bovine β chains (Fig. 2*B*). The deduced amino terminus of AP105a cannot be confirmed chemically, since β chains appear to be blocked (see also ref. 21). A second class of partial-length cDNA molecules (pTK613 and related shorter clones, Fig. 2*A*) encodes a protein similar to AP105a. We refer to this protein as AP105b (Fig. 2*B*). We note that the cDNA molecules for AP105b make alternative use of two polyadenylylation signals AATAAA (nucleotide positions 2863 and 5255; Fig. 2). AP105a and AP105b must be the products of different genes, since their DNA sequences show extensive third-position differences in codon usage, even in those regions where they display complete amino acid sequence identity. AP105b appears to be the major protein component b of the rat brain β chains, since it contains all the identifiable peptide sequences obtained from cleavage of either purified bovine β chains or a pool of bovine b, c1, and c2 chains (Fig. 2*B*). In contrast, the sequence of AP105a shows differences from the sequence of some fragments, such as T3b, T5d, T63, T96, T97, and CN6 (Fig. 2*B*), even though their yields are comparable to the other aligned peptides. Since we do not obtain peptides whose sequences correspond exclusively to AP105a, we conclude that it is a minor protein component of the β chains. The possibility that AP105a is either β' or b*, the immunologically related β chain variants found in bovine brain AP-1 and AP-2 complexes (8), seems unlikely, as β' and b* are significantly larger (≈115 kDa) than b (104 kDa) when analyzed by $NaDodSO_4$/PAGE (8). A computer search (22) shows that the sequences of AP105a and AP105b do not contain elements similar to AP50 or AP17, nor to other published sequences.††

Proteolytic Dissection of AP-2 Complexes and the Bipartite Organization of the α and β Chains. Analysis of the amino-terminal sequences from defined fragments of the large chains, combined with the primary structure determination of the β chains, has allowed us to map points of protease accessibility and to establish the polarity and domain structure of the large chains in AP-2 complexes. Several groups have shown that AP complexes are very susceptible to elastase, which generates a distinct cleavage pattern from the large chains while leaving AP50 and AP17 intact (23, 24). Fig. 1 shows the time-course $NaDodSO_4$/PAGE analysis of a cleavage pattern obtained by treating AP-2 complexes with trypsin. After brief proteolysis two groups of peptides appear with the concomitant disappearance of the large 100- to 115-kDa chains.

The first group of fragments contains a relatively stable fragment of 63 kDa and a more labile peptide of 60 kDa, which upon further digestion is quantitatively cleaved into a 58-kDa species. We note that at this point (Fig. 1, lane 9) the intensity

††Protein Identification Resource (1988) Protein Sequence Database (Natl. Biomed. Res. Found., Washington, DC), Release 16.



B

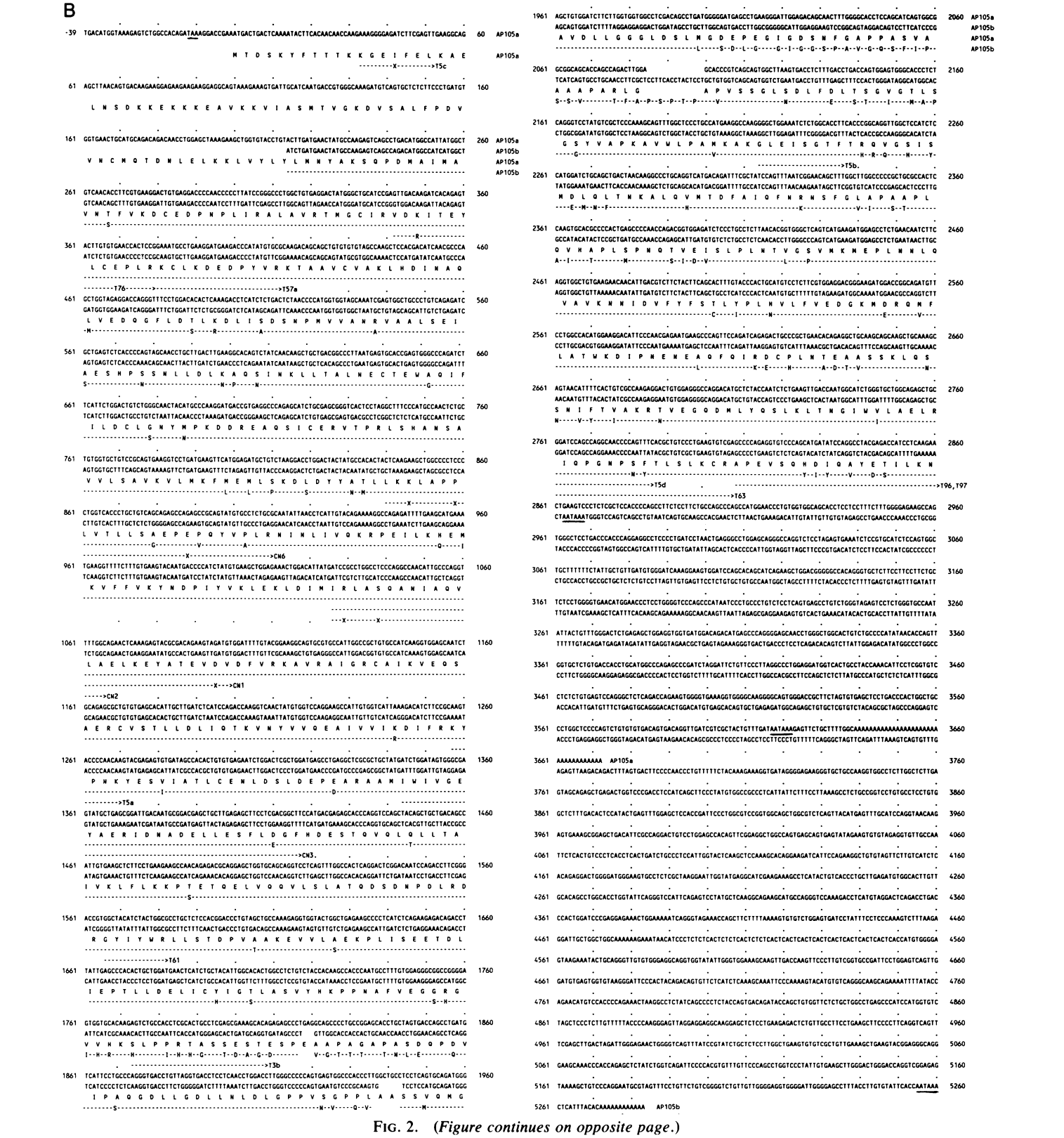


FIG. 2. (*Figure continues on opposite page.*)

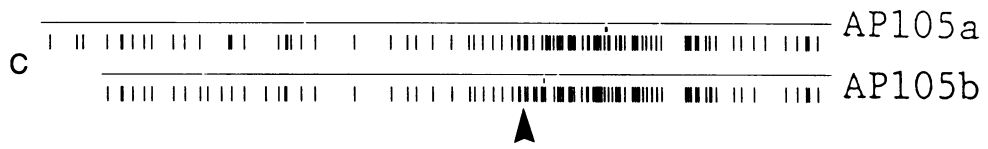


FIG. 2. Nucleotide and amino acid sequences from rat brain cDNA clones of two large β chains from AP-2 complexes. (*A*) Schematic representation of the sequenced rat cDNA clones isolated from λgt10 (λ) and plasmid (p) rat brain libraries. Clone pTK41 corresponds to the β chain protein designated AP105a. The independent partial-length clones pTK91, pTK92, pTK112, and pTK613 encode AP105b, the more prevalent b member of the brain β chains; their DNA sequences are identical. The coding regions (heavy line) have been aligned at their amino ends. Polyadenylylation signals (arrow) and poly(A) stretches are indicated. bp, Base pairs. (*B*) Compiled nucleotide and amino acid sequences for AP105a and AP105b. The open reading frame of AP105a is preceded by a stop codon TAA (position −12) and is followed by a 3′ untranslated region of 779 base pairs including a polyadenylylation signal (AATAAA; position 3617) and a poly(A) stretch. Due to isolation of partial length clones, the open reading frame of AP105b lacks about 70 amino acids at its amino terminus. Alternate polyadenylylation signals (AATAAA) are used by pTK91 and pTK92 (position 2863) and by pTK112 and pTK613 (position 5255). The corresponding 3′ untranslated regions span 26 and 2415 base pairs, respectively. Identifiable amino-terminal sequences from peptides of b chains and of a pool of b, c1, and c2 chains isolated from bovine brain AP-2 complexes are shown (horizontal arrows). Amino acids that differ from the deduced sequence of AP105b or whose determination was uncertain (x) are indicated. The single-letter amino acid code is used. (*C*) Vertical bars show the distribution of proline and glycine residues along the primary structure of AP105a and AP105b. The arrow points to the major site of tryptic cleavage of the prevalent b chain in AP-2 complexes. Its location defines the boundary between the amino- and carboxyl-terminal domain of the β chains.

of the stained bands is comparable for the 58- and 63-kDa fragments and AP50. Fragments of similar size generated by elastase treatment of AP-2 complexes remain associated with AP50 and AP17 in the AP core (24). The amino terminus of the 63-kDa fragment (peptide TO) is blocked, suggesting that it derives from the amino-terminal portion of the β chains. The related 60- and 58-kDa fragments (peptides T1 and T2) share the same amino-terminal sequence, which is identical to the amino-terminal sequences of the four α chains a1, a2, c1, and c2. We thus conclude that in the intact complex the amino-terminal domains of the α and β chains are located at the AP core.

The second group of fragments generated by tryptic digestion of AP-2 complexes contains peptides of 39 and 34 kDa as well as others of smaller size. These peptides are similar in size to the small fragments released from AP cores upon digestion of AP complexes with elastase (24). They must be derived from the carboxyl-terminal part of the chain since they appear simultaneously with the 63- and 58-kDa amino-terminal fragments. Determination of the amino-terminal sequence of the 39-kDa species indicates two sequences of similar molar yields. One of them (peptide T3b) is identified in the sequence of AP105b and maps to the carboxyl side of Lys-591 (Fig. 2*B*). It correctly predicts a fragment of 38 kDa measured from the carboxyl terminus of the intact β chain; the size of this fragment is complementary to the length of the corresponding 63-kDa amino-terminal domain. The other sequence from the 39-kDa species, as well as those from the other small fragments, cannot be found in the primary structures of the β chains. We suggest that they come from the carboxyl-terminal domains of the α chains.

The ease by which the large chains are cleaved into well-defined fragments suggests that the principal cleavage site is located on a relatively exposed section of the AP-2 complexes. We find that along the primary structure of both β chain molecules, the distribution of proline and glycine residues is not uniform (Fig. 2*C*) and that their content is relatively high between amino acid positions 577 and 728. This connecting segment contains a number of negatively charged residues, but few positively charged ones, and it is precisely where the cleavage site defining the boundary between the amino and carboxyl domains is located (Figs. 2*B* and 3). An accessible connecting segment is probably also present in the α chains, given the similar cleavage pattern of the α and β chains (Figs. 1 and 3).

Correspondence Between the Bipartite Organization and the Constant and Variable Domains of the α and β Chains. Comparison between the predicted amino acid sequences of AP105a and AP105b (Fig. 2*B*) establishes an overall similarity of ≈89%, allowing for conservative changes, and shows that the β chains have a relatively invariant amino-terminal domain and a much more variable carboxyl-terminal domain. The sequences of the amino-terminal domains (residues 1–580) can be aligned without gaps and differ at only 35 positions. The sequences of the terminal carboxyl domains (residues 581–949) require three gaps for alignment and differ at 135 positions. These constant and variable domains correspond precisely to the amino and carboxyl domains of the β chains defined earlier on the basis of their proteolytic sensitivity. A similar distinction appears to hold for the α chains. Fig. 1 shows that the α chains vary in size from 100 to 115 kDa, but that upon tryptic digestion all the amino-terminal domains are quantitatively reduced to one peptide of 58 kDa. We have not detected any other large fragments, in particular around 75 kDa, that could have arisen from the a1 or a2 chains. The simplest explanation, summarized in Fig. 3, is that the amino-terminal domains of the α chains are very similar to each other. In addition, the variability responsible for the different sizes of the intact molecules lies to the

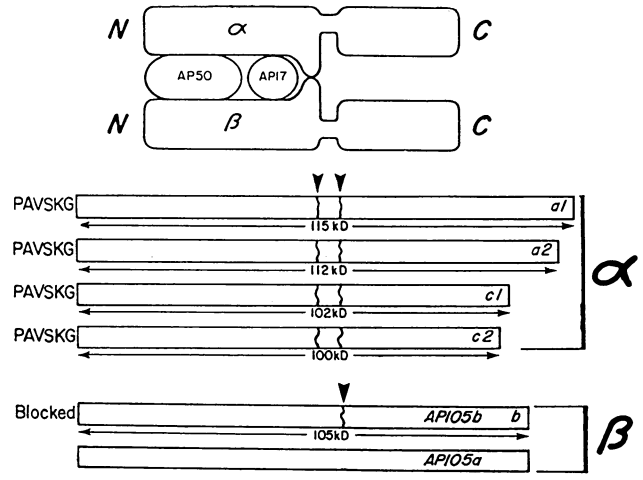


FIG. 3. Domain organization of the large α and β chains in AP-2 complexes that is consistent with relationships of their primary structures and structural domains (see *Discussion*). Assignment of amino-terminal sequences (single-letter code) of the intact large chains is indicated. Arrows point to the location of major tryptic cleavage sites that define the boundary between the two main domains of the large chains. The carboxyl-terminal domains are more variable and may, therefore, be responsible for the selection of those membrane-bound proteins (receptors) destined for coated-pit and coated-vesicle entrapment. The less variable amino-terminal domains, together with the unique AP50 and AP17 medium and small chains, define the AP core.

carboxyl-terminal side of the tryptic cleavage point that defines the 58-kDa amino-terminal domain.

DISCUSSION

Our results, combining the analysis and proteolytic cleavage experiments with the deduced primary structures of two β-type clathrin-associated large chains, can be summarized as follows. (*i*) The large chains in the AP-2 complexes have two distinct protease-resistant domains linked by a readily cleaved connecting segment. (*ii*) The amino-terminal domains of the two β chains we sequenced are significantly more similar to each other than are their carboxyl-terminal domains. Moreover, proteolytic cleavage of the α chains produces amino-terminal fragments of uniform size. The variability of AP large chains thus appears to reside in the carboxyl-terminal domains. (*iii*) The AP core, defined as the complex of one part of the large chains together with the medium and small chains, contains the amino-terminal domains of the large chains. These results are summarized in Fig. 3.

The diagram in Fig. 3 has the following features that depend on results and interpretations from other laboratories. (*i*) Each complex contains two large chains, one medium chain, and one small chain. This composition corresponds to the model of Virshup and Bennett (12), which is derived from careful cross-linking experiments. Our own analysis of the staining ratio of bands in cleaved AP-2 complexes (Fig. 1) is also consistent with this picture. Acceptance of alternative proposals (9, 11, 25) in which each complex contains two each of the large, medium, and small chains would not significantly alter our major functional conclusions. (*ii*) Each complex contains one α chain and one β chain. Ahle *et al.* (8) have shown that polyclonal antibodies against the β chains immunoprecipitate AP-2 complexes containing α chains. A more rigorous experiment performed with the analogous AP-1 complex demonstrated that it also contains one γ chain and one β′ chain. (*iii*) Chemical cross-linking experiments (11, 12, 26) show contacts between the large chains within an AP complex as well as an absence of contacts between the medium and small chains. Contacts between large chains and medium and small chains have not been analyzed in detail, and particular interactions cannot therefore be specified. (*iv*) AP complexes contain two appendages that correspond to the carboxyl-terminal domains of the large chains. The appendages were visualized by Heuser and Keen (27) as two globular structures extending from one end of the brick-shaped AP complexes. They showed that the appendages, removed by mild elastase treatment, correspond to the proteolytic fragments that we have now assigned to the carboxyl-terminal domains of the α and β chains.

Formation of coated pits and coated vesicles leads to the selective entrapment of proteins, such as membrane-bound receptors destined for intracellular vesicular traffic. It is therefore believed that some major element of the coat must provide the specific link with the cytoplasmic domains of the entrapped molecules. Receptors vary widely. If different linking elements are needed to select different classes of receptors, then clathrin chains are not good candidates; only one gene codes for the clathrin heavy chain in rat (14) and in yeast (28), and there is no evidence for differential processing of its RNA. Two genes code for the mammalian light chains, and their messages are differentially processed in a tissue-specific manner (18, 29). Their actual heterogeneity is restricted, however, and every tissue analyzed has a limited set of light chains. Thus, it is the AP complexes, not clathrin, that are generally thought to be the agents responsible for specific receptor interactions (30, 31). Since AP50 and AP17 appear to be unique species (ref. 10; S. Frucht and T.K., unpublished results), the heterogeneous group of large chains is the obvious candidate for the selector function.

Although heterogeneous in sequence and size, all the large chains have a similar two-domain organization. One domain is relatively invariant; the second is variable both in sequence and in length. Moreover, the constant domains interact with the other invariant proteins of the AP complex, AP50 and AP17. Thus the complex is divided into a constant core and variable appendages. It is plausible to propose that the constant part interacts with the other uniform component of coated vesicles—the clathrin lattice. Comparison of the effects of AP complexes on assembly of intact and light-chain free clathrin (T.K., unpublished result) shows that it is indeed the invariant heavy chain to which the APs bind. This proposal is also consistent with the retention of cores on the lattice of clathrin/AP-2 coats after cleavage of the APs with trypsin (T.K. and W.M., unpublished results) and with the specific binding of AP cores to preformed clathrin cages (32). By contrast, the variable carboxyl-terminal domains dissociate from proteolyzed coats (T.K. and W.M., unpublished results) and they do not bind to clathrin cages (32). In addition their sequences vary sufficiently to provide a set of recognition sequences for other proteins. Therefore, we propose that these are the selector domains responsible for the specific entrapment of membrane bound proteins in coated pits and coated vesicles.

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