We have utilized a rabbit reticulocyte lysate coupled transcription-translation system to express the large subunits of the clathrin associated protein-2 (AP-2) complex so that their individual functions may be studied separately. Appropriate folding of each subunit into N-terminal core and C-terminal appendage domains was confirmed by limited proteolysis. Translated ω2 subunit bound to both assembled clathrin cages and immobilized clathrin trimers, confirming and extending earlier studies with preparations obtained by chemical denaturation-renaturation. Translated α2 exhibited rapid, reversible and specific binding to clathrin cages. As with native AP-2, proteolysis of ω2 bound to clathrin cages released the appendages, while cores were retained. Further digestion revealed a 29-kDa ω2 clathrin-binding fragment that remained tightly cage-associated. Translated α2 also bound to immobilized clathrin trimers, although with greater sensitivity to increasing pH than the translated β2 subunit. Clathrin binding by both the α and β subunits is consistent with a bivalent cross-linking model for lattice assembly (Keen, J. H. (1987) Cell Biol. 105, 1989). It also raises the possibility that the α-clathrin interaction may have other consequences, such as modulation of lattice stability or shape, or other α functions.

Receptor-mediated endocytosis is a multi-step process involving membrane invagination, coated pit formation, and budding of these pits to form coated vesicles (2). A major protein implicated in endocytosis is clathrin, a triskelion-shaped protein that forms the structural basis for the regular polygonal lattice of coated pits and vesicles (1, 3). These coated membranes also contain additional protein components that have been referred to as assembly, adaptor, or associated proteins (APs). One probable function of APs is to promote polymerization of the clathrin lattice at defined sites and times. APs are also likely to interact with receptor cytoplasmic tails resulting in the selective inclusion of various receptors into coated pits (reviewed in Refs. 2, 4, 5).

APs vary in structure and intracellular localization. The best characterized examples include AP-1, a Golgi-associated heterotetramer consisting of γ, β1, AP47, and AP19 polypeptides; AP-2, a plasma membrane-associated heterotetramer of α, β2, AP50, and AP17 polypeptides; and AP-3/AP180, a neuron-specific monomer (2, 4). This study concerns the AP-2 complex and focuses on the interactions of its α subunit with clathrin. Two genetically distinct isoforms of α subunit exist: αα, an isoform which is expressed ubiquitously, and αω, an isoform believed to be expressed primarily in neurons. The isoforms are 84% identical and differ predominantly in their C-terminal portions. The αω isoform contains a unique 42 amino acid insert beginning at position 704 (6).

Although AP-2-clathrin interactions have been studied in detail (1, 7–13), it has been difficult to ascertain the contributions of individual AP-2 subunits. Fractionation of AP-2 polypeptides with urea and guanidinium chloride was used to study these interactions, indicating that the α and β2 subunits alone were necessary and sufficient for coat assembly activity (13). Ahle and Ungewickell (7), using mild denaturation to purify ω2 subunit from AP-2, demonstrated that the former was capable of competitively inhibiting AP-2 binding to preassembled clathrin cages. This work was extended by Gallusser and Kirchhausen (14) who demonstrated that recombinant ω2 subunit purified by denaturation-renaturation from Escherichia coli inclusion bodies was capable of promoting clathrin assembly. Collectively, these results support the hypothesis that the ω2 subunit plays an important role in AP-2-driven clathrin assembly in vivo, but the role of the β subunit remains undefined.

We have previously reported that an αω/AP50/AP17 complex prepared by mild denaturation-renaturation was capable of binding to preformed clathrin cages, suggesting that one or more of the other subunits, most likely the α subunit, also recognizes and binds clathrin (13). We adopt the approach of in vitro translation of the individual large AP-2 subunits to further explore this issue. The findings reported here indeed demonstrate that the α subunit can bind tightly to clathrin, consistent with a role in coat assembly or other coat-associated functions.

MATERIALS AND METHODS

The TnT rabbit reticulocyte lysate transcription-translation kit and pSP65 cloning vector were purchased from Promega. Translabel was obtained from ICN Biomedicals, Inc. Sepharose CL-4B and Superose 6B resins were from Sigma, and CNBr-activated Sepharose CL-4B was purchased from Pharmacia Biotech Inc. Clathrin and assembly proteins were prepared from calf brains as described previously (1, 15). L-1-tosyl-aminomethyl-2-phenylethyl chloromethyl ketone-trypsin was from Worthington Biochemical, Inc. HEPES was purchased from Boehringer Mannheim. All other chemicals were reagent grade or better.

Buffers used are as follows: Buffer A: 0.1 M sodium MES, 1.0 mM EGTA, 0.5 mM MgCl2, 0.02% NaN3, pH 6.5; Buffer B, Buffer A: 1.0 M Tris-HCl, pH 7.0, glyceral (4:5:4.9:1 (v/v)); Buffer C, 5 mM sodium MES, 2 mM CaCl2, pH 6.13; Buffer T, 100 mM dipotassium tartrate, 10 mM HEPES, 1 mM EGTA, 0.5 mM MgCl2, pH 7.0.

In Vitro Transcription-Translation—Polypeptides were expressed in vitro using a TnT rabbit reticulocyte lysate transcription-translation kit. The αω cDNA, kindly provided by M. S. Robinson (Oxford University) (6) was subcloned into the EcoRI site of the pSP65 vector (Promega), under the sp6 promoter. Constructs containing inserts in both
α Chain of the AP-2 Adaptor

The sense and antisense orientations were confirmed with restriction enzyme analysis and purified on cesium chloride gradients.

The cDNA template for the C-terminal deletion mutant \( \alpha_2\text{C1-605} \) was produced by restricting the pSP65\( \alpha_2 \) sense construct with Aval (\( \alpha_2 \) nucleotide 2021). The mutant was then generated by runoff transcription-translation. Full-length \( \beta_2 \) subunit was transcribed from the pBlueScript SK+ T3 promoter, using a construct kindly provided by T. Kirchhausen (Harvard University) (16). Luciferase was translated with the sp6T7 control construct (Promega). \( \beta_2 \)-Galactosidase was produced using a pSP65-based construct provided by V. Gurevich (Thomas Jefferson University). Translation-translation reaction mixtures were assembled according to the manufacturer's instructions and incubated 90–120 min. Proteins were translated in the presence of \( ^{35} \text{S} \) Translabel: although the reagent contained both \( ^{35} \text{S} \) L-methionine and -cysteine, a longerautoradiographicexposure was required for comparable detectability on film. As a negative control, we translated \( \alpha_2 \) in the presence of the antisense \( \alpha_2 \) construct, obtaining a slightly attenuated yield of protein (lane 3). The expressed \( \beta_2 \) subunit (lane 4, \( M_r = 105,133 \)) was readily resolved from translated \( \alpha_2 \) by gel electrophoresis. Like translated \( \alpha_2 \), the \( \beta_2 \) polypeptide comigrated with a corresponding Coomassie-stained band of AP-2. Finally, in some experiments, translated luciferase (lane 5, \( M_r = 62,000 \)) and \( \beta_2 \)-galactosidase (lane 6, \( M_r = 116,000 \)) were utilized as controls.

Proteolysis of in Vitro Translated \( \alpha_2 \) and \( \beta_2 \) Polypeptides—Limited proteolysis of AP-2 reveals two major stable protein domains: one containing 60–66 kDa N-terminal core domains of the \( \alpha \) and \( \beta_2 \) subunits associated with intact AP50 and AP17, and the other consisting of 30–40 kDa domains corresponding to smaller C-terminal appendages of the \( \alpha \) and \( \beta_2 \) subunits (10, 12, 17, 18, and data not shown).

Similarly, proteolysis of the in vitro translated \( \alpha_2 \) or \( \beta_2 \) subunits alone yielded fragments of 58–66 kDa and \( \sim 40 \) kDa (Fig. 2). Identical results were obtained when translated proteins were cleaved in the presence of carrier AP-2, as assessed by comigration of Coomassie Blue-stained and radiolabeled bands on SDS-PAGE (data not shown). Quantitative analysis of the changes in the full-length \( \alpha_2 \) product and the appendage domain confirm a precursor-product relationship (Fig. 3). The coincidence of the two curves in Fig. 3 demonstrates that at all tryptic concentrations the fraction of full-length \( \alpha_2 \), cleaved is virtually identical to that of appendage generated. Furthermore, from the published sequence of the \( \alpha_2 \) cDNA (6), the C-terminal 40 kDa portion of \( \alpha_2 \) (i.e., amino acids 610–977) is predicted to contain five of the 17 methionine residues of \( \alpha_2 \), or 27% of the total \( \alpha_2 \) radiolabel. In close agreement with this prediction, 24% of the undigested full-length counts were found in the 40-kDa proteolytic product upon complete digestion of full-length \( \alpha_2 \). Hence, we conclude that the initial cleavage of translated \( \alpha_2 \) by trypsin occurs in a region corresponding to the originally exposed linker of an \( \alpha \) subunit in the intact AP-2 complex.

The translated \( \beta_2 \) subunit was slightly more resistant to proteolysis initially than the \( \alpha_2 \) subunit, as has been observed by others (17). The initial proteolytic susceptibility of translated \( \alpha_2 \) resulting in the generation of cores and appendages (Fig. 2, lanes 2 and 3) was comparable to that of bovine brain \( \alpha_2 \) in an AP-2 complex as assessed by digestion of translated \( \alpha_2 \) in the presence of AP-2 (data not shown). However, further digestion of both translated subunits revealed significant differences from those in brain AP-2. The 58–66 kDa core domains of translated \( \alpha_2 \), and especially of \( \beta_2 \), were much more labile than...
translated β2 subunit did bind to clathrin cages (data not shown). As shown in Fig. 4, the majority of translated α polypeptide cosedimented with the clathrin cages (lane 13), while a small amount of translated and apparently aggregated α polypeptide was sedimented by low speed centrifugation (lane 12). Typically, sedimentation of translated αa in the absence of cages, taken as a measure of nonspecific binding, was 10–15% of the total binding (lane 10).

By incubating the in vitro translation mixture with increasing concentrations of clathrin cages we obtained a dissociation constant of $1.1 \times 10^{-7}$ M (Fig. 5). From the asymptote of the binding curve, the maximal $\alpha$ fraction bound was 0.71, implying that not all of the translated protein was capable of binding clathrin. The binding was not inhibited by 1 mM phytic acid, a potent inhibitor of AP-2 self-association (9), further evidence that binding was specific and not due to aggregation or self-association (data not shown).

Clathrin cage binding by translated αa subunit was inhibited by saturating quantities of AP-2, confirming that the interaction was specific (Fig. 6). From the apparent IC50 and the published $K_d$ for the AP-2-clathrin interaction of $10^{-8}$ M (17, 20), we calculate a $K_d$ for the αa-clathrin interaction of $0.7 \times 10^{-7}$ M, in reasonable agreement with the value estimated by direct binding.

Both clathrin-clathrin and AP-clathrin interactions are readily reversed by high concentrations of protonated amines such as Tris-HCl (1, 6, 15). The α-clathrin interaction was also reversible. Brief (5 min) treatment of the sedimented cages, to which translated αa was bound, with 500 mM Tris-HCl, pH 7, followed by a high speed spin released most ($>80\%$) of the αa into the supernatant. Furthermore, the solubilized αa again cosedimented with the clathrin cages reformed by stepwise dialysis of the dissociated preparation into Buffer C and then into Buffer A (data not shown).

Binding of Translated Proteins to Clathrin Trimers Immobilized on Sepharose CL-4B Beads—We used clathrin-Sepharose to assess the ability of α and β subunits to bind clathrin triskelia (Fig. 7). Underivatized Sepharose CL-4B bound little if any of either of the translation products. The translated β2 polypeptide bound tightly to clathrin-Sepharose, providing the first direct demonstration of its ability to bind disassembled clathrin as well as cage structures. Binding of the αa polypeptide was also observed, although it was not as complete as that of the β2 polypeptide under these conditions. Interestingly, while β2 bound with relatively little sensitivity to pH, αa binding to clathrin trimers was considerably more sensitive to increasing pH throughout the range 6.5–7.5.

Functional Domains of Translated Proteins—Previous studies have shown that following controlled proteolysis, only the large core fragments of AP-2 retain an ability to bind to clathrin...
The fraction bound was determined by excision and counting of the high speed pellet and supernatant bands and was corrected for nonspecific sedimentation. The data were fit to rectangular hyperbolas (Kaleidagraph) yielding values of Kd for the full-length αa, and Kd = 3.1 × 10⁻⁷ M and 36% maximal binding (r = 0.955) for αa(1–605).

In contrast to the failure of either core or appendage domain to bind appreciably with clathrin cages, we carried out related experiments to evaluate the functional properties of domains of isolated α subunit and its individual domains. We observed that only full-length αa bound clathrin cages de novo in contrast the 58–66- and 40-kDa proteolytic products remained predominantly in the supernatant (data not shown).

Alternatively, to assess the ability of the isolated N-terminal region of translated αa to bind to preformed clathrin cages, we also produced a truncated polypeptide, designated αa(61–605), by runoff transcription-translation (Fig. 1). This protein did bind in a saturable manner to clathrin cages, although the apparent binding affinity (Kd = 3 × 10⁻⁷ M) was somewhat lower that of the full-length protein (Fig. 5). Only 36% of the total protein was capable of binding, suggesting either that a greater proportion of the translated protein was misfolded or that a binding equilibrium had not been established.

In contrast to the failure of either core or appendage domain of translated and digested αa to bind, when cages with bound αa were incubated with trypsin the core fragments were preferentially retained by the cages while the appendage domain was released into the supernatant (Fig. 8). A similar result has been obtained with native AP-2 (12). Interestingly, on more vigorous proteolysis of the cage-bound translated αa a discrete 29-kDa cage-associated fragment became evident and was prominent only in the presence of clathrin cages (compare Fig. 8, lane 2, and 3, with lane 1).
with Fig. 2). This fragment likely corresponds to the clathrin-binding domain of the α subunit.

**DISCUSSION**

AP-2 is capable of binding clathrin trimers with high affinity, an interaction representing an initial step in the coat assembly process. Following treatment with urea or guanidinium chloride, the AP-2 complex has been fractionated by gel filtration or hydroxylapatite chromatography, yielding partially purified α, β, and 50 kDa/17 kDa subunits. Of these, only the large α and β2 subunits of AP-2 were required for clathrin coat assembly in vitro (13). Dissociated β2 subunits from such preparations were shown to bind to clathrin cages but could not alone sponsor clathrin assembly (7). Recent studies using recombinant protein have reported that β2 alone is capable of inducing clathrin assembly (14), and β1 has been implicated in clathrin recruitment in the trans-Golgi network (22). Previous work from this laboratory suggested that α subunits could bind to clathrin trimers and cages (23). However, these experiments suffered from potential limitations in that the α fractions contained small quantities of β and 50-kDa/17-kDa polypeptides, preventing an unambiguous assignment of clathrin binding activity to the α subunit.

To further examine the issue of clathrin-binding subunits, we have translated the α2 and β2 subunits of AP-2 in vitro in a rabbit reticulocyte lysate system to assess their respective clathrin binding capabilities de novo. The approach of in vitro translation has several important advantages. The individual subunits are generated without resort to the strong denaturants that make it extremely difficult to be certain that the native state has been reattained. In contrast, the translation system produces polypeptides in a physiological environment with the appropriate folding factors, more closely resembling the intracellular milieu. Further, readily detectable radioactive polypeptides are generated that can be used at tracer levels (= 10^{-14} m) in functional assays. This is a major advantage in the study of AP-2 structure and function because the protein and its subunits are prone to aggregation and self-association (8) even at relatively low protein concentrations (8 M). Finally, the study of individual polypeptides of multi-subunit proteins by in vitro transcription-translation (24) may be particularly appropriate for the APs. Although these proteins have been assumed to function only as intact tetrameric complexes, there is recent evidence that the AP50 functions independently of the AP-2 complex as an activator of the vacuolar proton pump (25, 26). The structural and functional attributes of the isolated α and β polypeptides reported here and previously (7, 14) suggest that they, too, could have independent roles.

Our results show that readily detectable amounts of AP-2 α2 and β2 polypeptides can be expressed in a functional form in vitro. Both appear to assume the proper secondary and tertiary conformation by folding into the core and appendage domains that are characteristic of the intact AP-2 protein. The fragments obtained on limited proteolysis correspond well to those expected from the large subunits of bovine brain AP-2, though there are differences. While the C-terminal appendage fragment is resistant to further proteolysis, the core fragments appear to be more heterogeneous and extremely labile. This is consistent with the proposed quaternary structure of isolated AP-2 (10, 21). The α and β2 C-terminal appendages do not display stable intermolecular contacts with other subunits of AP-2 and likely function as independently folded and stable domains. In contrast, in native AP-2 protein the N-terminal core domains of α and β are in proximity to each other and to the AP50 and AP17 polypeptides. These interactions do not occur with the translated polypeptides. Consequently, the in vitro translated subunits may be relatively unprotected and more prone to proteolysis, yielding the results seen in Fig. 2. Upon proteolysis of cage-bound α2, the appendage fragments are preferentially released, while the core is almost entirely retained. Of particular interest is the appearance of a novel 29-kDa fragment when cage-bound α2 is proteolyzed (Fig. 8). The appearance of this band correlates well with the disappearance of the core 58–66-kDa fragments, suggesting that further digestion is blocked by tight association and stabilization by clathrin. Conversely, if dissociated this fragment may be more rapidly degraded: in the absence of clathrin, heterogeneous bands of this size are barely detectable (Fig. 2). It seems likely that this fragment comprises part of a discrete clathrin-binding domain within the α subunit.

In contrast to the tight retention of the core and 29-kDa fragment when the full-length protein is proteolyzed, α2 (1–605) and proteolytic core fragments generated prior to cage binding interact with much lower affinity. This may indicate that the appendage and/or C-terminal linker regions of α2 are required to maintain the free core domain in a conformation in which it is able to interact with clathrin. Alternatively, the appendage or linker regions may interact with clathrin directly.

The observation that both α2 and β2 subunits of AP-2 have clathrin-binding sites supports the concept that coat assembly proceeds by bivalent binding and stabilization of overlapping clathrin triskelia in a conformation that leads to polygon formation, essentially the cross-linking model proposed earlier (1, 2). Whether this hypothetical mechanism extends to other proteins such as AP-3/AP180, auxillin (27, 28) and a novel AP-20 (29) that have been reported to promote clathrin assembly in vitro remains to be determined. In any case, the expanding group of proteins capable of promoting polymerization suggests that coat assembly may be invoked by different effectors under a variety of different circumstances in vivo.

While both α2 and β2 subunits bind strongly to assembled clathrin lattices, α2 subunit binding to clathrin trimers is much more sensitive to pH in the physiological range than is the β subunit. This seems unlikely to be a consequence of liability of the isolated α2 conformation in solution, as we detect no change in either the proteolysis pattern or susceptibility of translated α2 subunit over this pH range (data not shown). The increased affinity of the α subunit for clathrin with decreasing pH correlates with the increased ability of AP-2 to drive coat formation with decreasing pH (21), arguing for a role of the α subunit in lattice assembly. In addition, cytoplasmic acidification to pH 6.3–6.5 also results in “freezing” of clathrin lattices with increased curvature (32) thereby arresting receptor-mediated endocytosis (19, 21, 30, 31). These observations suggest that the α-clathrin interaction may also be involved in lattice shape changes during vesiculation and endocytosis, or conceivably, that through this binding clathrin may affect other α functions.

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Note Added in Proof—We have recently found that the translated mouse γ subunit of the AP-1 complex binds to clathrin cages with Kd ~ 0.15 μM, comparable to the α2 results shown in Fig. 5. In view of published results (22), this suggests that AP-1, like AP-2, can bind bivalently to clathrin.

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α Chain of the AP-2 Adaptor

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