The assembly of clathrin-coated buds on the Golgi requires the recruitment of the heterotetrameric AP-1 adaptor complex, which is dependent on both guanine nucleotides and the small GTP-binding protein ADP-ribosylation factor (ARF). Here, we have investigated the structural domains of the AP-1 complex necessary for ARF-mediated translocation of the adaptor complex onto Golgi membranes and the subsequent recruitment of clathrin onto the membrane. Controlled proteolysis of purified AP-1, derived from bovine adrenal coated vesicles, was used to generate AP-1 core fragments composed of the amino-terminal trunk regions of the α1 and γ subunits and associated μ1 and σ1 subunits, and lacking either the β1 subunit carboxyl-terminal appendage or both β1 and γ subunit appendages. On addition of these truncated fragments to AP-1-depleted adrenal cytosol, both types of core fragments were efficiently recruited onto Golgi membranes in the presence of GTPyS. Recruitment of both core fragments was inhibited by the fungal metabolite brefeldin A, indicative of an ARF-dependent process. Limited tryptic digestion of recruited, intact cytosolic AP-1 resulted in the quantitative release of the globular carboxyl-terminal appendage domains of the β1 and γ subunits. The adaptor core complex remained associated with the Golgi membranes. Recruitment of cytosolic clathrin onto the Golgi membranes was strictly dependent on the presence of intact AP-1. Tryptic removal of the β1 subunit appendage prevented subsequent clathrin recruitment. We conclude that the structural determinants required for the ARF-mediated binding of cytosolic AP-1 onto Golgi membranes are contained within the adapter core, and that the carboxyl-terminal appendage domains of the β1 and γ subunits do not play any role in this process. Subsequent recruitment of cytosolic clathrin, however, requires an intact β1 subunit.

The clathrin-coated vesicle exhibits a well defined structural organization, and the major protein components have been purified and characterized (1–4). In addition to the principal coat protein clathrin, two major adaptor complexes have been identified. One type is restricted to the trans-Golgi network (TGN) and Golgi-derived coated vesicles and termed AP-1. The second, localized to the plasma membrane and endocytic clathrin-coated vesicles, is termed AP-2. Adaptors are heterotetrameric complexes, composed of one related and one unique ~100-kDa subunit, and two additional components, the ~50-kDa μ and ~20-kDa σ subunits. Thus, the Golgi AP-1 adaptor complex is composed of γ, β1, μ1, and σ1 subunits, and the related AP-2 is composed of α, β2, μ2, and σ2 subunits. The β1 and β2 subunits are very similar in structure (5–8), while the α and γ subunits are the most distantly related adaptor subunits (4, 9). Electron microscopy has revealed that the AP-2 adaptor has a large globular core domain and two exposed globular head or appendage domains (9, 10). Controlled proteolysis of both AP-1 and AP-2 (11–14) has shown that the core is composed of the amino-terminal trunk regions of either the α and β2 or the β1 and γ subunits as well as the intact μ and σ subunits, while the appendages represent the 30–40-kDa carboxyl-terminal regions of the large subunits.

Adaptors are believed to facilitate clathrin-coated vesicle formation by combining a clathrin binding domain and a membrane association domain within an oligomeric protein complex. When it became apparent that the β1 and β2 subunits were highly related, it was suggested that these subunits might contain a common domain capable of binding to clathrin (5). Indeed, the purified β2 subunit interacts directly with preformed clathrin cages (15), and, more recently, it has been shown that recombinant β-type subunits alone can induce clathrin polymerization (16). Further attempts to define the clathrin binding site more precisely indicated that proteolytically generated cores can bind to preformed clathrin cages (12), suggesting that the clathrin binding site is located in the amino-terminal trunk domain. However, in buffer conditions that prevent adaptor self-association, tryptic removal of the β subunit appendage results in the release of both appendage and core fragments from clathrin-coated vesicles (14). This suggests that both the trunk and appendage domains of the β-type subunits are required for high affinity clathrin binding (14, 16).

Before clathrin binding and polymerization can occur, however, the adaptors must associate stably with the appropriate

The abbreviations used are: TGN, trans-Golgi network; AP-1, Golgi-specific heterotetrameric adaptor complex; AP-2, plasma membrane-specific heterotetrameric adaptor complex; ARF, ADP-ribosylation factor; BFA, brefeldin A; GTPyS, guanosine 5′-O-(thiotriphosphate).

The nomenclature that we have used to distinguish between the different domains and subunits of the AP-1 adaptor is as follows. The appendage refers to the globular carboxyl-terminal head domain and the hinge region of either the β1 or γ subunit. The hinge connects the appendage to the adaptor core, consisting of the 60–70-kDa amino-terminal trunks of both the β1 and γ subunits and the intact μ1 and σ1 subunits.

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intracellular membrane compartment. According to the simplest model for the interaction of adaptors with membranes (1, 3, 4, 17, 18), it was assumed that they attach directly to the cytoplasmic portions of selected receptors. Indeed, there is evidence that adaptors can interact directly, albeit weakly, with the cytoplasmic regions of certain proteins sorted into clathrin-coated vesicles (17–22). This simple model, while attractive, failed to explain the restricted localization of AP-1 within the cell and why AP-1 does not bind to cytoplasmically oriented trafficking motifs in receptors transiting through other intracellular compartments (2–4). Furthermore, the dramatic effect of brefeldin A (BFA) on the intracellular localization of AP-1 also suggested that clathrin coat formation is highly regulated (23, 24).

Recently, the recruitment of cytosolic AP-1 onto purified Golgi membranes was reconstituted in vitro (25, 26). Adaptor binding was found to be dependent on GTP and antagonized by BFA. ARF, a small GTP-binding protein, was identified as the GTP-requiring component (25, 26). Coatomer-coated vesicle formation also proceeds by the initial recruitment of ARF (27, 28), explaining the sensitivity of both clathrin- and coatomer-coated vesicles to BFA (29, 30). BFA sensitivity has yet been discerned (25, 26), we proposed that ARF may be required to ensure the selective interaction of AP-1 with a specific docking protein in the context of the TGN membrane. While our model overcomes the limitations of the direct association model, the structural determinants on the AP-1 complex that are recognized by the putative docking protein remain to be identified.

The role of the α and γ subunit appendages has been probed using a chimera-based approach (29). Swapping the α and γ subunit head and/or hinge regions did not appear to affect either the assembly or targeting of the AP-1 or AP-2 chimeric complexes. Here, we have taken a different approach to directly assess the structural features of the AP-1 heterotetramer required for Golgi membrane association and subsequent clathrin recruitment. Defined trypsic fragments were produced by controlled proteolysis (8, 14), and this has enabled us to follow the ARF-dependent recruitment of the different fragments onto Golgi membranes. We have found the translocation of the AP-1 core complex to be indistinguishable from intact AP-1. This provides the plausible explanation that targeting of the ARF protein remain to be identified.

**EXPERIMENTAL PROCEDURES**

**Materials—**α,β,γ-Pi (GTP (>3,000 Ci/mmol) was obtained from ICN. GTP-S was purchased from Boehringer Mannheim. Aprotinin, ATP, benzamidine, BFA, dithiothreitol, leupeptin, soybean trypsin inhibitor, triton X-100, and Tween 20 were from Sigma. BFA was stored as a 5 mg/ml stock solution in ethanol at 20°C. Sprague-Dawley rats were purchased from SASCO, and the frozen bovine adrenal glands were obtained from PelFreeze. BA 83 nitrocellulose membranes were obtained from Schleicher and Schuell, and Sepharose 4B, CNBr-activated Sepharose 4B, and M, markers for electrophoresis were obtained from Pharmacia Biotech Inc. Theslyphenylalanyl chloride-methyl ketone-treated trypsin was purchased either from Cooper Biomedical or Worthington. The crude homogenate was centrifuged sequentially at 10,000 × g for 10 min, 100,000 × g for 10 min, and 100,000 × g for 1 h. The clathrin-containing fractions were pooled separately and concentrated approximately 10-fold by ultrafiltration using a Amicon YM 30 filter. After centrifugation at 12,000 × g for 15 min, the clathrin-enriched fraction was stored at 4°C.

Endogenous AP-1 was removed from the clathrin-depleted adrenal cytosol by immunodepletion (26). Briefly, cytosol was passed over an immobilized mAb 100/3 column equilibrated in buffer C on ice, and the loading effluent was resupplied to the column several times, before similar passages over a second mAb 100/3 column equilibrated in buffer C on ice. The resulting AP-1-depleted cytosol was analyzed on immunoblots using mAb TD.1 and mAb 100/1. The clathrin triskelae eluted well ahead of the adaptors, and fractions after the clathrin peak were pooled, concentrated by precipitation with 60% ammonium sulfate, and dialyzed against 25 mM Hepes-KOH, pH 7.0, 125 mM potassium acetate, 5 mM magnesium acetate, and 0.05% sodium azide. Fractions of 20 ml were collected, and the elution positions of clathrin and AP-1 were determined on immunoblots using mAb TD.1 and mAb 100/1. The clathrin-enriched fraction was stored at 4°C.

**Cell and Tissue Culture and Immunofluorescence—**NIH 3T3 fibroblasts were grown in 10-cm round glass Petri dishes coated with 1.2-mm glass cover slips in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. For permeabilization, the cells were washed first in phosphate-buffered saline, then twice in 25 mM Hepes-KOH, pH 7.2, 125 mM
p-sheet potassium acetate, 2.5 mM magnesium acetate, 1 mg/ml glucose (buffer E) and then frozen on dry ice. The cells were thawed, and the endogenous cytosol was removed by dipping the coverslip into a beaker of buffer E 10 times at room temperature. Gel-filtered whole rat liver cytosol, at a concentration of ~5 mg/ml in buffer D, was supplemented with 100 μM GTPγS and coated vesicle-derived adrenal AP-1 or proteolytic fragment to give a total adaptor concentration of ~50–70 μg/ml. After addition of the cytosol, the cells were warmed to 37 °C for 15 min and then washed by dipping the coverslip into a beaker of buffer E 10 times. The cells were then fixed for 5 min at room temperature with 3.7% formaldehyde in PBS and double-labeled with R461 and mAb 100/3 as described in detail previously (5).

Controlled Tryptic Digestion—Tryptic digestion of Golgi membranes was carried out in buffer C at a membrane concentration of 1 mg/ml. Triton X-100 and trypsin were added on ice as indicated in the figures, and digestion was performed at 37 °C for 10 min, followed by chilling and addition of excess soybean trypsin inhibitor. For some experiments, the membranes were then collected by centrifugation at 15,000 × g for 10 min at 4 °C, the supernatant fractions were concentrated by precipitation with methanol/chloroform (36), and both supernatant and pellet fractions were analyzed by immunoblotting. Proteolysis of the purified adrenal AP-1 was performed in 25 mM Hepes-KOH, pH 7.0, 125 mM potassium acetate for 1 h at 30 °C using a protein/trypsin ratio of 1:30 for the quantitative removal of the β subunit appendage. The reaction was stopped by adding excess soybean trypsin inhibitor, and the sample was used for the binding studies without further purification. For the preparation of appendageless AP-1 cores, the digestion was carried out at 30 °C using a trypsin to substrate ratio of 1:20. Residual AP-1 containing an intact γ subunit, which we have found difficult to cleave quantitatively with trypsin (14), was removed by immunoadsorption on a mAb 100/3 column. The unbound material was concentrated to approximately 0.5–1.0 mg/ml in a Centricon 30 before use in the binding assays. The polypeptide composition of all the fragments used in this study was verified on Coomassie Blue-stained gels.

Electrophoresis and Immunoblotting—Discontinuous SDS-polyacrylamide gel electrophoresis was carried out as described previously (26). Proteins were transferred onto nitrocellulose membranes at 110 V for 70 min in a buffer of 15.6 mM Tris, 120 mM glycine, pH ~8.3, equilibrated to 4 °C. Following transfer, the membranes were blocked and probed with antibodies as described previously (26).

[32P]GTP Overlays—GTP-binding proteins immobilized on nitrocellulose were preincubated for 60 min at room temperature in 50 mM Tris-HCl, pH 7.5, 5 mM magnesium acetate, 1 mM EDTA, 1 mM dithiothreitol, and 0.3% Tween 20 (buffer D), supplemented with 200 μM ATP and 2 μCi/ml [32P]GTP, and then incubated for an additional 60 min, followed by four washes in buffer D. The GTP-binding proteins were visualized by autoradiography at ~80 °C using an intensifying screen.

RESULTS

The goal of this study was to identify the structural regions of the heterotetrameric AP-1 adaptor complex which are involved in the ARF-dependent recruitment onto Golgi membranes and the subsequent recruitment of cytosolic clathrin. To distinguish between the endogenous rat liver Golgi-associated AP-1 and exogenously added cytosolic AP-1, using a species-specific mAb directed against the γ subunit, we had to modify our existing AP-1 binding assay by substituting bovine adrenal gland cytosol for rat liver cytosol. To limit our analysis to the initial events in clathrin-coated vesicle assembly, clathrin-depleted adrenal cytosol (26) has been used for several of the experiments described below.

Recruitment of Bovine Adrenal AP-1 onto Golgi Membranes—Recruitment of AP-1 onto Golgi membranes is preceded by the recruitment of ARF (25, 26). While only very low levels of ARF are found on purified Golgi membranes (37, 38) (Fig. 1, lane a), the level increased markedly upon incubation with clathrin-depleted adrenal cytosol in the presence of GTPγS (lane c). Several other uncharacterized low molecular weight GTP-binding proteins were also recruited onto the Golgi membrane. The accumulation of ARF was accompanied by recruitment of cytosolic AP-1 onto the Golgi membrane fraction. When recruitment was followed after pretreatment with BFA, decreased levels of both ARF and AP-1 were observed in the membranes, and the extent of recruitment varied according to the time of addition of BFA (Fig. 1, lanes d–g). These results are analogous to those obtained using rat liver cytosol (26) and reiterate the sensitivity of both ARF and AP-1 membrane association to BFA.

FIG. 1. GTP-dependent association of ARF and AP-1 with Golgi membranes. Tubes containing 50 μg/ml rat liver Golgi, 5 mg/ml clathrin-depleted adrenal cytosol, 100 μM GTPγS, and 50 μg/ml BFA in a final volume of 400 μl were prepared on ice as indicated in the figure. The asterisk denotes addition after 10 min of incubation at 37 °C. All reactions were terminated after 20 min, and the membrane fractions were analyzed on immunoblots with mAb 100/3 (upper portion) or by [32P]GTP overlay (lower portion). The ~80-kDa band detected with mAb 100/3 represents an endogenous proteolytic degradation product of the γ subunit of the adrenal AP-1. In the lower panel, the ~22- to ~30-kDa GTP-binding proteins represent an uncharacterized group of low molecular mass GTP-binding proteins while the band migrating slightly ahead of the 20-kDa standard is ARF, based on both co-migration with purified ARF and sensitivity to BFA. The upper and lower portions originate from a single blot, and the position of the molecular size markers is indicated on the left.

Recruitment of AP-1 by adrenal cytosol onto the Golgi membrane fraction was digested into analogous appendage and trunk tryptic fragments (Fig. 2, A and B). As noted previously (8, 14), the β subunit was rapidly cleaved from the intact AP-1 complex, followed by much slower cleavage and release of the carboxyl-terminal γ subunit appendage. The aminoterminal trunks of both the β and γ subunits, as well as the μ1 and α1 subunits, collectively comprising the AP-1 core fragment, proved relatively resistant to trypsin. We found that the AP-1 recruited from adrenal cytosol onto the Golgi membrane fraction was digested into analogous appendage and trunk tryptic fragments (Fig. 2, A and B). As noted previously (8, 14), the β subunit was significantly more sensitive to trypsin than the γ subunit. Extensive tryptic degradation of α-mannosidase II, a luminal Golgi marker, was observed only in the presence of Triton X-100 (Fig. 2C, lane f). This demonstrated that during the assay and subsequent proteolysis, the Golgi membranes remained correctly oriented and sealed, and that AP-1 was recruited onto the cytoplasmic face of these membranes. Furthermore, membrane association of AP-1 in the absence of clathrin did not appear to result in major conformational changes as reflected by gross alterations in proteolytic sensitivity.

To ascertain whether the AP-1 appendages, the core, or both remained membrane-associated, the Golgi fraction was sedimented following digestion, and the resulting supernatant and pellet fractions were analyzed (Fig. 3). On untreated membranes or membranes that contained excess trypsin inhibitor prior to addition of trypsin, the recruited AP-1 remained membrane-associated during the 10-min digestion at 37 °C (lanes b and h). This is in contrast to endogenous Golgi-associated AP-1,
which dissociates from the membranes on incubation at 37°C (26). The stability of the recruited exogenous AP-1 is probably a consequence of the GTPyS, which we believe traps the AP-1 irreversibly on the membranes. In the presence of low concentrations of trypsin and Triton X-100 as indicated in the figure. After digestion at 37°C for 10 min, the samples were analyzed by immunoblotting with mAb 100/1 (panel A), AE/1 (panel B), and anti-α-mannosidase II (panel C). Note that the ~68-kDa doublet recognized by the anti-β subunit antibodies represents endogenous proteolytic degradation products.

Introduction of Purified AP-1 and AP-1 Core Fragments—While the above data indicate that the carboxyl-terminal appendage domains of the β1 or γ subunits are not required to retain the recruited AP-1 core complex on the Golgi membrane in the presence of GTPyS, they do not reveal whether these regions are required for the initial recognition and recruitment of the AP-1 complex onto the Golgi. We therefore used purified AP-1, derived from adrenal clathrin-coated vesicles, to prepare defined tryptic fragments lacking either the β1 subunit appendage or both the β1 and γ subunit appendages. The recruitment of these fragments was then followed in a modified binding assay in which the endogenous AP-1 was first removed from the adrenal cytosol by immunodepletion, and then either intact AP-1 or the tryptic fragments were added back to this depleted cytosol. The results of a typical experiment are presented in Fig. 4.

From a comparison of the supernatant fractions from incubations containing either control or AP-1-depleted cytosol, it is apparent that the immunodepletion had quantitatively removed AP-1 (Fig. 4, panels A and B, lanes b and c compared to lanes a and e). Consequently, no recruitment of adrenal AP-1, as judged by the presence of an exogenous γ subunit, was evident in the pellet fractions (panel D, lane e) of incubations containing the depleted adrenal cytosol. However, when probed with either mAb 100/1 (panel C) or GD1/1 (panel E), two closely migrating β-type subunits were observed on the Golgi membranes incubated with the AP-1-depleted cytosol and GTPyS (panels C and E, lane e). The slower migrating β form corresponds to the β1 subunit of residual endogenous rat liver Golgi-associated AP-1, which has not completely dissociated from the membrane during the course of the assay (26). The partner γ subunit can be seen in panel F (lanes a and e). Because this γ subunit was detected with the AE1 antibody (panel F) but not the species-specific mAb 100/3 (panel D), this membrane-associated AP-1 must be of rat origin. Note also that more of this endogenous AP-1 was observed on the membranes incubated together with the AP-1-depleted adrenal cytosol than on the Golgi membranes incubated without cytosol (panels C, E, and F, lane e compared to lane a). This demonstrates that in the presence of AP-1-depleted cytosol, the endogenous rat AP-1 that had dissociated from the Golgi can rebind to the mem-
branes in an ARF-dependent manner. The lower \(\beta\)-type band remains to be identified definitively. Additional experiments have shown that no \(\alpha\) subunit had been recruited, excluding the possibility that the lower band was a \(\beta\)2 subunit. The unidentified band might be a degradation product of the endogenous Golgi-associated \(\beta\)1 subunit or, less likely, a free monomeric \(\beta\) subunit.

When purified coated vesicle-derived AP-1 was added into the depleted cytosol to levels approximating 1 to 5 times the estimated endogenous concentration, the level of exogenous AP-1 recruited onto the membrane was similar to that of the cytosolic form of AP-1 (Fig. 4, panels C and D, lane g compared to lane c). In both cases, however, the majority of the AP-1 adaptors remained in the supernatants (panels A and B), which is consistent with our previous observation that another cytosolic component limits the extent of AP-1 recruitment (26). When the truncated AP-1 tryptic fragment, lacking the \(\beta\)1 subunit appendage, was added to the depleted cytosol together with GTP\(\gamma\)S, the resulting Golgi membranes showed that the \(\sim 60\)-kDa \(\beta\)1 trunk was membrane-associated (panel C, lane i).

On a duplicate blot probed either with mAb 100/3 (panel D) or AE/1 (panel F), the intact \(\gamma\) subunit was also seen to be membrane-bound (lane i), indicating that the tryptic \(\beta\)1 fragment was indeed part of a heterotetrameric complex. Again, the bulk of the added fragments remained in the supernatant fraction (panels A and C). BFA effectively antagonized the translocation of the \(\beta\)1-appendageless core in the presence of GTP\(\gamma\)S (panels C–F, lane k). Analysis of the blots with GD/1, an antiserum raised against a conserved peptide epitope in the \(\beta\) subunit hinge, that recognizes the \(\beta\)1 appendage (Fig. 3), revealed that the free \(\beta\)1 subunit appendage had not been recruited onto the membrane (panel E, lane i). Thus, the translocation of AP-1 onto the TGN appeared to proceed normally in the absence of the \(\beta\)1 subunit head and hinge regions.

**Subcellular Localization of Recruited Exogenous AP-1**—Independent confirmation of the specific targeting of the exogenous AP-1 adaptors onto the TGN was obtained using a cell-based recruitment assay (23, 24). Permeabilized NIH 3T3 fibroblasts were incubated at 37 °C with whole rat liver cytosol that had been supplemented with exogenous adrenal coated vesicle-derived AP-1. After washing, the cells were prepared for indirect immunofluorescence microscopy. The distribution of
clathrin in control cells, incubated with AP-1-supplemented cytosol but lacking added nucleotides is shown in Fig. 5a. A dispersed peripheral punctate staining pattern with a juxtaposed concentrated perinuclear staining was typical and represents cell surface- and TGN-derived clathrin-coated pits and vesicles, respectively (2, 5, 39). The same cells, double-labeled with the species-specific mAb 100/3, exhibited barely discernible perinuclear staining (Fig. 5b). While the overall clathrin staining pattern was not altered by adding 100 μM GTPγS to the donor cytosol (Fig. 5c), a dramatic enhancement of AP-1 staining in the perinuclear Golgi region was evident (Fig. 5d). Since little exogenous AP-1 was detected in the perinuclear area in the absence of GTPγS, and little of the recruited AP-1 was associated with the peripheral clathrin-coated regions, direct binding of exogenous AP-1 to vacant sites on perinuclear clathrin lattices appears unlikely. We have also performed additional experiments using the α subunit-specific mAb AP.6 and found no evidence for recruitment of the AP-2 adaptor complex onto the TGN (data not shown). This suggests that authentic recruitment of the intact AP-1 onto the TGN was being observed.

Fig. 5. Subcellular localization of recruited exogenous AP-1 and tryptic core fragment. Permeabilized NIH 3T3 cells were incubated with rat liver cytosol supplemented with purified adrenal AP-1 (a and b), purified adrenal AP-1 and 100 μM GTPγS (c and d), or β1 subunit appendageless adrenal AP-1 plus 100 μM GTPγS (e and f) at 37 °C for 15 min. The cells were then prepared for immunofluorescence analysis using polyclonal anti-clathrin light chain antibodies (lanes a, c, and e) and anti-γ subunit mAb 100/3 (b, d, and f) antibodies.

The effect of removing the β1 appendage on the binding of the exogenous AP-1 complex added to rat liver cytosol with GTPγS is shown in Fig. 5f. Strong perinuclear staining was observed, and the recruitment of the trypsinized AP-1 complex was indistinguishable from the intact coated vesicle-derived AP-1. Taken together, our data indicate that trypsin removal of the β1 subunit appendage had no effect on AP-1 recruitment onto the Golgi membrane fraction.

Having established that the globular carboxyl-terminal and intact hinge regions of the β1 subunit were not required for TGN recruitment, we next examined any role played by the γ subunit appendage in this process. A tryptic AP-1 core fragment, lacking both β1 and γ subunit appendages, was generated by trypsinization and removal of remaining intact γ subunit-containing adaptors by immunoadsorption on mAb 100/3 and then added to the AP-1-depleted cytosol. The appendageless AP-1 core prepared in this way remains as an assembled heterotetramer (14), but since removal of the γ subunit appendage abolishes reactivity with both mAb 100/3 and AE/1, we were only able to follow the fate of the β subunit trunks. Nevertheless, our experiments showed that the appendageless AP-1 core was recruited onto the Golgi membranes as efficiently as the intact AP-1 complex (Fig. 6, panel B, lane g compared to lane c). Again, recruitment of the core fragment was inhibited by BFA (panel B, lane i). Similar levels of recruitment of the different cores were observed over a range of concentrations (Figs. 4 and 6), so major differences in affinity between intact AP-1 and the core fragments appears unlikely. Taken together, these experiments show that neither appendage domain is essential for either recruitment or membrane

Fig. 6. Recruitment of appendageless AP-1 core complex onto Golgi membranes. Tubes containing either 5 mg/ml clathrin-depleted (Control) adrenal cytosol (lanes b and c), 5 mg/ml clathrin- and AP-1-depleted (Depleted) adrenal cytosol (lanes d and e), or 5 mg/ml depleted cytosol plus 5 μg/ml truncated adrenal AP-1 lacking both β1 and γ appendages (lanes f-i) were prepared together with 20 μg/ml Golgi membranes and 50 μg/ml BFA as indicated in the figure. All the tubes contained 100 μM GTPγS, added either at the beginning of the assay, or, when BFA was present, after 10 min at 37 °C. Reactions were terminated after 20 min at 37 °C, and the Golgi membrane pellets were recovered by centrifugation. Aliquots corresponding to 1/80 of each supernatant (panel A) and 1/2 of each pellet (panel B) fraction were analyzed on immunoblots using anti-β subunit mAb 100/1.
**Fig. 7. Recruitment of cytosolic clathrin onto Golgi membranes.** Tubes containing 5 mg/ml whole rat liver (Control) cytosol (lanes b, c, e, and f) or 5 mg/ml clathrin-depleted rat liver (Depleted) cytosol (lanes a, d, and g) were incubated with 10 µg/ml clathrin- and AP-1-containing Golgi membranes. The control cytosol caused a more substantial recruitment of clathrin to the Golgi membranes compared to the clathrin-depleted cytosol, as evidenced by the higher intensity of clathrin bands in the lanes containing whole rat liver cytosol. The origin is marked at the top, and the molecular weights are indicated at the bottom (97K, 68K, 43K, 30K).

**Fig. 8. Dissociation of membrane-bound clathrin following tryptic removal of the β1 subunit appendage.** Clathrin- and AP1-containing Golgi membranes were preincubated with varying concentrations of trypsin inhibitor (0, 2, 10, 10 µg/ml) and treated with 0.5 µg/ml trypsin. The membranes were then subjected to SDS-PAGE, and clathrin bands were visualized. The trypsin inhibitor prevented the dissociation of membrane-bound clathrin, as indicated by the persistence of clathrin bands in the lanes containing the inhibitor. The origin is marked at the top, and the molecular weights are indicated at the bottom (97K, 66K, 43K, 30K).
AP-1 Adaptor and Clathrin Binding to Golgi Membranes

FIG. 9. Clathrin recruitment is prevented following tryptic removal of the β1 subunit appendage domain of Golgi-associated AP-1. AP-1-containing Golgi membranes were prepared using clathrin-depleted rat liver cytosol and GTPγS, recovered by centrifugation and then incubated at 37°C with either excess soybean trypsin inhibitor and 2 µg/ml trypsin (lanes b–d) or 2 µg/ml trypsin (lanes e–g). After 10 min, the membranes were again recovered and then used for the second step reaction. Tubes containing untreated Golgi (lane a), mock-digested Golgi (lanes b and d), or trypsinized Golgi (lanes e and g) membranes and 0.1 mg/ml Sepharose 4B clathrin-enriched fraction (lanes a, c, d, f, and g) were prepared on ice. 20 µg/ml BFA was added to each tube followed by incubation at 37°C for 15 min. The membranes were pelleted and resolved on duplicate 12.5% SDS-polyacrylamide gels, transferred to nitrocellulose, and analyzed by immunoblotting with a mixture of anti-clathrin mAb TD-1 and anti-β-subunit mAb 100/1 (panel A) or anti-γ-subunit AE1 (panel B).

part of the hinge domain, was unable to recruit clathrin from a soluble pool.

DISCUSSION

Adaptors are thought to play a pivotal role in the assembly of clathrin-coated vesicles by determining the intracellular site of coat nucleation and by promoting assembly of the growing lattice. To perform this regulatory role, recruited cytosolic clathrin adaptors must establish multiple contacts with components within the membrane and with the cytosolic clathrin, and it is likely that this is the underlying reason for the structural complexity of the adaptor heterotetramer. In this study, we have delineated the regions required for the ARF-dependent membrane association of AP-1 and the subsequent recruitment of cytosolic clathrin triskelia onto the TGN. We found that an AP-1 adaptor core, lacking both the β1 and γ subunit appendages, was fully competent to bind to Golgi membranes. Binding of the cores was dependent on ARF·GTP and strongly inhibited by BFA, as also observed for intact AP-1 complex (41). By contrast, we found that clathrin could not be recruited onto Golgi-associated AP-1 lacking only the β1 appendage. Therefore, if the γ subunit of AP-1 binds to cytosolic clathrin triskelia first, the affinity of this initial interaction is not sufficient to retain clathrin on the TGN in the absence of an intact β1 subunit. Thus, while it is possible that the adaptor cores contain a second clathrin binding site, which is independent of the one that involves the β appendage, it seems to play a secondary role in the early recruitment steps of clathrin onto TGN-associated AP-1.

Our data on the requirement of an intact β1 subunit for the recruitment of clathrin differ from the results of a recent study showing that clathrin could assemble onto elastase-generated, plasma membrane-associated, AP-2 cores (43). The most obvious difference between the two systems is the adaptor population being studied. AP-1 and AP-2 adaptors might utilize different domains for clathrin recruitment. However, given the high degree of sequence and structural homology between the β1, β2, and β1-like subunits (5–8), this appears unlikely. The differences may rather be related to the fact that, under our experimental conditions, we have analyzed de novo formation of a coated bud on the TGN, while the AP-2 binding system is more likely to reflect rebinding of clathrin to pre-existing coated pit structures that had been previously disrupted by nonphysiological manipulation of pH and salt conditions. This makes these two systems difficult to compare.

At present, the available evidence supports the notion that AP-1 initially associates with the Golgi by binding to a TGN-specific docking protein in an ARF-regulated manner (23–26). The results of our controlled proteolysis experiments indicate that this association of AP-1 with the putative docking protein occurs through the core domain. We have also shown that the β1 subunit is involved in clathrin recruitment. What then is the function of the γ subunit appendage? The considerable amino acid sequence divergence between the appendages of the α, β, and γ subunits has prompted the suggestion that the appendages might encompass a receptor binding domain (6, 7, 12, 13, at most, a passive role in determining the intracellular targeting of AP-1 (29). Our work now provides independent biochemical confirmation of this conclusion and, in addition, establishes that the appendage of the β1 subunit is not required for the ARF-dependent membrane association of AP-1.

Although we have not yet established whether GTPyS inhibits the complete assembly of a coated vesicle or the budding process, clearly the analog does not interfere with the recruitment of cytosolic clathrin triskelia. Our observation that an intact β1 subunit is required for this recruitment of clathrin is a strong argument for the selective association of this subunit with clathrin. This idea was initially proposed when it was determined that the β-type subunits of AP-1 and AP-2 were highly related, both by peptide mapping and immunological criteria (5) and by sequencing (6–8). Our data are also consistent with the finding that under conditions that reduce aggregation of adaptors, cleavage of the β1 subunit in the hinge region was sufficient to release the truncated AP-1 core, together with the appendage, from purified coated vesicles (14). Furthermore, a β1-like subunit, purified from bacteria expressing the recombinant protein, induces clathrin coat assembly in vitro (16). Together, these observations all implicate the β1 subunit in clathrin association.

The β2 subunit of AP-2 also interacts directly with clathrin (14–16). Weaker interactions between the AP-2 adaptor core and clathrin cages have been reported, but these interactions failed to induce clathrin assembly (11–14). The α subunit of AP-2 also appears to bind to clathrin (41, 42), and it has been proposed that this is the first contact that is established between these two molecules (41). By contrast, we found that clathrin could not be recruited onto Golgi-associated AP-1 lacking only the β1 appendage. Therefore, if the γ subunit of AP-1 binds to cytosolic clathrin triskelia first, the affinity of this initial interaction is not sufficient to retain clathrin on the TGN in the absence of an intact β1 subunit. Thus, while it is possible that the adaptor cores contain a second clathrin binding site, which is independent of the one that involves the β appendage, it seems to play a secondary role in the early recruitment steps of clathrin onto TGN-associated AP-1.
s of ARF-GTP. Several lines of evidence, including the data shown here, support the notion that the clathrin-AP-1 interaction would be maintained by the β1 subunit (14-16). Polygo­nally arranged clathrin would remain membrane-associated due to multiple, cooperative low affinity adaptor-receptor inter­actions, docking protein-AP-1 interactions at the growing edges of the lattice, and perhaps adaptor-adaptor interactions, as exemplified by AP-2.

Upon budding and removal of the clathrin coat by the uncoating ATPase Hsc70 (3, 45, 46), the interaction between individual adaptors and receptors may not be sufficiently strong to maintain adaptors on the membrane. If this were the case, upon removal of the clathrin, the remaining coat components would be released from the budded vesicle surface without necessitating additional uncoating factors. However, the multivalency of the proposed adaptor-membrane association may result in only slow release of the adaptors (47, 48) and could be regulated by specific cytoplasmic factors (49). We have not yet examined what requirements the clathrin has to meet in order to be recruited by membrane-bound AP-1 and if there is any difference between cytosolic and coated vesicle-derived clathrin as has been observed for AP-2-mediated clathrin recruitment (50). These and other questions concerning the role of light chains and clathrin domains can now be studied using our membrane binding assay.

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