Coatomer, a seven-subunit hetero-oligomeric complex, is the major component of the COP-I membrane coat of transport vesicles of the early secretory pathway. We have followed the assembly of this complex in vivo by pulse-chase experiments and immunoprecipitation of native coatomer subunits and found that it is an ordered process that takes 1–2 h to complete. During assembly, direct interactions between α, β′- and δ-COP, β- and δ-COP, and γ, ε, and δ-COP occur. Coatomer, once it has assembled, is stable with a half-life of ~28 h. No significant amounts of partial coatomer complexes have been detected. The only subunit to exist at steady state out of the complex is ε-COP, which has a similar half-life to coatomer subunits within the complex. Assembly is inhibited by brefeldin A, suggesting that it may be a regulated process. These results describe for the first time in vivo assembly of a coat protein complex involved in membrane traffic and extend our knowledge of how coatomer is structured.

COP-I-coated vesicles mediate membrane transport of proteins between distinct compartments of the early secretory pathway. Evidence obtained using both in vitro and in vivo systems has implicated COP-I vesicles in biosynthetic transport to and through the Golgi complex and more recently in retrograde transport from the Golgi complex to the endoplasmic reticulum (1, 2). Interestingly, COP-I vesicles also play a role in the endocytic pathway, although the precise steps involved are presently unclear (3, 4).

The COP-I coat is composed of two components, coatomer and ADP-ribosylation factor, ARF (5, 6). Coatomer is a complex composed of seven subunits termed α- (160 kDa) (7, 8), β- (107 kDa) (9), β′- (102 kDa) (10, 11), γ- (97 kDa) (12), δ- (60 kDa) (13), ε- (36 kDa) (14), and ζ- (20 kDa) (15) -COP (for coat protein). ARF is a small GTPase regulating membrane traffic, which must first bind in its GTP-bound state to membranes, for coatomer recruitment to occur (16–19). Brefeldin A (BFA) inhibits nucleotide exchange on ARF and consequently blocks coatomer recruitment to occur (16–19). Brefeldin A (BFA) in which must first bind in its GTP-bound state to membranes, for coatomer association, direct interactions between α, β′- and ε-COP, γ- and ζ-COP, and β- and δ-COP (24). Here we have investigated coatomer assembly and find that the same subunit-subunit interactions occur during assembly in vivo. Assembly takes about 1–2 h and is inhibited by BFA. We have also found that coatomer, once it has formed, is very stable and that no significant amounts of partial coatomer complexes exist within interphase cells at steady state.

**MATERIALS AND METHODS**

**Antibodies**—Antibodies against β-COP and ε-COP were prepared and affinity purified as described (24, 25). Rabbit antisera against α-, γ-, and δ-COP were obtained from Drs. Harter and Wieland (University of Heidelberg, Heidelberg, Germany). Rabbit antisera against ε-COP (14) and coatomer (15) were provided by Drs. Rothman (Memorial Sloan-Kettering Cancer Center, New York) and Sheff (Yale University School of Medicine, New Haven, CT). Monoclonal CM1A10 anti-coatomer antibody (17) was purified from ascites using protein G-Sepharose.

**Cell Culture and Metabolic Labeling**—Vero cells (ATCC CCL81) were maintained as described earlier (26). Dishes (6 cm) of nearly confluent cells were starved of methionine (Met) and cysteine (Cys) by incubation of 30 min in Met/Cys-free MEM. Cells were then pulsed for 5 min with 20 μCi/ml of [35S]Met/Cys-free medium. The chase was initiated by washing the cells with normal MEM and incubation in this medium for the appropriate time.

For analysis of coatomer half-life, cells were diluted 1:20 in 6-cm dishes 1 day before labeling. For labeling, cells were incubated in Met/Cys-free labeling medium (9 parts Met/Cys-free MEM, 1 part normal MEM) containing 15 Ci/ml [35S]met for 10 h. The cells were then washed 2 × in Met/Cys-free medium containing 5 mM met and chased in this medium for the appropriate time.

**Lysis and Immunoprecipitation**—Cells were washed twice with ice-cold HB (150 mM NaCl, 20 mM Tris, pH 7.5, 2 mM EDTA) and lysed in 500 μl of immunoprecipitation buffer (IP; HB containing 0.5% Triton X-100) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 0.5 mM aprotinin, 2 μg/ml pepstatin A, 2 μg/ml aprotinin, 0.5 μM leupeptin) for 15 min on ice. The lysates were cleared for 10 min at 15,000 rpm in a microcentrifuge, 20 μl of protein A-Sepharose (Pharmacia Biotech Inc., 50% slurry in IP) was added to the supernatants and incubated for 1 h at 4°C on a rotating wheel. The beads were removed by centrifugation, and specific antibodies were added to the supernatant. Typical antibody amounts used were as follows: 2 μg of affinity-purified anti-β′-COP or anti-ε-COP, or 0.5 μl of anti-ζ-COP antiserum. After incubation for 1 h at 4°C, 20 μl of protein A-Sepharose was added and the mixture incubated for further 2 h at 4°C. The beads were collected by centrifugation and washed 5 × with 1 ml of IP and then 1 × with 1 ml of phosphate-buffered saline. Proteins were eluted from the beads by boiling in sample buffer and subjected to SDS-PAGE analysis.

To immunoprecipitate monomeric ε-COP we first immunodepleted coatomer from the samples (since the anti-ζ-COP antibodies recognize both monomeric and coatomer-associated ε-COP). Samples were first incubated with anti-ε-COP antibodies and 20 μl of protein A-Sepharose as described and the beads removed by centrifugation. The supernatants were incubated again with 2 μg of CM1A10 and 20 μl of protein A-Sepharose.
Assembly of Coatomer in Vivo

**RESULTS**

Assembly of Coatomer in Vivo—To study assembly of coatomer in vivo we employed a pulse-chase approach. Cells were radiolabeled for 5 min with [35S]Met/Cys, chased for various lengths of time with unlabeled amino acids, and cell lysates immunoprecipitated with antibodies directed against different coatomer subunits. Immunoprecipitated proteins were analyzed by SDS-PAGE and fluorography.

In initial experiments we studied the incorporation of β-COP into coatomer. 5 min after labeling, β-COP appears to be monomeric (Fig. 1A). During the chase other radiolabeled COPs associate until at 1–2 h coatomer assembly is complete. There is an order of association, with δ-COP associating at an earlier time (5–20 min) than α-, β', and γ-COP (20–60 min), and ε-COP associating last (40–120 min; Fig. 1, A and B). To ensure that we observe indeed assembly (and not exchange of labeled COPs, for example), we analyzed the density of β-COP on sucrose gradients during the chase period (Fig. 2). At early times (0–20 min) β-COP sediments near the top of the gradient at the position expected for monomeric protein. During the chase it shifts to fraction 11 (~13 S) concomitant with the appearance of other labeled COPs. What we observe during the chase therefore reflects assembly of coatomer subunits. After 1 h virtually no monomeric β-COP remains, showing that it is all incorporated into coatomer or that what remains is degraded. Importantly the subunit stoichiometry at 120 min is the same as that obtained from an overnight metabolic labeling (data not shown). This indicates that each pool of unassembled subunit is the same and that there is not unequal incorporation of unlabeled subunits into the complex. The one exception is ζ-COP where the amount of labeled protein is less than that observed with overnight labeling. This can, however, be explained by there being a significant pool of monomeric unlabeled protein in the cell at steady state (Ref. 15; see also Fig. 6).

The radiolabeled coatomer formed during these pulse-chase experiments is functional and can be recruited onto membranes in a GTP-dependent manner (data not shown). β'-COP associates at early time points exclusively with α- and δ-COP. After 5-min chase ~40% of α-COP and 20% of δ-COP has already associated with β'-COP (Fig. 3, A and B). β', γ', and ε-COP associate more slowly, and as expected, assembly

**FIG. 1. Assembly of β-COP into coatomer.** Cells were pulse-labeled for 5 min with [35S]methionine/cysteine and then chased for the indicated times before lysis and immunoprecipitation with antibodies against β-COP (anti-EAGE). A, proteins were separated on a 12% gel (top) or on a 7% gel containing 6 M urea (bottom) and detected by fluorography as described under "Materials and Methods." Molecular weight standards are in kilodaltons. B, quantitation of immunoprecipitated COPs expressed as percentage of amount precipitated after 240-min chase (average of two experiments). Note that although there was an increase in labeled β-COP during the first 5 min of chase due to continued protein synthesis (it could be blocked by cycloheximide), assembly occurred after this time.

**FIG. 2. Sucrose gradient analysis of β-COP during assembly into coatomer.** Cells were pulse-labeled with [35S]methionine/cysteine and chased for the indicated times before lysis and fractionation on a 5–30% sucrose gradient. Fractions were collected from the top and immunoprecipitated with anti-EAGE antibodies. Immunoprecipitated proteins were separated by SDS-PAGE using a 12% gel and detected by fluorography.

followed by peroxidase-conjugated secondary antibodies (Cappel) at 1:2,500. Peroxidase labeling was detected by ECL (Amersham).
is complete at 120 min. Identical results were obtained with CM1A10 antibody (data not shown), demonstrating its previously unknown antigen to be \( b_9 \)-COP. The interaction between \( b_9 \)- and \( a \)-COP seen at an early stage in assembly is entirely consistent with \textit{in vitro} data showing that \( a \)-, \( b_9 \)-, and \( e \)-COP interact directly within coatomer (24).

Immunoprecipitations with antibodies to \( z \)-COP showed that it associates at early time points exclusively with \( g \)-COP and \( d \)-COP. After 5-min chase; 50\% of \( g \)- and \( d \)-COP is complexed with \( z \)-COP (Fig. 3, C and D). \( a \)-, \( b_9 \)-, and \( b_9 \)-COP are next with \( e \)-COP having the slowest association kinetics. Again, these findings support \textit{in vitro} data showing that \( a \)-, \( b_9 \)-, and \( e \)-COP interact directly within coatomer (24).

Coatomer Assembly Is Inhibited by Brefeldin A—Addition of BFA to mammalian cells rapidly results in the removal of coatomer from membranes, leading ultimately to membrane tubularization and fusion (28). To study whether coatomer assembly may be regulated by levels of soluble coatomer in the cell we used BFA to inhibit binding of coatomer to membranes to increase its soluble pool. Addition of 1 \( \mu \)g/ml BFA almost completely abolished incorporation of \( b \)-COP into coatomer (Fig. 4). BFA also inhibited association of \( z \)-COP with other COPs and blocked the incorporation of \( b_9 \)-COP into coatomer, although the interaction between \( a \)- and \( b_9 \)-COP was not affected (data not shown). This effect of BFA was apparent within 20 min of the chase and did not appear to be due to nonspecific toxic effects, since overall protein synthesis was normal, as was synthesis of \( b \)-COP, at least when measured during the 5-min pulse period (data not shown). BFA did also not cause coatomer subunits within the preformed complex to dissociate (not shown).

Coatomer Is a Stable Complex in Vivo—To study coatomer stability \textit{in vivo} exponentially growing cells were radiolabeled with \(^{35}\)S-methionine for 10 h and then chased in medium containing excess unlabeled methionine. Coatomer was immunoprecipitated from cells lysed at 8-h intervals during the chase and analyzed by SDS-PAGE (Fig. 5A). Coatomer is degraded slowly; a logarithmic scale graph of the decay of labeled protein gives a half-life of approximately 28 h (Fig. 5C). The stoichiometry of radiolabeled COPs remains the same at all times during the chase showing that each subunit is degraded at the same rate and suggesting that subunits in the complex do not exchange with newly synthesized COPs. In support of this suggestion, we have failed to observe incorporation of transfected, tagged \( b \)-COP into pre-existing coatomer complexes \textit{in vivo} (data not shown). We also examined the stability of monomeric \( z \)-COP, the only COP to exist in significant amounts as a free subunit at steady state (Ref. 15; Fig. 6), by first immunodepleting coatomer from samples prior to immunoprecipitation with anti-\( z \)-COP antibodies. Monomeric \( z \)-COP is degraded at a similar rate to \( z \)-COP in coatomer with a
half-life of $\sim 32$ h (Fig. 5, B and C).

No Partial Coatomer Complexes Exist in the Cell at Steady State—Recent results have demonstrated that coatomer is recruited onto endocytic membranes (3). Interestingly, $\delta$- and $\gamma$-COP were not detected on endosomes, suggesting that either the antibodies did not cross-react with endosomal $\delta$- and $\gamma$-COP, or that partial complexes lacking these subunits bind to endosomes (3, 29). To determine whether coatomer subcomplexes exist in cells, we analyzed the distribution of each COP on a sucrose density gradient. There was a major peak for each subunit at fraction 11 ($\sim 13$ S), the position of coatomer (Fig. 6). There was no detectable $\alpha$, $\beta$, $\beta^\prime$, $\gamma$, and $\delta$-COP and only small amounts of $\varepsilon$-COP at lower densities. Identical results were obtained with cytosol and Triton X-100 cell extracts that had been prepared in different buffers (data not shown). This shows that these subunits do not exist in partial complexes at steady state. There was a significant amount of $\zeta$-COP in fractions 2 and 3, corresponding to monomeric protein. This agrees with the findings of Kuge et al. (15) and shows that this is the only COP to exist in significant amounts separately of the COP-complex.

**DISCUSSION**

We have shown that coatomer assembly *in vivo* takes $\sim 1$–2 h and that during assembly the following subunits interact directly with each other, $\alpha$-COP with $\beta^\prime$- and $\delta$-COP, $\beta$-COP with $\delta$-COP, and $\gamma$-COP with $\zeta$- and $\delta$-COP (Fig. 7). This corroborates results obtained during *in vitro* disassembly of coatomer showing direct interactions of $\alpha$-COP with $\beta^\prime$- and $\varepsilon$-COP, of $\gamma$-COP with $\zeta$-COP, and of $\beta$-COP with $\delta$-COP (24). The strongest subunit interactions within coatomer are therefore also those that form earliest during assembly of the complex, thus suggesting that partial complexes form early during assembly and are then brought together to form coatomer.

We have recently found that a related form of COPI is essential for endosome function (3, 4). Interestingly, some (e.g. $\zeta$-COP) of the coat subunits involved in endocytosis appear to be significantly more conserved with coatomer involved in transport between the intermediate compartment and the cis-Golgi network than others ($\gamma$- and $\delta$-COP (3)). It has thus been tempting to speculate that various forms of coats can be generated by exchange of coatomer subcomplexes from a soluble pool of different “modules.” Variation of these modules would define specific functions of coatomer or target coatomer to different subcellular sites. Since partial coatomer complexes

**Fig. 4.** Coatomer assembly is inhibited by brefeldin A. Cells were pulse-labeled for 5 min with $[^{35}S]$methionine/cysteine and then chased for the indicated times in the presence or absence of 1 $\mu$g/ml brefeldin A before lysis and immunoprecipitation with anti-EAGE antibodies. Immunoprecipitated proteins were separated by SDS-PAGE using a 12% gel (top) or a 7% gel containing 6 M urea (bottom) and detected by fluorography.

**Fig. 5.** Coatomer is a stable complex *in vivo*. Exponentially growing cells were labeled for 10 h with $[^{35}S]$methionine and chased in the presence of excess unlabeled methionine for the indicated times. A, cells were lysed and immunoprecipitated with anti-EAGE antibodies. Proteins were separated on a 12% gel (top) or a 7% gel containing 6 M urea (bottom). The middle portion is a longer exposure of the bottom of the 12% gel. B, lysates were immunodepleted of coatomer as described under “Materials and Methods” and immunoprecipitated with anti-$\zeta$-COP antibodies. C, the intensities of coatomer ($\alpha$-COP) and monomeric $\zeta$-COP were quantitated and graphed on semi-logarithmic plots. Curves yield half-lives of 28 and 32 h for coatomer and monomeric $\zeta$-COP, respectively.
are transient and not found in significant amounts in the cell at steady state, exchange of such modules must either be very rapid, or more likely, coatomer once assembled maintains its structure and subunit composition during its lifetime. Since heterogeneity of coatomer subunits has been described at the biochemical and post-translational level (29), we speculate that variety of coats is produced by stable assembly of different combinations of distinct partial coatomer complexes. Thus endosomal coatomer may have similar subunit composition and structure to the previously characterized coatomer, with differences predominantly in (some of) its subunits. The finding that complexes with different protein compositions did not co-immunoprecipitate in the pulse-chase experiments, even though some of the antibodies used did cross-react with endosomal COPs, also supports this conclusion. We cannot be sure at the moment, however, if we are observing the assembly of one or more types of coatomer complex in the pulse-chase experiments.

Most likely the three subcomplexes of coatomer (α-, β-, and ε-COP; γ- and ζ-COP; β- and δ-COP) are responsible for different coatomer functions within the context of the seven-subunit complex (see Fig. 7). These different functions include ARF-regulated binding of coatomer to specific organelar membranes, interaction of coatomer with defined cargo, and assembly of coatomer into a coat. It has been shown that the α-, β-, ε-COP trimer is a membrane binding subcomplex of coatomer (24) and interacts with a KKXX motif present on cargo transported between the endoplasmic reticulum and Golgi complex (7, 23). Interestingly, binding of this subcomplex to membranes is not regulated by GTP (24). Furthermore, antibodies to ζ-COP prevent binding of coatomer to membranes; yet, this COPI subunit appears not to be directly involved in coatomer membrane interaction (15). Finally, antibodies directed against the “EAGE epitope” of β-COP stabilize coatomer membrane binding. We conclude therefore that the α-, β-, ε-COP trimer is involved in binding of coatomer to membranes and that β-COP may be involved in the (ARF-dependent) regulation of this interaction. More detailed experiments using purified coatomer subcomplexes should help their further characterization in coatomer specific function.

It is possible that chaperones are involved in coatomer assembly. Possible candidates are a protein at ~70 kDa, which transiently co-immunoprecipitates with β-, β′-, and ζ-COP (Figs. 1A and 3, A and C), as well as a doublet at ~45 kDa, which appears transiently associated with assembling ζ-COP (see Fig. 3C). However, the 45-kDa doublet was not found associated with other assembling subunits and appeared variably, like the 70-kDa protein, between experiments and so we cannot at the moment exclude them to be cross-reacting contaminants. Further experiments are required to clarify the specificities of these putative interactions. We could not detect any other proteins associated in stoichiometric amounts with assembling coatomer subunits using different lysis conditions or chemical cross-linking (data not shown). Although it is possible that chaperones associate transiently with coatomer subunits during assembly, it is also tempting to speculate that δ-COP may function in this capacity. It is indeed striking that δ-COP associates with several other COPs independently early during coatomer assembly. Alternatively, δ-COP may associate rapidly with these other COPs during coatomer assembly, because it has a central position in the complex and interacts with several subunits. Further experiments should help elucidate more precisely the exact role of δ-COP in the process of coatomer assembly.

The finding that the stoichiometry of radiolabeled COPs remains during a prolonged chase is consistent with en bloc binding of coatomer to membranes (14). The fact that monomeric ζ-COP is also long lived in the cell suggests that it may function independently of the coatomer complex. What might this function be? One possibility, that it regulates the rate of assembly of coats by binding to the coatomer binding site on membranes, does not appear to be true, since ζ-COP does not interact on its own with the coatomer binding site on membranes (15). Its function therefore remains unclear at the moment and awaits further experiments.

How might BFA inhibit coatomer assembly? One possibility is that assembly occurs on membranes and that BFA exerts its effect by preventing membrane binding of newly synthesized COPs. This seems unlikely, however, since the same assembly intermediates were found in cytosol preparations devoid of membranes (not shown). Alternatively, since BFA also inhibits membrane traffic, assembly might be regulated by the rates of vesicular transport. However, aluminum fluoride, an activator of trimeric G proteins, which in contrast to BFA, increases binding of coatomer to membranes (30), and also inhibits secretion, did not inhibit assembly (not shown). We therefore favor the possibility that assembly may be regulated by levels of soluble coatomer within the cell. One might imagine a mechanism whereby a sensor in the cytoplasm measures the amount of soluble coatomer and if this becomes too high, as in the case of BFA, then assembly is inhibited. Such a mechanism would provide a means of regulating the amount of coatomer within the cell and would resemble in principle other feedback mechanisms used to regulate levels of protein complexes in the cell, as in the case of tubulin for example (31). Further work is clearly necessary to determine if such a mechanism does exist for coatomer and how it might function.

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FIG. 7. Model for assembly of coatamer. For details, see “Discussion.” Touching subunits disassemble together.