A Role for Calcium in Stabilizing Transport Vesicle Coats*

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Calcium has been implicated in regulating vesicle fusion reactions, but its potential role in regulating other aspects of protein transport, such as vesicle assembly, is largely unexplored. We find that treating cells with the membrane-permeable calcium chelator, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis(ace-toxyethyl ester) (BAPTA-AM), leads to a dramatic redistribution of the vesicle coat protein, coatomer, in the cell. We have used the cell-free reconstitution of coat-protem 1 (COPI) vesicle assembly to characterize the mechanisms of this redistribution. We find that the recovery of COPI-coated Golgi vesicles is inhibited by the addition of BAPTA to the cell-free vesicle budding assay. When coatomer-coated membranes are incubated in the presence of calcium chelators, the membranes “uncoat,” indicating that calcium is necessary for maintaining the integrity of the coat. This uncoating is reversed by the addition of calcium. Interestingly, BAPTA, a calcium chelator with fast binding kinetics, is more potent at uncoating the coatomer-coated membrane than EGTA, suggesting that a calcium transient or a calcium gradient is important for stabilizing COPI vesicle coat. The primary target for the effects of calcium on coatomer recruitment is a step that occurs after ADP-ribosylation factor binding to the membrane. We suggest that a calcium gradient may serve to regulate the timing of vesicle uncoating.

Calcium is known to be an essential second messenger in numerous cell-signaling events. One particularly well characterized role for calcium is in signaling via G protein-coupled receptors that activate phospholipase C to produce IP3 and diacylglycerol (1). This signaling pathway utilizes calcium stores that are maintained in the ER by the action of a sarco-/endoplasmic reticulum calcium ATPase (SERCA) (1, 2). Increased IP3 levels cause the IP3-receptor calcium channels on the ER to open, generating a transient rise in cytosolic calcium. Increased calcium levels act through a number of different effectors, such as calmodulin, to regulate downstream processes.

Calcium also plays a well characterized role in regulating membrane fusion reactions. During calcium-regulated exocytosis, the opening of voltage-gated calcium channels on the plasma membrane results in an increase in cytosolic calcium levels that triggers vesicle fusion at the cell surface and the release of cargo. This process has been best characterized for synaptic transmission (3). Besides regulated exocytosis, calcium may play a more widely utilized role in membrane fusion reactions. Studies on ER to Golgi transport in yeast and in semi-intact (perforated) cells have indicated a clear role for calcium in this trafficking step (4–7). More recent studies have implicated calcium in homotypic vacuolar fusion (8), in late endosome-lysosome heterotypic fusion and the reformation of lysosomes from hybrid organelles (9), in fusion between endosomal compartments (10), and in the brefeldin A-induced retrograde transport from the Golgi apparatus to the endoplasmic reticulum (11). Calmodulin has been shown to mediate the effects of calcium in fusion reactions (8, 12).

Although many studies have focused on the role of calcium in the late secretory pathway, an emerging body of literature is implicating calcium in the regulation of protein trafficking through the Golgi apparatus. For instance, it is now appreciated that the Golgi apparatus is an insitol 1,4,5-trisphosphate-sensitive Ca2+-store (13) and that proteins involved in sequestering and releasing calcium are localized to the Golgi apparatus (14–16). These proteins include a SERCA-like ATPase and IP3 receptor channels (13, 17). One very interesting protein in this regard is CALNuc (nucleobindin), a calcium-binding protein found in the lumen of the Golgi apparatus (15). CALNuc, an EF-hand protein, has been shown to be the major calcium-binding protein in the Golgi and to be involved in maintaining SERCA-sensitive and IP3-sensitive calcium stores (16). The clearest evidence for the role of calcium in regulating protein trafficking in the Golgi has come from a study using the cell-free reconstitution of intra-Golgi trafficking (18). In that study, the calcium chelator BAPTA and agents that disrupt cellular calcium stores were found to be potent inhibitors of Golgi transport. Interestingly, the regulation of Golgi transport by calcium was also found to involve calmodulin, as has been noted for the effects of calcium on endosomal fusion (12).

Endosome fusion, vacuolar fusion, and Golgi trafficking are inhibited to a greater extent by BAPTA than EGTA (8–10, 18). Because BAPTA has considerably faster association and dissociation kinetics for calcium than EGTA (19), these findings are often interpreted to indicate that a calcium transient or a calcium gradient is required (18). BAPTA has a faster on-rate for calcium and therefore would be able to buffer a calcium...
transient or gradient that would not be affected by a chelator with a slower on-rate, such as EGTA.

The studies described above indicate that calcium plays a fundamental role in regulating protein trafficking through membrane fusion reactions. In this study, we have examined whether calcium plays a more general role in regulating vesicular transport using the formation of COPI-coated transport vesicles at the Golgi apparatus as a model system. 

**EXPERIMENTAL PROCEDURES**

**Antibodies and Materials**—The following antibodies were used in this study: monoclonal anti-ARF antibody MA3–060 (Affinity BioReagents Inc., Golden, CO), monoclonal antibodies to β-COP, M3A5 and mAD (Sigma), monoclonal anti-ε-COP (20), monoclonal anti-α-mannosidase II (Berkeley Antibody Co., Richmond, CA), and anti-syntaxin 6 (Transduction Laboratories, Lexington, KY). Rat and rabbit liver Golgi membranes and bovine-brain cytosol were prepared as described previously (21). BAPTA, EGTA, and TPEN were from Sigma, and BAPTA-AM was from Molecular Probes (Eugene, OR). All other materials were reagent grade and were purchased from commercial sources. 

**Golgi Binding and Vesicle Budding Assays**—Golgi binding assays were carried out by sedimentation or by flotation as described previously (22). For the two-step experiments involving separate incubations with ARF1 and a coatomer source, Golgi membranes were incubated as described except that the cytosol was replaced with the constitutively active mutant human ARF1(Q71L) that had been expressed in *Escherichia coli* 

The vesicles were stripped from the membrane by incubating in HSSB (25 mM HEPES, pH 7.2, 2.5 mM magnesium acetate, 250 mM potassium chloride, 0.2 mM succrose) for 10 min on ice. Following the incubation, the membranes were centrifuged at 15,000 × g for 15 min. The supernatant was loaded onto a 35% (w/w) sucrose cushion (25 mM HEPES, pH 7.2, 2.5 mM magnesium acetate, 250 mM potassium chloride, 35% (w/w) sucrose) and centrifuged at 350,000 × g for 30 min. The 35% sucrose pellets were resuspended in Laemmli sample buffer and analyzed by Western blotting.

"Uncoating" and "Recoating" Incubation—A two-stage assay was used for the uncoating experiments. The coatomer-bound Golgi membranes were isolated as described above (stage 1) and then reincubated at 37°C for varying lengths of time in the presence or absence of chelators in a reaction mixture identical to the one used for coatomer binding (stage 2). The reaction mixture was then subjected to centrifugation at 15,000 × g for 20 min at 4°C. Both the pellet and the supernatant were processed for Western blotting as described above. The effect of BAPTA on COPI vesicles was assessed by incubating the 250 mM KCl supernatant from budding reactions (see above) with BAPTA for 10 min at 37°C prior to isolating the vesicles over the 35% sucrose cushion. For the recoating experiments, the coatomer-bound Golgi membranes were incubated at 37°C for 20 min, following which CaCl2 (final concentration, 5 mM) or an equivalent volume of water was added to the reaction mixture, and the incubation was continued for another 20 min at 37°C. The reaction mixtures were then centrifuged and the pellets prepared as described above for the “float-up” binding assay.

**Western Blotting**—Proteins were fractionated by SDS-polyacrylamide gel electrophoresis and blotted onto polyvinylidene difluoride membranes using the standard protocol for the Bio-Rad minigel and blotting apparatuses. Following the transfer, the membranes were dried and incubated with appropriate dilutions of the indicated primary antibodies. The signal was visualized using horseradish peroxidase-conjugated secondary antibodies (Bio-Rad) and ECL (Amersham Pharmacia Biotech). Where indicated, the signals were quantified by densitometry using Scan Analysis software (Biosoft, Cambridge, United Kingdom).

**Immunofluorescence**—Chinese hamster ovary (CHO) and NRK cells

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**FIG. 1. Calcium chelation causes redistribution of coatomer without affecting the localization of α-mannosidase in whole cells.** NRK cells were incubated with 0.1% Me2SO (control) (A–C) or 50 μM BAPTA-AM (D–F) in serum-free α-MEM for 90 min at 37°C. The cells were then immunolabeled with anti-α-mannosidase II antibodies (A and D) or ε-COP antibodies (B and E) followed by the corresponding antibodies conjugated to Texas Red and fluorescein isothiocyanate, respectively. Confocal microscope images show loss of the perinuclear, Golgi localization of ε-COP (compare E to B) but not α-mannosidase II (compare D to A) after treatment with BAPTA. Merged images (C and F) show near complete overlap of α-mannosidase II with ε-COP in untreated cells (C) but not in BAPTA-treated cells (F). The bar equals 10 μm.
were grown on coverslips for 20–24 h in α-minimum Eagle’s medium (α-MEM) with 5% fetal bovine serum, washed with serum-free medium, and then treated with or without 50 μM BAPTA-AM dissolved in anhydrous Me2SO (final concentration, 0.1%) or 0.1% anhydrous Me2SO in serum-free medium for varying lengths of time. For the expression of the ARF1-GFP fusion protein, human ARF1 was cloned using EcoRI and BamHI sites into the pEGFP vector. NRK cells were transfected with this ARF1-GFP construct using Lipofectin® as per the manufacturer’s instructions (Life Technologies, Inc.). Following the treatment, the cells were washed with PBS and fixed with 3% paraformaldehyde in PBS for 20 min at room temperature. After the cells were rinsed with PBS, the aldehyde groups were quenched with 50 mM ammonium chloride in PBS for 10 min at room temperature. The cells were washed with PBS and then permeabilized with 0.1% Triton X-100/PBS for 4 min at room temperature. After 1–3 washes with PBS containing 0.2% normal donkey serum and 0.2% Tween-20 (PBS-DT), the nonspecific binding sites were blocked with PBS-2.5% donkey serum for 20 min, and the cells were incubated simultaneously with fluorescein isothiocyanate– and/or Texas Red-conjugated secondary antibodies diluted in PBS-DT for 20–60 min at room temperature. The cells were washed three times with PBS-DT and incubated simultaneously with fluorescein isothiocyanate– and/or Texas Red-conjugated secondary antibodies diluted in PBS-DT for 30–45 min at room temperature. The cells were washed three times with PBS-DT, rinsed with water, mounted in 50% PBS/50% glycerol/10 mg/ml n-propyl gallate, and examined by confocal (Bio-Rad) microscopy.

RESULTS

Calcium plays an important role in regulating vesicle fusion reactions within the cell. Although it has been suggested that calcium or calcium gradients may regulate vesicle formation or targeting (25), this possibility has remained largely unexplored. We have studied whether calcium regulates vesicular transport at the Golgi apparatus by examining the effects of calcium chelators on COPI vesicle assembly in whole cells and in vitro.

Chelating Calcium Leads to a Redistribution of Coatomer in Cells—We initially investigated whether intracellular calcium levels regulate Golgi structure and function by assessing the distribution of coatomer, with a progressive diminution in the extent of Golgi localization occurring over 30–90 min (data not shown). Analysis of the distribution of coatomer at various times following the addition of 50 μM BAPTA-AM showed a gradual redistribution of coatomer, with a progressive diminution in the extent of Golgi localization occurring over 30–90 min (data not shown).

Fig. 2. BAPTA inhibits COPI-coated vesicle production. Rat liver Golgi membranes were incubated with cytosol and 20 μM GTPγS alone (lane 2) or in the presence of 5 mM BAPTA (lane 3) or 5 mM EGTA (lane 4). Following the incubation, the membranes were isolated and the vesicles were extracted from the Golgi membrane pellets with 250 mM KCl. Vesicles were isolated by sedimentation through a 35% sucrose gradient. The pellets were processed for Western blotting using anti-β-COP antibody. An incubation carried out without GTPγS was included as a control (lane 1).

Fig. 3. BAPTA inhibition of coatomer binding to Golgi membranes is due to chelation of calcium and not zinc. A. Golgi membranes were incubated with (lanes 2–4) or without (lane 1) 20 μM GTPγS and bovine brain cytosol in the presence of 5 mM BAPTA (lane 3) or 5 mM BAPTA plus 5 mM calcium chloride (lane 4) as detailed under “Experimental Procedures.” The membranes were isolated by sedimentation and processed for Western blotting using antibodies against β-COP. B. Golgi membranes were incubated with cytosol, 20 μM GTPγS, and varying concentrations (shown in mM) of BAPTA (lanes 2–6) or TPEN (lanes 7–10) as indicated. The membranes were isolated by centrifugation and processed for Western blotting using antibodies against β-COP as for Fig. 2. TPEN did not decrease the amount of β-COP associated with the Golgi membrane at concentrations ranging from 0.05 to 1 mM, whereas comparable concentrations of BAPTA caused a marked decrease in the amount of coatomer bound to the Golgi membranes (compare lanes 4 and 5 to lanes 9 and 10).

Calcium chelators are membrane-permeable analogs of the fast-acting calcium chelator BAPTA. Incubating NRK cells with BAPTA-AM caused a complete redistribution of the coatomer in comparison with untreated cells (Fig. 1, compare E and B). Despite the redistribution of coatomer, the juxtanuclear α-mannosidase II-positive Golgi structures remained largely unaffected (Fig. 1, A and D). Merged images show a good

Fig. 4. Calcium chelation inhibits COPI vesicle production. Rat liver Golgi membranes were incubated with cytosol and GTPγS alone (lane 2) or in the presence of 5 mM BAPTA (lane 3) or 5 mM EGTA (lane 4). Following the incubation, the membranes were isolated and the vesicles were extracted from the Golgi membrane pellets with 250 mM KCl. Vesicles were isolated by sedimentation through a 35% sucrose gradient. The pellets were processed for Western blotting using anti-β-COP antibody. An incubation carried out without GTPγS was included as a control (lane 1).

Fig. 5. BAPTA inhibition of coatomer binding to Golgi membranes is due to chelation of calcium and not zinc. A. Golgi membranes were incubated with (lanes 2–4) or without (lane 1) 20 μM GTPγS and bovine brain cytosol in the presence of 5 mM BAPTA (lane 3) or 5 mM BAPTA plus 5 mM calcium chloride (lane 4) as detailed under “Experimental Procedures.” The membranes were isolated by sedimentation and processed for Western blotting using antibodies against β-COP. B. Golgi membranes were incubated with cytosol, 20 μM GTPγS, and varying concentrations (shown in mM) of BAPTA (lanes 2–6) or TPEN (lanes 7–10) as indicated. The membranes were isolated by centrifugation and processed for Western blotting using antibodies against β-COP as for Fig. 2. TPEN did not decrease the amount of β-COP associated with the Golgi membrane at concentrations ranging from 0.05 to 1 mM, whereas comparable concentrations of BAPTA caused a marked decrease in the amount of coatomer bound to the Golgi membranes (compare lanes 4 and 5 to lanes 9 and 10).
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ARF1 (28, 29). COPI-coated vesicles can be extracted from the Golgi membrane with a high salt (250 mM KCl) wash and then isolated by sedimentation (22, 30). We carried out vesicle-budding reactions in the presence of BAPTA and EGTA. Following the incubations, we quantitated the recovery of COPI-coated vesicles. As shown in Fig. 2, the appearance of coatomer in the pellet fraction was completely dependent upon the addition of GTPγS to activate ARF (lanes 1 and 2). The addition of 5 mM BAPTA to the binding reaction completely blocked the appearance of COPI-coated vesicles in the pellet fraction (Fig. 2, lane 3). However, EGTA addition had little or no effect (Fig. 2, lane 4). The inhibition of COPI-coated vesicle assembly by BAPTA, but not EGTA, is similar to the effects of these chelators on calcium-regulated membrane fusion events and intra-Golgi membrane transport (9, 10, 18, 31).

A previous study has indicated that the effect of BAPTA on endosome fusion results from the chelation of zinc (32). There-
the inhibition of coat binding by BAPTA, together with the lack of inhibition by TPEN, indicates that BAPTA exerts its effects by chelating calcium.

**BAPTA Causes Coatomer-coated Vesicles to Uncoat**—The effects of BAPTA on vesicle recovery in vitro suggested that the redistribution of coatomer observed when whole cells were treated with BAPTA-AM resulted from its effects on vesicle assembly rather than a redistribution of the vesicles themselves. The inability to recover coatomer-coated vesicles in the cell-free system, as shown in Fig. 2, could result either from an inhibition of coatomer binding to the membranes or from an increase in the rate at which coatomer dissociates from the membrane. In order to help resolve this issue, we performed binding assays in which BAPTA was added to the reactions at various times after initiation of the incubation at 37 °C. As shown in Fig. 4, BAPTA reduced the levels of bound coatomer even when it was added 15 min after starting the reaction. Assessment of the kinetics of coatomer binding to the Golgi membranes showed that near maximal recruitment of coatomer to the membranes had already occurred after 5 min (Fig. 4, inset). The finding that BAPTA addition reduced coatomer levels, even after maximal recruitment of coatomer on the membranes, suggested that BAPTA was causing the preassembled coatomer to dissociate.

We tested directly whether chelation of calcium affects the stability of coatomer preassembled on the membrane using two-stage binding reactions (Fig. 5A). In the first stage, Golgi membranes were incubated with cytosol and GTPγS to load coatomer onto the membrane. The coatomer-bound membranes were reisolated and then incubated in the second stage with or without BAPTA. In the absence of BAPTA, the coatomer remained associated with the Golgi and was recovered with the membrane pellets by centrifugation after the second-stage incubation (Fig. 5A, lanes 1 and 2). In contrast, inclusion of BAPTA in the second stage caused the dissociation of a large fraction of the Golgi-bound coatomer such that upon centrifugation, it was recovered in the supernatant fraction and not in the Golgi membrane pellet (Fig. 5A, lanes 3 and 4). This result indicated that the addition of BAPTA does not necessarily block the recruitment of coatomer to the Golgi membranes, but it leads to the uncoating of previously assembled coat from the Golgi membranes and vesicles.

It is striking that the differential effects of BAPTA and EGTA are observed in both membrane fusion assays and in stabilization of coatomer on Golgi membranes. This difference in the effects of these two chelators is often attributed to the faster exchange rate of BAPTA for calcium (~100 times) than that of EGTA, and it is interpreted to suggest that a localized calcium transient or a calcium gradient that is more sensitive to the fast binding kinetics of BAPTA is involved (18, 31, 33). Chelation should be a function of both the association rate and the concentration of the chelator. If the difference in calcium binding kinetics between BAPTA and EGTA is responsible for the differential inhibition of coatomer binding to the membranes in the presence of these agents, one would predict that higher EGTA concentrations would produce effects similar to those observed with BAPTA. With this in mind, we reexamined whether EGTA has an effect on coatomer association using the two-stage uncoating assay.

Measuring coatomer dissociation from the Golgi allows smaller effects on coat stability to be observed, because it is much easier to detect a small change in the amount of coatomer released into the supernatant than it is to detect a similar change in the amount of total coatomer bound to the Golgi membrane. Hence, Golgi membranes were preloaded with coatomer, reisolated, and then incubated with 5 mM BAPTA or 20 mM EGTA for varying lengths of time (Fig. 5B). The amount of free coatomer in the supernatant was assessed by immunoblotting. The addition of 5 mM BAPTA led to a rapid uncoating of the membranes, with a t1/2 of ~2 min. As expected from the results of single-stage Golgi binding assays (see Figs. 2 and 3A), the BAPTA-induced uncoating was largely inhibited by the presence of calcium. The addition of 20 mM EGTA to the coatomer-loaded Golgi membranes also led to a release of a significant amount of the coatomer from the Golgi membranes, albeit at a much slower rate (t1/2 > 60 min) than with BAPTA. Although EGTA was much less effective at uncoating the membranes than BAPTA, its effect was also mediated by chelation of calcium because it could be reversed by the inclusion of calcium in the second-stage incubation. Chelation of zinc by TPEN did not cause coatomer release from the Golgi membranes in this more sensitive assay, confirming that zinc does not play a role in maintaining the integrity of the coat on the membranes (data not shown).

If the calcium-sensitive uncoating of the assembled coat plays a role in the regulation of membrane traffic, one would expect the integrity of the coat on the coated vesicles to be sensitive to calcium chelation. To determine whether vesicles themselves were uncoating as opposed to the dissociation of a non-vesicle-associated coatomer pool from the Golgi membranes, we examined the effects of BAPTA on isolated COPI-coated vesicles. Golgi membranes were incubated with GTPγS and cytosol to cause budding, and the vesicles were stripped off the Golgi membranes with a high salt wash. The vesicle-enriched supernatant was then incubated in the presence or absence of BAPTA, and the COPI-coated vesicles were isolated by sedimentation. As shown in Fig. 5C, incubation of isolated coated vesicles with BAPTA prevented the recovery of coatomer-coated vesicles (lanes 1 and 2) compared with incu-
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Calcium Regulation of Coatomer Binding Occurs at Physiologically Relevant Calcium Concentrations—If cellular calcium plays a regulatory role in the interaction of coatomer with the Golgi membrane, the reaction would be expected to occur at a physiologically relevant calcium concentration, and it should be reversible. To address the reversibility of coatomer uncoating, we incubated coatomer-bound Golgi membranes with BAPTA at 37 °C for 20 min to cause release of an appreciable amount of coatomer from the membranes. Free calcium equivalent to the molar concentration of BAPTA was then added to the reaction mixture, and the incubation was continued at 37 °C for another 20 min. Fig. 6 shows that a significant fraction of the coatomer released from the membranes by BAPTA reassociates with the Golgi membrane upon addition of calcium (compare two right bars). The coatomer released from the membranes by BAPTA is also competent to bind naive (i.e. previously unincubated) Golgi membranes, but only in the presence of activated ARF (data not shown). These results indicate that the addition of BAPTA does not lead to an irreversible conformational change or denaturation of coatomer released from the membranes. Therefore, the released coatomer is competent to rebind Golgi membranes and thus participate in the assembly of a “new” vesicle.

In order to estimate the concentration of calcium that allowed reassociation of released coatomer with the Golgi membranes, we calculated free calcium levels present in the incubations during the recoating experiment. We find that coatomer begins reassociating with the membrane at a free calcium concentration in the range of 100–500 nM. Calcium concentrations in this range are typically found within cells (1). Our results showing that calcium-sensitive uncoating is reversible, that calcium regulation of coat assembly occurs at calcium concentrations normally found in cells, and that calcium sensitivity can be demonstrated both in vitro and in whole cells are all consistent with a physiologically relevant role for calcium in regulating vesicle coat assembly and/or disassembly.

ARF Binding Is Not the Primary Target of Calcium Regulation—The calcium sensitivity of coatomer binding to the membranes could result directly from the properties of coatomer or from the effects of calcium on the upstream mechanisms that regulate coat binding. The activation of ARF and the binding of ARF to the Golgi membranes is the critical regulatory event for coatomer recruitment (34, 35). In order to identify which aspect(s) of vesicle assembly was affected by BAPTA, we performed Golgi binding assays in which ARF and coatomer levels were determined simultaneously in the presence and absence of the chelator (Fig. 7). Fig. 7A shows that the addition of BAPTA almost completely blocked the binding of coatomer to Golgi membranes (lanes 3 and 4). The effects of BAPTA on ARF binding were much less pronounced (Fig. 7A, compare lanes 3 and 4 with lane 2), although in a few experiments, a small reduction in ARF binding to the membranes was observed following BAPTA addition (data not shown). Because coatomer binding to the membrane was inhibited in all experiments even when ARF was unaffected, it is likely that the effects of calcium chelation on coatomer association occur at a step subsequent to ARF binding.

In order to further investigate the site of BAPTA action, i.e. at the ARF binding step or the coatomer binding step in vesicle assembly, we performed two-stage incubations in which ARF binding and coatomer binding to Golgi membranes were carried out sequentially (Fig. 7B). We utilized Q71L-ARF1, which behaves as a constitutively activated mutant due to a markedly reduced GTPase activity (36). The addition of BAPTA during the binding of Q71L-ARF to the Golgi membrane in the first stage had only a small effect on the subsequent binding of coatomer (lanes 3 and 4). By contrast, when BAPTA was added only during the second stage, i.e. during the coatomer binding step, coatomer binding was almost completely blocked (lanes 5 and 6). As expected, inclusion of BAPTA during both stages also inhibited coatomer binding effectively (lanes 7 and 8). Addition of calcium to the second-stage incubation prevented BAPTA-induced inhibition of coatomer binding to Golgi membranes primed with Q71L-ARF (lanes 9 and 10), consistent with results shown above.

If coatomer and ARF are differentially sensitive to BAPTA, as indicated by our in vitro data, then treatment of cultured cells with BAPTA is expected to affect the distribution of coatomer independently from ARF. We analyzed the effects of calcium chelation on these proteins by transiently transfecting NRK cells with ARF1 fused to green fluorescent protein (ARF-GFP) and then treating them with BAPTA-AM. The merged images show that the overlap between ARF1-GFP-labeled Golgi membranes and coatomer is completely abolished by treatment of the cells with BAPTA-AM (Fig. 8, compare C and F). Whereas ARF was slightly dispersed after treatment with BAPTA-AM (Fig. 8D), coatomer was completely redistributed.
in transfected NRK cells (Fig. 8E). The coatamer was dispersed despite the fact that ARF-GFP remained largely associated with the Golgi. Together, these experiments show that BAPTA acts to block coatamer assembly on the Golgi membrane, likely at a step subsequent to ARF binding to the membrane.

**DISCUSSION**

In this article, we provide evidence that calcium is required for the stable interaction between Golgi membranes and the COPI vesicle coat protein coatamer. Our findings indicate that calcium is required in addition to the previously described factors, the small GTPase ARF and the dilysine motifs of vesicle membrane proteins (37, 38), to allow the formation of COPI-coated vesicles. Thus, along with its described role in regulating vesicle fusion, calcium may act earlier to regulate vesicle assembly or disassembly. This regulation of vesicle coating or uncoating could be responsible, at least in part, for the calcium-dependent regulation of protein transport between Golgi cisternae that has been observed in a cell-free Golgi transport assay (18).

**BAPTA Acts through Chelating Calcium**—We show that the calcium chelator BAPTA has dramatic effects on the interaction between the vesicle coat protein (coatamer) and Golgi membranes. BAPTA has also been found to block membrane fusion reactions and protein trafficking through the Golgi apparatus. Interestingly, in each of these cases, it was found that BAPTA was a much more potent inhibitor than another calcium chelator, EGTA. There are three possible explanations for this finding. First, the difference between BAPTA and EGTA may result from the fact that BAPTA binds calcium 100 times faster than EGTA (19, 33). Therefore, if calcium gradients or transients are important, they may be buffer only by chelators with rapid binding kinetics. A second possible explanation for these results is that BAPTA acts by chelating another metal that binds with higher affinity to BAPTA than to EGTA. A third possibility is that the effects of BAPTA are mediated by a mechanism other than chelating metals. For example, BAPTA could bind directly to a protein and inhibit its function.

Several lines of evidence indicate that the effects of BAPTA on coatamer binding presented in this study are mediated by a reduction in calcium levels. First, the effects of BAPTA could be reversed by adding calcium, but not zinc or magnesium, to the binding reaction. Second, a metal chelator with specificity toward zinc was shown to have no effect on coatamer binding. Importantly, although BAPTA was more effective than EGTA, EGTA did have a clear effect on coat stability when studied using the more sensitive membrane-uncoating assay. The difference in the uncoating rates upon BAPTA addition versus EGTA addition approximately correlates with the difference in the association rate for calcium.

**Golgi Calcium Stores May Be Important for Vesicular Transport**—Previous studies have shown that vesicle uncoating is triggered by the hydrolysis of GTP on ARF (36). We now find that reduced calcium levels can also lead to vesicle uncoating. This indicates that there are two potential mechanisms that could be used by cells to regulate the timing of vesicle uncoating. Based on our results, a sufficiently high calcium level must be maintained for coatamer to stably associate with Golgi membranes. This would indicate that there may be mechanisms regulating calcium levels around the Golgi apparatus during COPI vesicle assembly. Consistent with this notion, studies on the distribution of free calcium within the cell indicate that it is in fact highest in the Golgi region (39). Golgi calcium stores appear to be substantial and can contribute significantly to calcium-mediated signaling events (40, 41). The Golgi-localized calcium-binding protein CALNUC (15) could be important for maintaining this high calcium level around the Golgi. Given that a large calcium store is maintained by the Golgi, a constant release or leakage of calcium would create a gradient of calcium in the cell with the highest levels at the Golgi, as seen by Wahl et al. (39).

It was proposed over 10 years ago that a calcium gradient existing between the Golgi and the ER could play an important role in regulating vesicular trafficking between these organelles (25). Our data showing that COPI transport vesicles uncoat at low calcium levels suggests a role for such a gradient. It is possible that a calcium gradient serves to define the timing of vesicle uncoating. When vesicles are forming in close proximity to the Golgi cisternae, the high calcium levels would allow stable coat association with the membrane. As a fully formed vesicle moves away from the Golgi membrane, either by diffusion or by a mechanism directed by the cytoskeleton, it will move down the calcium gradient until it reaches a point at which the calcium concentration is not sufficient to maintain the interaction between the coat and the membrane. This could provide an additional regulatory mechanism to ensure that vesicles do not uncoat and fuse with the donor organelle. Instead, the coat would only dissociate from the vesicle once it...
has reached an “uncoating zone” with a lower calcium concentra-
tion at a safe distance from the donor cisternae.

Finally, because calcium is utilized by many signaling pro-
cesses in the cell, our findings suggest a mechanism through
which the regulation of coated vesicle-mediated trafficking
could be connected to other aspects of cellular regulation, such
as growth and differentiation.

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A Role for Calcium in Stabilizing Transport Vesicle Coats
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