Regulation of GTP hydrolysis on ADP-ribosylation factor-1 at the Golgi membrane

Running title: GTP hydrolysis on ARF1 at the Golgi membrane

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Summary

The interaction of the coatmer coat complex with the Golgi membrane is initiated by the active, GTP-bound state of the small GTPase ARF1, whereas GTP hydrolysis triggers coatmer dissociation. The hydrolysis of GTP on ARF1 depends on the action of members of a family of ARF1-directed GTPase-activating proteins. Previous studies in well-defined systems indicated that the activity of a mammalian Golgi membrane-localized ARF GAP (GAP1) might be subjected to regulation by membrane lipids as well as by the coatmer complex. Coatmer was found to strongly stimulate GAP-dependent GTP hydrolysis on a membrane-independent mutant of ARF1, whereas we reported that GTP hydrolysis on wild type, myristoylated ARF1 loaded with GTP in the presence of phospholipid vesicles was coatmer-independent.

To investigate the regulation of ARF1 GAPs under more physiological conditions, we studied GTP hydrolysis on Golgi membrane-associated ARF1. The activities at the Golgi of recombinant GAP1 as well as coatmer-depleted fractions from rat brain cytosol resembled those observed in the presence of liposomes, but unlike in liposomes, GAP activities on Golgi membranes were approximately doubled upon addition of coatmer. By contrast, endogenous GAP activity in Golgi membrane preparations was unaffected by coatmer. Cytosolic GAP activity was partially reduced following immunodepletion of GAP1, indicating that GAP1 plays a significant though not exclusive role in the regulation of GTP hydrolysis at the Golgi. Unlike the activities of the mammalian proteins, the S. cerevisiae Glo3 ARF GAP displayed activity at the Golgi that was highly dependent on coatmer. We conclude that ARF GAPs in themselves can efficiently stimulate GTP hydrolysis on ARF1 at the Golgi, and that coatmer may play an auxiliary role in this reaction, which would lead to an increased cycling rate of ARF1 in COPI-coated regions of the Golgi membrane.
Introduction

The budding of vesicles mediating the transport of proteins among different compartments of the secretory system is driven by the attachment of specialized protein complexes termed coats to the cytoplasmic surface of the donor membrane. These coat complexes also function in the sorting of appropriate cargo into the transport vesicle (reviewed in 1-3). At the Golgi, the heptameric coat termed coatamer drives the formation of COPI vesicles mediating retrograde transport of proteins to the endoplasmic reticulum (4,5) and possibly also anterograde trafficking (reviewed in 6,7).

The interaction of coatamer with the Golgi membrane is regulated by the small GTPase ARF1\(^1\). GTP-bound ARF1 triggers the recruitment of coatamer (8,9), apparently by direct interaction with its β and γ subunits (10). Coatamer also interacts with cytoplasmic tails of membrane proteins destined for retrograde transport to the ER bearing the KKXX retrieval signal (11) and of members of the p23/24 family of Golgi membrane proteins (12,13,14). The subsequent dissociation of coatamer from membranes depends on the hydrolysis of GTP on ARF1 (15,16). The cycles of GTP binding and hydrolysis on ARF proteins is controlled by two sets of cytosolic proteins, guanine nucleotide exchange proteins (GEFs) which facilitate GTP binding, and GTPase-activating proteins (GAPs) which are obligatory for GTP hydrolysis on ARFs (reviewed in 17,18). ARF GAPs are a family of proteins sharing a catalytic domain of 120-140 amino acids that includes a Cys\(_4\) zinc coordination motif. The first ARF GAP to be discovered, now termed GAP1, is a 45 KD protein that distributes between cytosol and the Golgi complex and functions in the regulation of membrane traffic through this organelle (19-23). S. cerevisiae contains two proteins (Gcs1 and Glo3) shown to possess ARF GAP activity (24,25). These two yeast GAPs form an essential pair with a redundant function in the ER-Golgi shuttle (25). Genetic evidence implicated both GAPs, but in particular Glo3, in the
regulation of retrograde traffic from the Golgi to the ER in yeast (25,26). Four additional proteins with an ARF GAP domain are predicted by the S. cerevisiae genome (27), and multiple ARF GAPs have been recently described in mammalian cells (28-38; reviewed in 39). Whereas some of these proteins are likely to regulate plasma membrane-associated processes, others may function at the Golgi (36,37), adding further complexity to the regulation of traffic through this organelle.

Since an ARF1 GAP activity is a prerequisite for the uncoating of COPI vesicles, a regulation of this activity may be necessary to ensure a proper timing of vesicle uncoating, as well as cargo segregation (2,3). Two types of factors have been reported to influence the activity of ARF GAPs in well-defined systems, lipids and the coat protein. ARF1 depends on a lipid environment for nucleotide exchange (40-42), and becomes tightly associated with lipid vesicles following GTP binding (41). Under these conditions, GAP1 activity depends on its association with the phospholipid vesicle, which in turn is increased by diacylglycerols (21). Studies pioneered by Goldberg utilizing a truncated, lipid-independent ARF1 mutant (∆17-ARF) as substrate showed that the activity of GAP1 is strongly stimulated by coatmer (43,44). Similar findings were subsequently described for yeast Gcs1, whereas the activity of the phosphoinositide-dependent ASAP1 ARF GAP was coatmer insensitive (45). We reported however that coatmer does not influence the activity of GAP1 and Gcs1 when myristoylated ARF1 is used as substrate in the presence of liposomes (45). These findings were interpreted to suggest that in the biological membrane, proximity between ARF1 and its GAP, which results from their mutual binding to membrane phospholipids, might be sufficient for the stimulation of ARF1 GTPase activity.

Even though studies in well-defined systems such as liposomes provided important clues to mechanisms that regulate GTP hydrolysis on ARF1, results of such studies may not fully represent regulatory mechanism operating at the Golgi. To investigate this issue, we
designed an assay that measures the activity of exogenous GAPs on Golgi membrane-bound ARF1. Our findings demonstrate that multiple ARF GAPs act at the Golgi membrane, and that their activities may be fine-tuned by the coatamer complex.

**Experimental Procedures**

**Chemicals:** $[^{\gamma}32P]$,GTP (3000 Ci/mmol) was purchased from NEN Life Science Products. Ni-NTA was from Qiagen, Valencia, CA. Brain phosphatidylserine was from Avanti Polar-lipids; egg phosphatidylcholine, egg phosphatidylethanolamine, PIP2, and brefeldin-A (BFA) were from Sigma.

**Preparation of proteins and antibodies:** Coatamer was purified from rabbit liver according to Pavel et al (46). Recombinant myristoylated ARF1 was prepared as described (47). S. cerevisiae Glo3p was expressed in E. coli and purified under denaturing conditions as described in (48), and was dialyzed overnight against 25 mM Tris pH 7.5, 100 mM NaCl, and 1 mM dithiothreitol. Full length GAP1 with a hexahistidine extension was expressed in insect cells using a baculovirus expression vector and purified as described (49). This protein was used to raise antibodies in rabbits by repeated injections of 0.1 mg GAP1 protein in Freund’s adjuvant. The antibodies (marked GAP1-807) were found to be directed against determinants at the carboxy terminal part, as these antibodies did not recognize a truncated GAP1 containing the first 257 amino acids. These antibodies were used in immunodepletion experiments, whereas for Western blot analysis we used rabbit anti-GAP1-257 antibodies (20).

**Golgi membrane preparation:** Golgi membranes were prepared from rat liver by a modification (50) of the procedure of Tabas & Kornfeld (51). Prior to use, the membranes were washed with 0.75 M KCl / 0.2 M sucrose / 25 mM MOPS pH 7.0 to remove peripheral membrane proteins, and were resuspended in 25 mM MOPS pH 7.0 / 0.2 M sucrose.
Measurement of GTP hydrolysis on Golgi membrane-bound ARF1: To load ARF1 with $[\gamma^{32}P]$-GTP at the Golgi membrane, salt washed membranes (0.15-0.20 mg/ml) were incubated at 30°C with 2 µM bovine myristoylated ARF1 in a GTP-loading mixture consisting of 4 µM $[\gamma^{32}P]$-GTP (approx. 200,000 cpm), 0.1 M KCl, 0.2 M sucrose, 2.5 mM MgCl$_2$, 25 mM MOPS pH 7.0, 1 mM dithiothreitol 1 mM ATP plus ATP-regenerating system (5 mM phosphocreatine and 50 µg/ml creatine phosphokinase), and 0.4 mg/ml bovine serum albumin, in a final volume of 50 µl. After 15-30 minutes, nucleotide exchange was stopped by the addition of 0.3 mM brefeldin A (from a 30 mM methanolic stock solution), followed by the rapid addition of ARF GAPs with or without coatomer (0.2 µM). When cytosolic fractions were employed as the source of GAP, unlabeled GTP (0.3 mM) was added along with BFA. At different times aliquots were removed for the determination of membrane-associated $[\gamma^{32}P]$-GTP by the nitrocellulose filtration assay (52). Experiments were repeated at least 4 times and a typical experiment is presented.

ARF GAP assay in the presence of liposomes: ARF1 (4 µM) was first loaded with $[\gamma^{32}P]$-GTP (1 µM) in the presence of 0.4 µm phospholipid vesicles containing 40% phosphatidylcholine, 30% phosphatidylethanolamine, and 30% phosphatidylserine, and GAP activity was assayed at 30°C as described in (45), except that the reactions were carried out in the presence of 100 mM KCl. The hydrolysis of $[\gamma^{32}P]$-GTP on ARF1 was determined by the charcoal absorption assay (49).

Fractionation of rat brain cytosol: Rat brain cytosol was prepared as described in Malhotra et al. (50) and was concentrated to 15-20 mg/ml by centrifugal ultrafiltration. One half-ml cytosol was loaded onto a 24 ml Superdex 200 gel filtration column (Pharmacia-LKB) equilibrated with 150 mM NaCl, 25 mM Tris pH 7.5, and 1 mM dithiothreitol. 0.5 ml fractions were collected and combined into pools, which were concentrated approximately 10 times.
Results

Determination of GTP hydrolysis on Golgi membrane-bound ARF1: In previous studies (16,53), GTP hydrolysis on ARF1 at the Golgi membrane was investigated by relying on endogenous ARF GAPs that are present in the membrane preparation. However, we found that under the conditions employed in these studies, Golgi membranes contain significant amounts of coatamer, which would complicate an analysis of coatamer effects on GAP activity. Additionally, since ARF GAPs are not integral membrane proteins, endogenous GAPs found in purified Golgi membrane preparations may not represent the full repertoire of GAPs that act at the Golgi. In this study we measured the effect of exogenous GAPs from both crude and recombinant sources on GTP hydrolysis on ARF1 bound to rat liver Golgi membranes.

Endogenous coatamer and most endogenous GAPs were removed from the membranes by salt wash, and the membranes were incubated in the presence of myristoylated ARF1 and [γ<sup>32</sup>P]-GTP. During this incubation, the nucleotide binds to the membrane in an ARF1-dependent and BFA-sensitive manner (Fig. 1), indicating that ARF1 has become loaded with [γ<sup>32</sup>P]-GTP through the action of a Golgi-bound GEP. Comparison of loading with [γ<sup>32</sup>P]-GTP and with [α<sup>32</sup>P]-GTP indicated that during the loading period up to 50% of ARF1-bound GTP is hydrolyzed; however, since the assay measures bound GTP and not release of Pi, the hydrolysis of GTP during the loading of ARF at the Golgi membrane does not increase the background of the assay. When nucleotide exchange was subsequently stopped by the addition of BFA, the amount of membrane-bound nucleotide decreased slowly, apparently reflecting residual endogenous GAP activity (Fig. 1); the rate of this reaction varied somewhat among different membrane preparations. Upon addition of purified recombinant GAP1 protein, the decrease in
membrane-bound radioactivity was strongly accelerated, thus providing a sensitive assay for the activity of exogenous GAP proteins at the Golgi membrane.

Role of coatomer in GTP hydrolysis on ARF1 at the Golgi: To study the role of coatomer, we first investigated its effect on GTP hydrolysis at the Golgi stimulated by recombinant mammalian GAP1. As shown in Fig. 2, addition of a GAP1 (28 or 65 nM) effectively stimulated GTP hydrolysis on Golgi membrane-bound ARF1. The addition of a relatively high concentration of purified coatomer (200 nM) caused a further increase in GTP hydrolysis; even though this effect was of low magnitude (1.5-2.5 fold stimulation), it was consistently observed. This effect of coatomer differed from the complete lack thereof in assays containing liposomes (45), but was much smaller than the effect observed with the lipid-independent ARF1 mutant (43). The slow decline in ARF1-bound [γ32P]-GTP in the absence of exogenous GAP, which apparently reflects endogenous GAP activity, was unaffected by the addition of coatomer. GTP hydrolysis in systems that did not receive coatomer was not due to an effect of endogenous coatomer as this was effectively removed by the salt wash of membranes (Fig. 2, inset).

In addition to GAP1, multicellular organisms contain multiple ARF GAPs that share a highly related catalytic domain but show little similarity in their non-catalytic parts. Some of these GAPs may also contribute to GTP hydrolysis on ARF1 at the Golgi membrane. Since all ARF GAPs are soluble proteins, it was of interest to examine the activity and regulation of GAPs present in the cytosol. For these experiments, we employed rat brain cytosol, which was found to possess high levels of ARF GAP activity. In order to test the effect of coatomer on cytosolic GAPs, we fractionated cytosol on a Superdex 200 column and prepared pools of coatomer-depleted fractions. As shown in Fig. 3A, the majority of GAP activity separated from coatomer, peaking in fraction III that represents proteins of molecular size centering around 200 KD. The distribution of GAP activity among different fractions paralleled the distribution
of GAP1, which showed a smeared migration that was shifted to sizes higher than the expected one (Fig. 3A); this behavior of GAP1 was previously observed (19), and is likely to represent aggregation rather than oligomerization (23). The addition of purified coatomer to the cytosolic pools (Fig. 3B) resulted in an approximately two-fold stimulation of GAP activity in the coatomer-free fractions (no. II-IV), and, as expected, had no effect on the fraction containing coatomer (no. I) and on unfractionated cytosol.

The similar magnitude of the effect of coatomer on cytosolic GAPs and on GAP1 raised the possibility that GAP1 might be the major active species in the cytosolic fractions. To assess the contribution of GAP1 to GAP activity in cytosol, we immunodepleted GAP1 from cytosol by employing a polyclonal antiserum raised against the full-length GAP1 protein. This antiserum was found to efficiently remove GAP1 from cytosol (Figure 4A). Depletion of GAP1 from a coatomer-free pool of cytosolic fractions resulted in a partial decrease in GAP activity on Golgi-bound ARF1 (Fig. 4B). This decrease was of similar extent both in the absence and in the presence of coatomer, and thus the depletion of GAP1 from cytosol had little effect on coatomer stimulation of GTP hydrolysis. Once again, endogenous GAP activity in the membrane preparation, which was somewhat higher in the experiment shown in Fig. 4B than that reported in Fig. 2, was unaffected by coatomer. When GAP activity was assayed on ARF1 bound to liposomes instead of Golgi membranes, GAP1 depletion from the cytosolic pool caused a somewhat more pronounced decrease in GAP activity (Fig. 4C). This suggests that the liposomes we employ (40% phosphatidylcholine/30% phosphatidylserine/30% phosphatidylethanolamine) provide a favorable environment for GAP1 activity, but might not be optimal for other cytosolic GAPs. As previously reported for GAP1 (45), GAP activity of the cytosolic fraction (either GAP1 depleted or mock-depleted) in the liposome assay was completely unaffected by coatomer. Depletion of GAP1 from unfractionated cytosol (which contains coatomer) affected GAP activities in a manner that resembled that shown in Fig. 4,
that is a 1.5 fold decrease in GAP activity on Golgi membranes, and a 2-3 fold decrease in activity in the liposome assay (data not shown).

Role of phosphoinositides: Several mammalian ARF GAPs were reported to depend on phosphoinositides for activity (28,29,34,54). It was of interest to examine whether GAPs in cytosol show phosphoinositide-dependent activity on Golgi membrane-bound ARF1. As shown in Fig. 5A, addition of phosphatidylinositol 4,5-bisphosphate (PIP2) to full cytosol caused a slight stimulation of GAP activity on Golgi membranes, but had little effect on endogenous GAP activity of the membranes. The phosphoinositide had virtually no effect on GAP activity in the liposome assay (Fig. 5B). Under the same conditions, PIP2 strongly stimulated the activity of the phosphoinositide-dependent ARF GAP ASAP1 both in Golgi membranes and in liposomes (data not shown), indicating that the conditions we employed were proper for the detection of the phosphoinositide effect. These findings suggest that under the conditions employed, GAP activity for ARF1 in brain cytosol is largely due to phosphoinositide-independent GAP proteins.

S. cerevisiae Glo3 shows high coatomer dependency at the Golgi: In the yeast S. cerevisiae, the Gcs1 and Glo3 proteins form an essential pair of ARF GAPs that function in a partially redundant manner in membrane traffic through the ER-Golgi shuttle (25). The Gcs1 protein shows high similarity to mammalian GAP1, whereas Glo3 is more distantly related to either Gcs1 or GAP1. When tested for its effect on Golgi-bound ARF-GTP, Gcs1 showed low GAP activity either in the absence of presence of coatomer (data not shown). Yeast Glo3 acted more efficiently in this assay ($SC_{50}=50 \text{ nM}$). Interestingly, Glo3 activity at the Golgi was strongly stimulated by coatomer (approximately 50-fold, Fig. 6A). When tested in the liposome assay (Fig. 6B), Glo3 acted at very low efficiency ($SC_{50}=1 \mu\text{M}$). Coatomer increased the activity of Glo3 in the presence of liposomes, but this effect was much smaller that the effect of coatomer on Glo3 activity in the presence of Golgi membranes. It is noteworthy that in a
previous study (25), we reported that Glo3 shows relatively high activity in the presence of mixed micelles containing dimyristoylphosphatidylcholine and cholate, while in the present study we employed a mixture of phospholipids and no detergent. Thus, the composition or the physical state of phospholipids may strongly influence Glo3 activity.

**Discussion**

GTPase-activating proteins acting on ARF1 are thought to constitute an important regulatory device in the processes of COPI vesicle formation and consumption. Biochemical studies in model systems indicated that the catalytic activity of ARF GAPs might be subjected to regulation by membrane lipids (21,35,55), by the coatomer complex (43) and by cytoplasmic tails of certain Golgi membrane proteins (44). However, it remained unclear whether and to what extent each of these factors contributes to the regulation of GAP catalytic activity in the context of the biological membrane. To address this issue, we have measured GTP hydrolysis on ARF1 that became bound to the Golgi membrane through the action of endogenous Golgi GEPs. We show that under these conditions, both recombinant GAP1 and crude cytosolic fractions from brain show considerable GAP activities on their own, and that these activities are approximately doubled upon addition of coatomer.

Previous studies (43,45,54) have shown that ARF GAPs display very low activity on a lipid-independent ARF1 mutant (Δ17-ARF1) as compared with their activity on myristoylated wild-type ARF1 in the presence of liposomes. These findings apparently reflect a very low intrinsic affinity of GAP for its substrate that is alleviated by their mutual binding to liposomes (45). Unlike with Δ17-ARF1, neither recombinant GAP1 (45) nor ARF GAPs in crude cytosolic fractions (present study) showed coatomer-dependent activity on wild type ARF1 in the presence of liposomes, reiterating our conclusion that coatomer plays an auxiliary rather than essential role in GAP-catalyzed GTP hydrolysis on ARF1.
Our findings that GAP1 and cytosolic GAPs display a similar efficacy on ARF1 bound to Golgi membranes as compared with ARF1 bound to liposomes (see Figs. 2, 4 & 5 and ref. (45)), suggest that like liposomes, the Golgi membrane provides a platform for a two-dimensional interaction between ARF and GAP. Such interaction could be brought about by the binding of GAP to Golgi membrane lipids and/or proteins. The finding that unlike in liposomes addition of coatomer causes some increase in the activity of exogenous GAPs at the Golgi may reflect differences in the efficacy of the interaction of GAPs with the different surfaces. While liposomes provide a protein-free surface that may be sufficient for the effective interaction between ARF and GAP (45), biological membranes are covered with the extracellular domains of glycoproteins and glycolipids that could render them less approachable to GAPs. Thus at the Golgi coatomer might act by further increasing the efficacy of interaction of GAP with its substrate. In support of this hypothesis, we found that endogenous ARF GAP activity that remained associated with Golgi membrane preparations was unaffected by coatomer (see Figs. 2 &4). Conceivably under circumstances where interaction of GAP with the Golgi membrane is not rate limiting, factors that may influence this interaction become ineffective. Thus, coatomer dependency of GAP activity in vivo may reflect the cycling rate of ARF GAPs on and off the Golgi membrane. While the influence of coatomer would be significant for fast cycling GAPs, GAPs that have long residence time at the Golgi membrane would show coatomer-independent activity.

In contrast to our observations with mammalian GAP1 and GAPs from tissue extract, we found that the activity of S. cerevisiae Glo3 at the Golgi is highly coatomer-dependent (see Fig. 6). This finding could be explained by assuming that unlike other GAPs that we have studied, Glo3 does not interact efficiently with the Golgi membrane, which could be due to the heterologous nature of this assay system. Interestingly, Glo3 was recently reported to form a complex with coatomer (56), and such interaction could further contribute to the large effect of
coatamer on Glo3 activity. A somewhat unexpected finding was that Glo3 showed very low activity when assayed in the presence of liposomes, and that this activity was only slightly stimulated by coatamer (see Fig. 5). Even though we employed liposomes containing phospholipids that are typical of biological membranes, the low activity of Glo3 under these conditions might reflect requirement for other lipids (and/or proteins) that are present in Golgi membranes.

Whereas *S. cerevisiae* cells possess six proteins with a predicted ARF GAP catalytic domain, human genome sequences predict 16 ARF GAP domain proteins, and many of these predicted proteins (or their orthologues from other animals) were demonstrated to possess ARF GAP activity (39). While the first ARF GAP to be identified (GAP1) was found to localize at the Golgi and regulate traffic through this organelle (19-23), more recent ectopic expression studies suggest that members of two additional subfamilies of ARF GAPs may act at the Golgi as well (36,37). Our analysis of GAP activities in crude cytosol provides some clues to the identity of GAPs that are catalytically active at the Golgi membrane. We found that significant GAP activity on Golgi membranes is retained in cytosol following immunodepletion of GAP1 (see Fig. 4), indicating that in addition to GAP1, other ARF GAPs are involved in the regulation of GTP hydrolysis at the Golgi. Additionally, our observation that PIP2 has only modest effect on the activity of cytosolic GAPs (Fig. 5) suggests that ARF GAPs whose activity is highly dependent of polyphosphoinositides (such as members of the ASAP family) are not major players at the Golgi. The role of additional ARF GAP subfamilies in GTP hydrolysis on ARF1 at the Golgi, as well the question of whether there exist mammalian GAPs that manifest high coatamer dependency as reported here for Glo3, will be topics of future studies.

In considering the role of GTP hydrolysis on ARF1, it is noteworthy that this G protein controls a number of processes other than the formation of COPI vesicles, including the
interaction of adaptor complexes with the Trans-Golgi network (57-60) and the regulation of the activity of the enzymes phospholipase-D (61,62) and PtdIns-4-OH kinase (63). Even though the lipid-modulating activities of these enzymes might take part in the process of vesicle formation, the regulation of these enzymes by ARF1 appears to take place independently of the process of vesicle budding (63,64). These different processes may have distinct ARF1 cycling rates due to modulation of GAP activity by specific effectors. In this regard, it is of interest to note that whereas coatomer may increase cycling on ARF1 at the Golgi, GGA proteins, which function as ARF1 effectors at the Trans-Golgi network, competitively inhibit the action of GAPs, thereby increasing their own residence time at the membrane (65). An additional level of regulation of the ARF cycle might be exerted by differential dependency of distinct ARF GAPs on effectors, as suggested by our findings of the high dependency of S. cerevisiae Glo3 on coatomer. In conclusion, we propose that ARF GAPs constitute a basic machinery for the catalysis of GTP hydrolysis on ARF1, and that effectors such as coatomer may participate in the fine-tuning of this reaction.
References


Footnotes

1Abbreviations used: ARF, ADP-ribosylation factor; GAP, GTPase activating protein; GEP, guanine nucleotide exchange protein; PIP₂, phosphatidylinositol 4,5-bisphosphate.

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Figure legends

Figure 1. An assay of GAP-catalyzed GTP hydrolysis on Golgi membrane-bound ARF1. ARF1 and salt-washed Golgi membranes were incubated at 30°C, singly or together, with [γ32P]-GTP in the presence or absence of BFA as indicated. At different times, the amount of bound nucleotide was determined by nitrocellulose filtration. After 30 min, GTP binding to the sample that received membranes plus ARF1 was stopped by the addition of BFA with or without GAP1 (50 nM) and incubation was continued for the indicated times.

Figure 2. Effect of coatomer on GAP1-catalyzed GTP hydrolysis on Golgi membrane-bound ARF1. Golgi membranes were preloaded with [γ32P]-GTP and loading was stopped as described in Fig. 1. At zero time, GAP1 (28 or 65 nM) and coatomer (200 nM) were added where indicated, and the amount of membrane-bound [γ32P]-GTP was monitored. Insert: Western blot analysis of Golgi membranes (unwashed or washed with 0.3 or 0.75 M salt) using the M3A5 anti-βCOP antibody.

Figure 3. A. Fractionation of rat brain cytosol on Superdex 200. Cytosol (0.5 ml containing 8 mg protein) was chromatographed on a 24 ml Superdex 200 column as described under Experimental Procedures. 0.5 ml fractions were collected and analyzed by Western blotting for βCOP and for GAP1. The positions of molecular weight markers are marked by arrowheads. B. Coatomer effect on GAP activity of cytosolic fractions. Pooled fractions of the Superdex 200 column (see panel A) were concentrated and tested for GAP activity on Golgi membranes (0.2-0.3 mg/ml final concentration of each fraction in the assay). Data represent the relative rate of the decrease in membrane-bound [γ32P]-GTP per mg of added protein as compared with dialyzed, unfractionated cytosol.

Figure 4. Effect of GAP1 depletion on GAP activity of a coatomer-depleted cytosolic fraction. A. Anti-GAP1 antibodies 807 were attached to protein A at a 1:2 v/v ratio. A pool of ten-fold-
concentrated Superdex 200 fractions (combined pools III and IV, Fig. 3A) was incubated for 4 hours at 4°C with the anti-GAP1 beads (1 volume of beads per 3 volumes of cytosolic pool), and depletion of GAP1 was followed by Western blot analysis. Lane 1, depleted cytosol (HC=heavy chain of antibodies that dissociated from the beads during incubation with cytosol); lane 2, mock-depleted cytosol. B. GAP activity on Golgi membranes of GAP1-depleted or mock-depleted cytosolic fractions (0.72 mg/ml) in the presence or absence of coatomer. C. Activity of the fractions (0.3 mg/ml) was assayed on ARF1 preloaded with GTP in the presence of liposomes (see Experimental Procedures).

Figure 5. Effect of PIP2 on GTP hydrolysis catalyzed by cytosolic GAPs. Unfractionated, dialyzed rat brain cytosol (0.35 mg/ml) was assayed for GAP activity on ARF1 preloaded with GTP in the presence of Golgi membranes (A) or liposomes (B) in the presence and absence of PIP2 (0.2 mM).

Figure 6. A. Effect of coatomer on GAP activity of S. cerevisiae Glo3. GTP hydrolysis was measured in the presence of the indicated Glo3 concentrations following 10 min of incubation with ARF1 preloaded with GTP in the presence of Golgi membranes (A) or liposomes (B).
Figure 1

![Graph showing the time (min) vs. membrane-bound [γ-32P]-GTP (nmol/mg protein) for different treatments: GM, ARF, GM+ARF, GM+ARF+BFA. The graph includes time points at 0, 10, 20, 30, 40, and 50 minutes. The treatments are indicated with different symbols: circles for GM, triangles for ARF, squares for GM+ARF, and diamonds for GM+ARF+BFA. The graph shows an increase in membrane-bound [γ-32P]-GTP for GM+ARF+BFA compared to GM, ARF, and GM+ARF. At 30 minutes, BFA is added, leading to a decrease in membrane-bound [γ-32P]-GTP for GM+ARF+BFA. The graph also shows the effect of GAP on GM+ARF+BFA, with GAP+ being indicated by dashed lines and GAP- by solid lines.](#)
Figure 3

A

β-COP
GAP1

66 440 232 66

OD$_{280}$

Elution volume

B

Relative rate of GTP hydrolysis

- coatomer
+ coatomer

Fraction
None cyt I II III IV V
Figure 4

A

B

Membrane-bound $[^{32}\text{P}]-\text{GTP}$, % of initial

Mock-depl
Mock-depl+coatamer
GAP1-depl
GAP1-depl+coatamer
Control
Control+ coatamer

C

32Pi released, % of ARF-bound $[^{32}\text{P}]-\text{GTP}$

Mock-depl
Mock-depl+coatamer
GAP1-depl
GAP1-depl+coatamer
Figure 5
Figure 6

A. Membrane-bound \( [\gamma^{32}P] \)-GTP, % of initial

B. \( ^{32}P \) released, % of ARF-bound \( [\gamma^{32}P] \)-GTP