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Studies on the inhibition of endosome fusion by GTPγS-bound ARF

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SUMMARY

Using a cell free assay, we have previously shown that ARF is not required for endosome fusion but that inhibition of fusion by GTPγS is dependent on a cytosolic pool of ARFs. Since ARF is proposed to function in intracellular membrane traffic by promoting vesicle biogenesis, and components of clathrin- and COP-coated vesicles have been localized on endosomal structures, we investigated whether ARF-mediated inhibition of early endosome fusion involves the recruitment or irreversible association of these proteins onto endosomal membranes. We now report that depletion of components of clathrin coated vesicles (clathrin, AP-1 and AP-2) or COPI vesicles (β COP) does not affect the capacity of GTPγS-activated ARF to inhibit endosome fusion. Inhibition of fusion by activated ARF is also independent of endosomal acidification since assays performed in the presence of the vacuolar ATPase inhibitor bafilomycin A1 are equally sensitive to GTPγS-bound ARF. Finally, in contrast to reported effects on lysosomes, we demonstrate that ARF-GTPγS does not induce endosomal lysis. These combined data argue that sequestration of known coat proteins to membranes by activated ARF is not involved in the inhibition of early endosome fusion and that its capacity to inhibit fusion involves other specific interactions with the endosome surface. These results contrast with the mechanistic action of ARF on intra-Golgi transport and nuclear envelope assembly.

Key words: ADP-ribosylation factor, Endosome fusion, Coat protein

INTRODUCTION

Members of the ARF (ADP-ribosylation factor) family of monomeric GTP-binding proteins are proposed to function in intracellular membrane traffic (Moss and Vaughan, 1995; Boman and Khan, 1995). ARFs have been characterized to function in secretory transport, participating in the formation of coatomer-coated vesicles (Bednarek et al., 1996; Schekman and Orci, 1996; Kreis et al., 1995). Coatomer or COP coats are heteroheptameric complexes, recruited from the cytosol for anterograde and retrograde traffic between the ER and the Golgi. More recently, subunits of the COPI complex, α, β, β′, ε and ζ, have been identified to associate with endosomes and to be involved in the formation of carrier vesicles to late endocytic compartments (Whitney et al., 1995; Aniento et al., 1996; Gu et al., 1997). ARFs also recruit clathrin and heterotetrameric clathrin adaptor protein (AP) complexes to the trans-Golgi network (TGN) (Stamnes and Rothman, 1993; Traub et al., 1993), immature secretory granules (Dittie et al., 1996) and endosomes (Seaman et al., 1993; West et al., 1997).

A role for ARF in endosomal traffic was first implicated in studies by Lenhard et al. (1992). When activated by the poorly hydrolyzable GTP analog GTPγS, myristoylated ARF1 was found to inhibit in vitro endosome fusion. Studies in our laboratory later revealed that while inhibition of endosome fusion by GTPγS was critically dependent on a cytosolic pool of ARFs, endosome-endosome fusion was still functional when cytosol was depleted of these GTP-binding proteins (Spiro et al., 1995). These findings correlate with observations made of ARF function in reconstituted Golgi transport. Taylor et al. (1994) described the inhibition of intra-Golgi transport by ARFs in the presence of GTPγS, but found that mechanistic properties of this cell-free activity were unaffected upon depletion of cytosolic ARFs. Elazar et al. (1994) reported similar observations and proposed that reconstituted intra-Golgi transport could occur via steps coupled to vesicle biogenesis, therefore requiring ARFs, or via a mechanism uncoupled from ARF activity. In addition, ARF-mediated inhibition was found to be coatomer dependent and could be reversed by the ARF nucleotide exchange factor inhibitor brefeldin A. More detailed studies by Happe and Weidman (1998) explored the reconstitution of cis-to-medial Golgi, medial-to-trans Golgi, and trans-Golgi-to-TGN transport and revealed that cytosolic ARFs inhibit each of these steps in the presence of GTPγS, but that these functions continue unperturbed in their absence. All of these results suggest that membrane traffic is blocked by activated GTPγS-bound ARFs in a manner that is coupled to its mechanistic role in coat recruitment, a process that is required for vesicle formation but not necessarily for fusion. However, the recent demonstration that membrane-bound ARF promotes leakage from rat liver lysosomes indicates that GTPγS-bound ARF could also inhibit membrane transport steps by promoting organellar lysis and disintegration (Sai et al., 1998; Arai et al., 1998). Two other

well described membrane fusion events blocked by GTPγS are nuclear envelope assembly in *Xenopus laevis* eggs (Boman et al., 1992) and homotypic vacuolar fusion in yeast (Haas et al., 1994). While a second membrane-bound GTPase has been implicated in addition to ARFs for the GTPγS effects on nuclear envelope membranes (Gant and Wilson 1997), the identity of the inhibitory GTPase(s) for vacuole biogenesis is unknown.

In contrast to the formation of Golgi-derived COPI vesicles which is stimulated by GTPγS and ARF (Ostermann et al., 1993), GTPγS blocks the generation of endosome-derived COPI-coated carrier vesicles destined to fuse with late endosomes (Aniento et al., 1996). Similarly, Kornfeld and coworkers (Traub et al., 1993; Zhu et al., 1998) have described the ARF-dependent recruitment of AP-1 and clathrin to Golgi membranes, but the failure to bud clathrin-coated vesicles. GTPγS-bound ARF in the absence of other cytosolic factors is able to activate coat binding sites on membranes (Stamnes et al., 1998; Zhu et al., 1998), however, the enhanced number of COPI coated vesicles generated in the presence of GTPγS were found to be depleted of cargo (Nickel et al., 1998). The observation that intra-Golgi transport and endosome fusion proceeds in the absence of ARF but that both are inhibited by GTPγS led us to question whether the mechanism of ARF inhibition involved the recruitment and/or irreversible association of cytosolic coat proteins with these membranes**.** Possible ARF-induced disintegration of endosomal compartments was also investigated. The results reported here demonstrate that inhibition of endosome fusion activity by GTPγS-bound ARF does not require cytosolic COPI, clathrin or the clathrin-associated complexes AP-1 and/or AP-2 and is not a result of perturbations in endosomal integrity. These results contrast with the mechanistic action of ARF on intra-Golgi transport and nuclear envelope assembly.

MATERIALS AND METHODS

Materials

Avidin-conjugated β-galactosidase (Av-βGal), biotin-insulin, holotransferrin (Tf), Protein A Sepharose CL-4B, bafilomycin A1, monoclonal antibody M3A5 against the β-subunit of COPI coatomer, and horse radish peroxidase (HRP)-conjugated rabbit anti-mouse antibody were purchased from Sigma. GTPγS and goat anti-human Tf was purchased from Boehringer Mannheim. [α-^{32}P]GTP (3000 Ci/mmol) was obtained from NEN-Life Science Products. Biotinylated Tf (B-Tf) was prepared as previously described (Wessling-Resnick and Braell, 1990). Rabbit anti-mouse antibody was from Jackson ImmunoResearch Laboratories. Human transferrin was from Boehringer Mannheim and was iodinated as previously described (Schonhorn and Wessling-Resnick, 1994).

Preparation of K562 cell cytosol and membrane fractions

K562 cells were maintained in α-minimum essential medium supplemented with 7.5% fetal bovine serum, 50 units/ml penicillin, and 50 μg/ml streptomycin. Post-nuclear supernatant (PNS) fractions were prepared as previously described (Wessling-Resnick and Braell, 1990). Briefly, K562 cells were washed in phosphate-buffered saline (PBS), resuspended in 25 mM Hepes, pH 7.5, 150 mM NaCl, 1 mg/ml dextrose, 1 mg/ml bovine serum albumin, and incubated with either 0.5 mg/ml Av-βGal or 100 nM B-Tf for 60 minutes at 20°C. After endocytosis was quenched on ice, the cells were washed and broken using a ball-bearing homogenizer in breaking buffer (20 mM Hepes, pH 7.4, 100 mM KCl, 85 mM sucrose, 20 μM EGTA). PNS was collected upon centrifugation at 800 ***g*** for 5 minutes at 4°C, and after dialysis against breaking buffer, aliquots were stored frozen at –80°C until use. Cytosol was prepared from K562 cells washed in PBS and broken in the same buffer, except after the post-nuclear supernatant centrifugation step, the PNS was subjected to ultracentrifugation for 15 minutes at 380,000 ***g*** at 4°C to remove membranous components. Supernatant cytosol was also dialyzed against breaking buffer and stored frozen at –80°C.

To collect membrane fractions containing endocytic vesicles, PNS was layered onto 0.25 M sucrose, 10 mM acetic acid, 10 mM triethylenolamine, 1 μM EDTA, pH 7.4, and membranes were collected off an underlying cushion of isotonic Nycodenz after centrifugation at 380,000 ***g*** for 5 minutes at 4°C to pellet vesicles. Alternatively PNS was centrifuged at 16,000 ***g*** for 20 minutes at 4°C. Isolated membranes were immediately employed in fusion assays described below.

Endocytic vesicle fusion assay

In vitro endosomal fusion assays were carried out as described by Wessling-Resnick and Braell (1990). PNS or separated membrane fractions containing endocytic vesicles with internalized Av-βGal and B-Tf were mixed on ice in a buffer containing cytosol, 1 mM MgATP, 50 μg/ml creatine kinase, 0.8 mM phosphocreatine, 10 μg/ml biotin-insulin, and 1 mM dithiothreitol. Details of the amount of cytosol in the assay mixture and the exact manipulation of vesicle fractions are found in the figure legends; all manipulations of cytosol at 37°C prior to fusion assays were carried in the presence of ATP, an ATP-regenerating system, and 1 mM dithiothreitol as indicated above. Reaction mixtures were held at 37°C for 30 minutes and fusion was terminated by 1:10 dilution of lysis buffer (10% Triton X-100, 1% sodium dodecyl sulfate, 50 μg/ml biotin-insulin). After microcentrifugation, clarified lysates were placed in microtiter wells coated with anti-Tf antibodies (1:100 dilution).

The complex between Av-βGal and B-Tf resulting from specific fusion events was measured as previously described (Wessling-Resnick and Braell, 1990). Briefly, after overnight incubation of the lysates at 4°C to capture the Av-βGal:B-Tf complex, microtiter wells were washed three times with PBS, followed by four washes with 10 mM Tris-HCl, pH 7.5, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 100 mM NaCl, 1 mM EDTA. The wells were incubated 20 minutes at 37°C in a final wash, then rinsed with PBS three times. Av-βGal activity was measured by incubating the wells with 250 μl aliquots of substrate solution (0.3 mM 4 methylumbelliferyl β-galactoside in 100 mM NaCl, 25 mM Tris-HCl, pH 7.4, 1 mM $MgCl_2$, 12 mM β-mercaptoethanol) for 1-3 hours. Samples were diluted with 5 volumes of 133 mM glycine, 83 mM Na_3CO_3, pH 10.7, and the fluorescence of the hydrolysis product was measured using an Hitachi F-2000 spectrophotometer (365 nm excitation, 450 nm emission). The fluorescence signal is directly proportional to the extent of probe colocalization detected by complex formation. Fusion measurements were adjusted for background by subtracting the amount of activity measured for samples incubated at 4°C.

Preparation of COPI-depleted cytosol

To prepare COPI-depleted cytosol, approximately 2×10^7 cells were washed 3 times with PBS and cell pellets were resuspended in 4-5 volumes of ice-cold buffer containing 30 mM KCl, 5 mM $MgCl_2$, 1 mM ATP, 20 μM creatine phosphate and 11 U/ml creatine phosphokinase (Taylor et al., 1992). PNS was prepared as described above. After adjusting the pH to 7.4, dithiothreitol and GTPγS were added to a final concentration of 1 mM and 200 μM, respectively, and the PNS was incubated at 37°C for 30 minutes. Cytosol was prepared by centrifugation at 380,000 ***g*** for 15 minutes at 4°C. The supernatant was removed and was subsequently dialyzed for two hours at 4°C against ice-cold breaking buffer. Cytosol was stored at –80°C until further use. This preparation was analyzed for the presence of COPI

coatomer subunits by western blotting with M3A5 antibody (1:1000) and GTP-binding proteins were identified by [α-^{32}P]GTP overlay assays (see below).

Immunodepletion of clathrin and APs from cytosol

To deplete clathrin, 110 μl packed Protein A-Sepharose beads were washed in breaking buffer and incubated for 90 minutes at 4°C with 121 μl mouse ascites containing anti-clathrin antibody X22 (Brodsky, 1985). The beads were extensively washed in breaking buffer prior to addition of 300 μl cytosol (10.8 mg/ml). This mixture was incubated for 3 hours at 4°C prior to collecting immunodepleted cytosol by centrifugation. Control cytosol was treated in the same way except that the beads were pre-incubated with non-immune mouse serum. The extent of immunodepletion was quantified by western blotting using monoclonal antibody CON1 which recognizes clathrin light chain (Nathke et al., 1992). To enhance specificity of detection, the latter antibody was pre-bound with HRP-labeled secondary antibody according to the method of Langstein and Schwarz (1997). Briefly, CON1 antiserum was diluted 1:175 in Tris-buffered saline (TBS) containing 5% milk fat and 0.1% Tween-20 and incubated overnight at room temperature with HRP-conjugated rabbit anti-mouse antibody (1:600). Unbound secondary antibody was then quenched by incubation for 3 hours at room temperature with 1.6 μl mouse serum (45 mg/ml). HRP activity was detected by enhanced chemiluminescence (ECL) according to the manufacturer's directions (Amersham).

Immunodepletion of clathrin AP β1 and β2 was performed using saturating amounts of monoclonal 10A antibody bound to Protein A-Sepharose beads covalently coupled with a 'bridging' rabbit anti-mouse antibody (Harlow and Lane, 1988). The Protein A complexes (110 μl packed beads) were incubated with 250 μl K562 cytosol (10 mg/ml) for 90 minutes at 4°C. Immunodepletion was assessed by western analysis with monoclonal 9A antibody (1:2,500). Monoclonal antibodies 9A and 10A have been previously characterized (Clairmont et al., 1997).

Subcellular fractionation of ^{125}I-Tf and Av-βGal bearing endosomes

Ten million K562 cells were washed in PBS and resuspended in 25 mM Hepes, pH 7.5, 150 mM NaCl, 1 mg/ml dextrose, 1 mg/ml bovine serum albumin. To label early endosomal compartments, 5.3×10^6 of the washed cells were incubated for 1 hour at 20°C with 0.4 μM ^{125}I-Tf (4.0×10^{16} cpm/mol). Internalization was then quenched on ice and cells were combined with the remaining unlabelled cells, washed and broken to prepare PNS as described for endosome fusion assays. Thirty μl aliquots of PNS were incubated at 4°C and 37°C for 30 minutes under fusion reaction conditions in the presence of 7.6 mg/ml cytosol that had been pre-incubated for 30 minutes at 37°C with or without 150 μM GTPγS. The assay mixtures were quenched on ice prior to centrifugation at 16,000 ***g*** for 10 minutes at 4°C to generate a low speed pellet (LSP). The supernatant was harvested and further centrifuged at 380,000 ***g*** for 30 minutes at 4°C to generate a high speed pellet (HSP) and high speed supernatant (HSS). The pellets were washed once with ice cold breaking buffer and radioactivity in the LSP, HSP and HSS was determined by gamma counting prior to solubilization in Laemmli buffer and SDS-polyacrylamide gel electrophoresis. The gels were dried and ^{125}I-Tf was detected by autoradiography. Identical experiments were carried out with Av-βGal labeled endosomes except that LSP and HSP were resuspended in breaking buffer containing 10% (v/v) lysis buffer; lysis buffer was also added to adjust HSS to 10% (v/v). βGal activity in each fraction was then measured using the fluorescence-based assay described above.

GTP overlay assays

Proteins were electrophoresed on 12% SDS-polyacrylamide gels and then transferred to nitrocellulose under non-denaturing conditions (25 mM Tris-HCl, pH 8.3, 192 mM glycine, 20% methanol). To analyze blots for the presence of small GTP-binding proteins, the nitrocellulose was incubated at room temperature for 1 hour in 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 2 mM dithiothreitol, 0.3% Tween-20, 0.3% bovine serum albumin, and 0.1 mM ATP, and then incubated in fresh buffer containing ^{32}P-GTP (0.8-1.5 μCi/ml) for 1 hour. The blot was then washed three times with the same buffer, dried, and exposed to film for autoradiography.

RESULTS

Inhibition of fusion by activated ARF does not depend on endosomal vacuolar ATPase activity

A discreet set of cytoplasmic proteins including COPI and ARF are recruited to endosomes in a GTPγS-dependent manner (Whitney et al., 1995). Previous studies have shown that association of COPI proteins to endosomal membranes and the binding of ARF to microsomal membranes is dependent on luminal acidification (Aniento et al., 1996; Zeuzem et al., 1992). Alkalinization of endosomal pH using inhibitors of the vacuolar ATPase reduces COPI association with early endosomes and inhibits the formation of carrier vesicles to late endosomes (Aniento et al., 1996). We therefore examined whether inhibition of endosome fusion by GTPγS, which is mediated by cytosolic ARFs (Spiro et al., 1995), could be reversed using the vacuolar ATPase inhibitor bafilomycin A1. Fig. 1 demonstrates

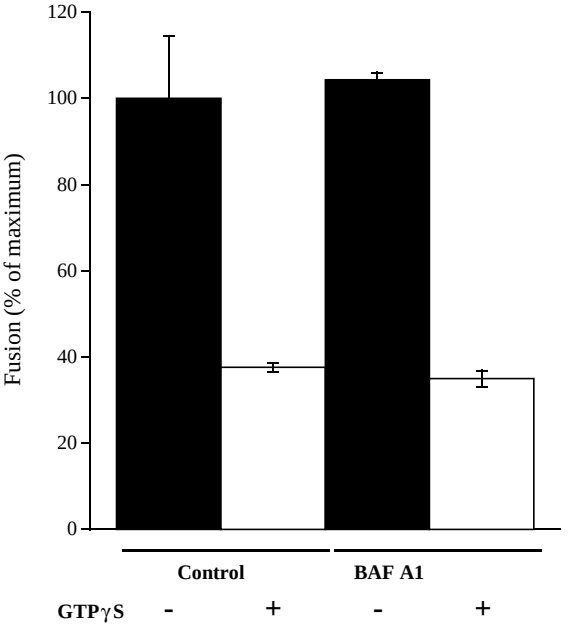

Fig. 1. GTPγS inhibition of endosome fusion persists under conditions that raise lumenal pH. Endosome fusion assays were prepared with 2.5 μl PNS fractions each containing Av-βGal or B-Tf labeled endosomes and K562 cell cytosol pre-activated for 30 minutes at 37°C with or without 100 μM GTPγS. Ten μM bafilomycin A1 (BAF A1) was added as indicated and mixtures were incubated for 10 minutes on ice. Fusion assays were then performed at 37°C under conditions described in Materials and Methods. Results are the mean (± s.d.) from duplicate samples from an individual experiment and are representative of data obtained on three separate occasions. Control experiments demonstrated that BAF A1 decreased GTPγS-dependent binding of βCOP to membranes as previously described (Gu et al., 1997).

that bafilomycin A1 has no effect on fusion and that assays performed with bafilomycin A1 are equally sensitive to GTPγS. These data agree with earlier studies indicating that bafilomycin A1 does not affect cell free endosome fusion activity in vitro (Clague et al., 1994; Pless and Wellner, 1996), suggesting that the observed effects of bafilomycin A1 in vivo are downstream of early endocytic fusion events. Thus, endosome-endosome fusion does not appear to involve coatomer-coated vesicle formation. Given that bafilomycin A1 releases endosome-associated COPI (Aniento et al., 1996), our results further suggest that inhibition of this activity by GTPγS is independent of coatomer recruitment.

Endosome fusion remains sensitive to activated ARF in the absence of coat proteins

To rigorously assess whether ARF-mediated inhibition of fusion in the presence of GTPγS involves the recruitment of coatomer to endosomal membranes, the ability of depleted COPI(-) cytosol to support endosome fusion was examined. The cytosolic heptameric coatomer complex is recruited en bloc to membranes by GTPγS-bound ARFs (Waters et al., 1991; Donaldson et al., 1992; Palmer et al., 1993; Hara-kuge et al., 1994). Therefore, PNS fractions were incubated with GTPγS such that coatomer proteins bound to membranes could be separated from cytosol by centrifugation (Taylor et al., 1992). Fig. 2A presents western blot analysis confirming >90% depletion of β-COP. COP(-) cytosol supports endosome fusion activity in a concentration-dependent manner identical to control COP(+) cytosol (Fig. 2B). However, COP(-) cytosol displays a significant decrease in GTPγS-mediated inhibition of fusion. Although this result suggests a role for COPI proteins in the mechanism of GTPγS inhibition, loss of inhibition could be explained by concomitant depletion of ARF with the coatomer complex. We have previously demonstrated that depletion of cytosolic ARFs does not affect endosome fusion, but does result in the loss of inhibition of this activity in the presence of GTPγS (Spiro et al., 1995). GTP overlay assays shown in Fig. 2A reveal that ARFs are in fact depleted from COP(-) cytosol. Therefore, fusion assays were performed with COP(-) cytosol supplemented with 50 ng purified ARF1 (Fig. 3); as expected, inhibition of fusion activity by GTPγS was restored to control levels for COP(-) cytosol. Thus, inhibition of endosomal fusion by GTPγS-bound ARFs does not appear to involve recruitment of coatomer, consistent with the bafilomycin A1 results shown in Fig. 1.

It has been demonstrated recently that coatomer stimulates ARF-GAP activity (Goldberg, 1999). Thus, one could argue that COPI might be required to release the inhibitory action of ARF1. Since GTPγS would mask these potential indirect effects of COPI, assays similar to those shown in Fig. 3 were carried out except that GTP was employed. However, the extent of fusion activity was unaffected by GTP for both control and COP(-) cytosol and the addition of ARF1 failed to inhibit the assay in the presence of GTP under both conditions (not shown). These data further support the idea that coatomer does not participate in inhibition of endosome fusion by activated ARF.

One might envision that cytosolic GTPγS-activated ARFs indirectly inhibit endosome fusion by recruiting and sequestering factors necessary for this activity to other membrane organelles. However, the fact that COP(-) cytosol fully supports fusion activity argues against this possibility since these components, like βCOP, would be eliminated or depleted from this preparation. The fact that addition of ARF by itself is sufficient to mediate inhibition further supports the idea that ARF-recruitable cytosolic components are unlikely to be involved in direct effects on endosomal membranes since these would also be exhausted from the COP(-) preparation. This evidence suggests, for example, that other factors known to be recruited to membranes by ARFs such as the members

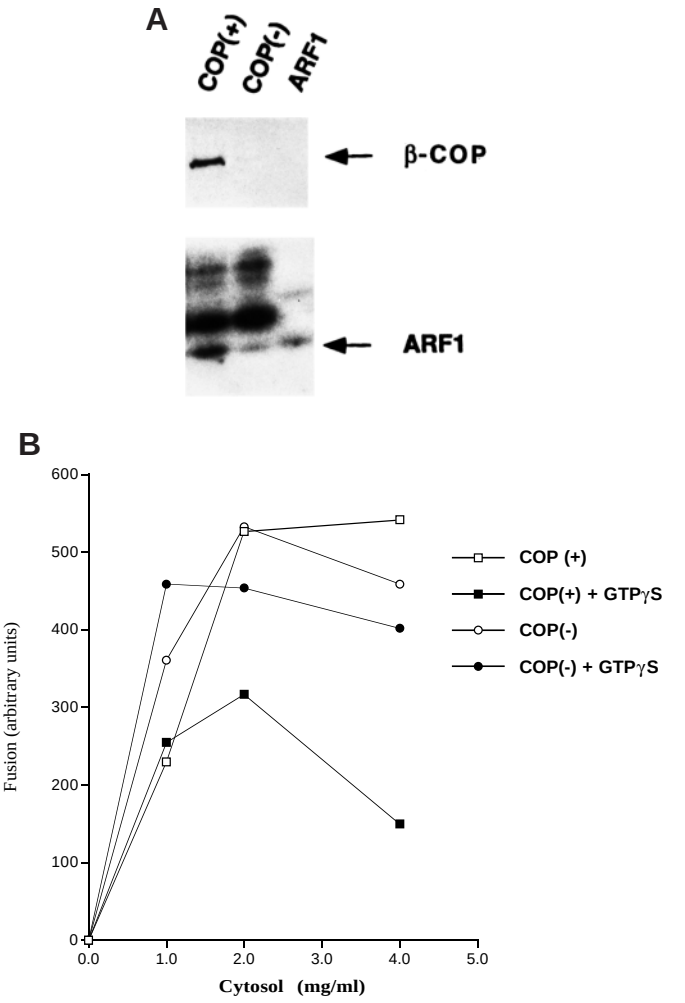

Fig. 2. Depletion of COPI does not alter endosome fusion activity. (A) Cytosol was depleted of coatomer upon incubation with membranes and GTPγS at 37°C as described in Materials and Methods. One hundred μg samples of COP(+) and COP(-) cytosol, along with 200 ng purified ARF1, were electrophoresed on a 12% SDS-polyacrylamide gel and then transferred to nitrocellulose under non-denaturing conditions. The nitrocellulose was cut into two portions; the higher molecular mass segment was incubated with a 1:1000 dilution M3A5 anti-βCOP and antibody binding was detected by ECL. The lower portion of nitrocellulose was analyzed for the presence of small mass GTP-binding proteins by the ^{32}P-GTP overlay assay as described in Materials and Methods and an autoradiograph of the blot is shown. (B) COP-depleted (circles) or wild-type (squares) cytosol was incubated for 30 minutes with (filled symbols) or without (open symbols) 100 μM GTPγS. Increasing amounts of each cytosol were added to fusion assays with K562 cell vesicles separated on a sucrose step gradient as previously described (Wessling-Resnick and Braell, 1990).

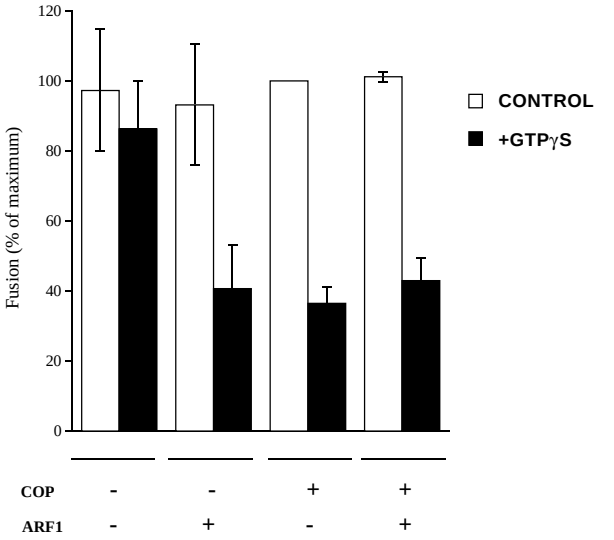

Fig. 3. ARF1 restores GTPγS inhibition to COPI depleted cytosol. COP(+) or COP(-) cytosol, with or without purified bovine ARF1 (50 ng/assay) as indicated, were incubated at 37ºC for 30 minutes with or without 100 μM GTPγS and then combined with separated membrane fractions at a final concentration of 3 mg/ml. The results are presented as percentage of maximal activity and are the means (±s.d.) determined from combined data of three independent experiments.

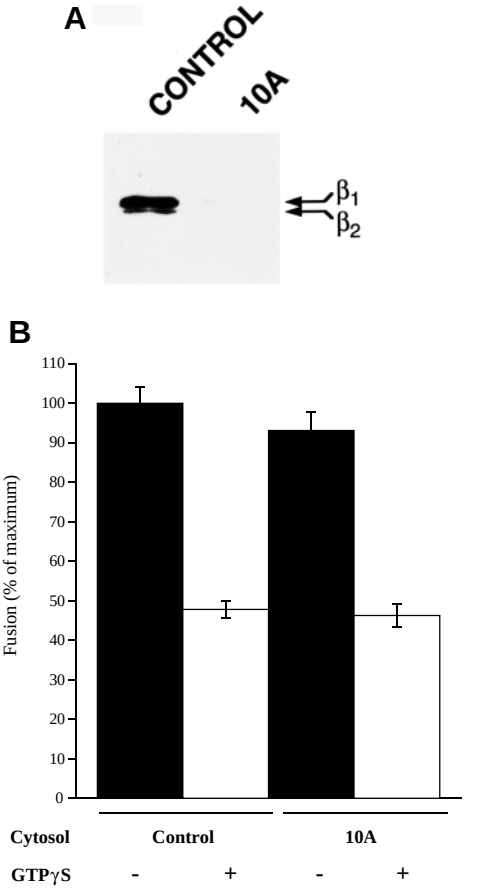

Fig. 4. GTPγS inhibition of endosome fusion persists in cytosol immunodepleted of the β subunits of AP-1 and AP-2. (A) K562 cell cytosol was incubated with Protein A-Sepharose beads covalently coupled with rabbit anti-mouse antibody and pre-incubated with either pre-immune serum (Control) or monoclonal antibody against the β1 and β2 subunits of AP-1 and AP-2 (10A). The beads were removed by centrifugation and 25 μg samples were electrophoresed on an 8% SDS-polyacrylamide gel. β subunits were identified by western blotting with a 1:2500 dilution of monoclonal 9A using ECL. (B) Membranes from PNS containing Av-βGal or B-Tf labeled endosomes were isolated by microcentrifugation and combined under standard fusion assay conditions with control or immunodepleted cytosol pre-incubated with or without 150 μM GTPγS. The final concentration of cytosol in the assay was 4.2 mg/ml. Data are from a single experiment with similar results obtained for at least two other independent immuno-depleted preparations.

of the clathrin associated complexes AP-1 and AP-2 (Stamnes and Rothman, 1993; Traub et al., 1993; Seaman et al., 1993; Dittie et al., 1996; West et al., 1997) are not involved in the mechanism of inhibition by GTPγS-bound ARF.

To confirm that AP-1 and AP-2 are not involved in endosome fusion or inhibition of this activity by GTPγS-activated ARFs, immunodepleted cytosol was prepared using monoclonal antibody 10A which recognizes both β1 and β2 subunits of the clathrin associated complexes (Clairmont et al., 1997). As shown in Fig. 4A, greater than 95% of both subunits were effectively removed. However, the immunodepleted cytosol fully supported fusion activity to control levels and, under these conditions, GTPγS inhibited endosome fusion to the same extent as control cytosol (Fig. 4B). This result is consistent with the presence of cytosolic ARFs in the immunodepleted cytosol, which was confirmed by GTP overlay blots (not shown).

Recently, a related class of adaptor complexes, AP-3, has been identified (Simpson et al., 1996, 1997; Dell' Angelica et al., 1997) and reported to be recruited to membranes by GTPγS (Simpson et al., 1996; Fáundez et al., 1998). Affinity purified antisera that recognize the δ subunit of AP-3 (a kind gift from Dr M. Robinson, University of Cambridge, UK) do not inhibit endosome fusion, suggesting that this complex is not involved (data not shown). Nonetheless, because the μ subunit of AP-3 interacts with clathrin (Dell' Angelica et al., 1998), and the other AP subunits enable clathrin association with membranes in a GTPγS-dependent manner (Dittie et al., 1996; Zhu et al., 1998), the effects of clathrin immunodepletion were further studied. Previous studies by Brodsky (1985) demonstrated the immunoprecipitation of clathrin heavy and light chains using anti-heavy chain antibody X22. As shown in Fig. 5A, X22 antisera effectively removed >90% of clathrin light chain from K562 cell cytosol. Fusion activity supported by the immunodepleted cytosol was slightly less than control (85% activity). However, endosome fusion maintained its sensitivity to GTPγS inhibition despite clathrin depletion (Fig. 5B). Thus, similar to the results shown for coatomer depletion (Fig. 2), these data argue that clathrin coats are not involved in endosome fusion or GTPγS inhibition of this activity. All of this information is compatible with the idea that GTPγS-activated ARFs do not require other cytosolic factors to mediate their effects on endosomal membranes.

Activated ARF does not disrupt endosomes

Recent reports have raised the question whether ARF can

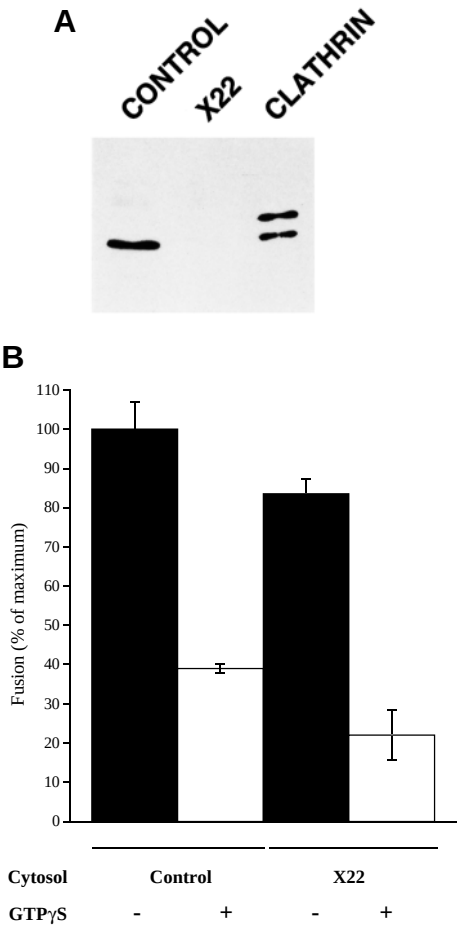

Fig. 5. GTPγS inhibition of endosome fusion persists in clathrin-depleted cytosol. (A) K562 cell cytosol was incubated with Protein A-Sepharose beads loaded with pre-immune serum (control) or monoclonal antibody against clathrin heavy chain (X22). After removing the beads by centrifugation, 150 μg samples were electrophoresed on an 11% SDS-polyacrylamide gel. Bovine brain clathrin light chains (LCa and LCb isoforms as noted by Brodsky, 1985) were identified by western blotting using a 1:175 dilution of monoclonal antibody CONI as described in Materials and Methods. (B) Membranes from PNS containing vesicles loaded with Av-βGal or B-Tf were isolated by microcentrifugation and combined with control or immunodepleted cytosol that had been pre-incubated with or without 150 μM GTPγS. The final concentration of cytosol in the assay was 6.3 mg/ml. Results are from a single experiment and represent data obtained from several independent immuno-depleted preparations.

promote organellar lysis (Sai et al., 1998; Arai et al., 1998). To rule out the possibility that ARF effects are due to non-specific disintegration or vesiculation of endosomes, we investigated the potential redistribution of internalized ^{125}I-Tf and Av-βGal in membrane fractions incubated with or without GTPγS treated cytosol. PNS fractions containing early endosomes labeled with ^{125}I-Tf or Av-βGal were incubated under fusion reaction conditions in the presence of untreated or GTPγS-treated cytosol. The distribution of the endosomal markers in low speed pellets (LSP), high speed pellets (HSP) and high speed supernatants (HSS) was monitored (Fig. 6). Greater than 95% of ^{125}I-Tf (A and B) and Av-βGal (C) was contained in LSP and HSP combined. A small amount of temperature-dependent transfer of both ^{125}I-Tf and Av-βGal from LSP to HSP was observed but there was no change in the minor amount of label present in HSS. Importantly, the distribution of label in all fractions was not significantly affected by GTPγS. The fact that ARF-GTPγS does not release ^{125}I-Tf or Av-β-Gal into HSS or significantly increase their distribution into HSP supports the idea that the inhibition of fusion does not result from endosomal lysis or fragmentation of these membranes.

DISCUSSION

Previous studies have shown that ARF is not essential for early endosome fusion but that GTPγS inhibition of fusion is entirely dependent on a cytosolic pool of these GTP-binding proteins (Spiro et al., 1995). Similar observations have been demonstrated for Golgi transport assays (Taylor et al., 1994; Elazar et al., 1994; Happe and Weidman, 1998). Since vesicle biogenesis and budding are dependent on the recruitment of coat complexes via activation of ARF, irreversible sequestration of these coat proteins onto endosomes by ARF-GTPγS could conceivably block downstream events to inhibit endosome-endosome fusion. Therefore, the potential role of coat proteins in the mechanism of ARF-mediated inhibition was investigated.

COPI components have been localized on endosomes (Whitney et al., 1995; Aniento et al., 1996) and CHO cells with a temperature-sensitive mutation in εCOP display pleiotropic defects in endocytosis (Guo et al., 1994; Darro et al., 1997). Moreover, the vacuolar proton ATPase inhibitor bafilomycin A1 has been shown to inhibit recruitment of βCOP to endosomes (Aniento et al., 1996), ARF binding to microsomes (Zuezem et al., 1992) and the formation of endosomal carrier vesicles that mediate transport from early to late endosomes (Clague et al., 1994; Aniento et al., 1996). Contradictory effects of bafilomycin A1 on cell free endosome fusion have been reported since studies have found that the drug either inhibits (Hammond et al., 1998) or has no effect (Clague et al., 1994; Pless and Wellner, 1996). Our results confirm the latter findings and are generally consistent with effects of this drug which appears to act on the endocytic recycling pathway (Johnson et al., 1993; van Weert et al., 1995; Presley et al., 1997). Our findings further reveal that endosome fusion remains sensitive to GTPγS inhibition in the presence of bafilomycin A1, indicating that inhibition by activated ARF is independent of luminal pH of the endosome and COPI recruitment. This idea is further supported by the more direct demonstration that COPI-depleted cytosol supports fusion activity and, when reconstituted with soluble purified ARF1, supports inhibition of endosome fusion by GTPγS.

Clathrin coats also have been localized to endosomes (Stoorvogel et al., 1996; Futter et al., 1998) although a functional role for these structures has yet to be identified. Immunodepletion of clathrin and adaptor proteins AP-1 and AP-2 did not affect endosome fusion activity or its inhibition by GTPγS. A suggested role for the clathrin adaptor proteins in endosome formation and fusion has been proposed by Beck et al. (1992) to explain AP-2 promoted aggregation of stripped clathrin coated vesicles and reconstituted liposomes. This effect, however, is specific for the AP-2 α subunit and is not

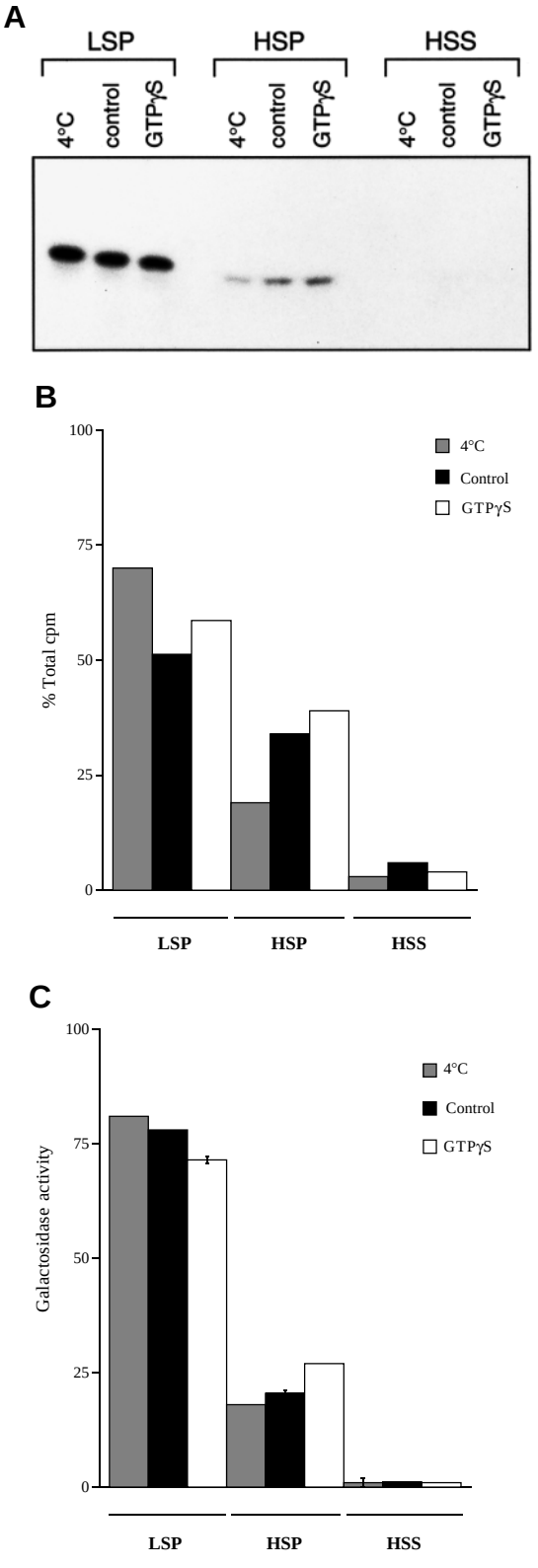

Fig. 6. Activated ARF does not cause endosomal lysis. (A) K562 cells were incubated for 1 hour at 20°C with 0.4 μM ^{125}I-Tf to label early endosomes. The cells were lysed and PNS fractions were incubated at 4° or 37°C for 30 minutes with control or GTPγS-activated cytosol prepared as described in Materials and Methods. Samples were then centrifuged at 16, 000 ***g*** to generate a low speed pellet (LSP) and supernatants were further centrifuged at 380,000 ***g*** for 30 minutes to generate a high speed pellet (HSP) and supernatant (HSS). LSP and HSP were solubilized in Laemmli buffer and entire fractions were loaded on 10% SDS-polyacrylamide gels together with 20% of the HSS fraction (215 μg). (B) ^{125}I-Tf present in the isolated fractions from the experiment shown in A was measured by gamma counting and normalized to total internalized cpm at the beginning of the experiment. Data are representative of results obtained on two different occasions. (C) PNS containing Av-βGal labeled early endosomes was incubated with or without GTPγS and LSP, HSP, and HSS fractions were isolated as in A. βGal activity in each fraction was quantified using the fluorescence-based assay described in Materials and Methods. Data are the means (± s.d.) determined for single experiment (n=2); similar results were obtained in a separate experiment.

cytosol-dependent or GTPγ-sensitive. Since aggregation rather than vesicle fusion was demonstrated in the latter study, the idea that aggregation is somehow inhibitory to fusion could not be ruled out. However, our results clearly demonstrate that neither AP-1 nor AP-2 are likely to be involved in endosome-endosome fusion.

While our studies demonstrate that cytosolic components of COP and clathrin coats are not required for inhibition of endosome fusion by GTPγS-bound ARFs, the possibility remains that coatomer or AP subunits pre-bound to endosomes mediate inhibition in response to ARF activation. However, ARF has been shown to promote coat formation rather than to stabilize existing complexes bound to membranes (Zhu et al., 1998). These observations argue against the idea the ARF exerts its inhibitory effects through membrane-associated coat components, but we can not exclude this possibility nor the idea that immunodepletion of a single coat component is not sufficient to relieve inhibition because multiple components (e.g. COPI, AP-1, AP-2 and AP-3) may be involved.

ARF is known to activate phospholipase D (PLD) (Liscovitch et al., 1994) and the proposal has been made that the generation of phosphatidic acid by this enzyme recruits coat proteins (Ktistakis et al., 1995). Stimulation of PLD by ARF promotes secretory vesicle budding (Chen et al., 1997; Tüscher et al., 1997), indicating that recruitment of AP-1 and clathrin may also be affected by negatively-charged lipids. Furthermore, West et al. (1997) have described the association of AP-2 with endosomal membranes in the presence of PLD. However, because exogenously-added PLD does not inhibit endosome fusion (Jones and Clague, 1996), GTPγS-ARF is unlikely to promote inhibition via its generation of phosphatidic acid. This idea is in general agreement with our findings that cell free endosome fusion is unaffected by depletion of known coat proteins.

What is the mechanism of ARF inhibition of endosome fusion? The data presented here clearly demonstrate that sequestration or recruitment of coat proteins to membranes by activated ARF is not involved. Our results distinguish ARF's effects on endosomes from its observed activity in intra-Golgi transport since inhibition of the latter by ARF is coatomer-dependent (Elazar et al., 1994). Like endosome fusion, vesicle fusion during nuclear assembly is not dependent on ARF but is inhibited by GTPγS-ARF (Boman et al., 1992; Gant and Wilson, 1997). However, unlike endosome fusion, nuclear assembly is still sensitive to GTPγS when assays are performed with ARF-depleted cytosol thus suggesting a role for a second

inhibitory GTPase. ARF is the sole inhibitory GTPase involved in endosome fusion since fusion assays are insensitive to GTPγS in the absence of ARF (Spiro et al., 1995). Moreover, our data indicate that ARF does not exert its effect by disrupting endosomes as has been reported for lysosomes (Arai et al., 1998). ARF-promoted lysosomal lysis was attributed to phospholipase participation (Sai et al., 1998); the observed differences may reflect variation in the presence and levels of phospholipases distributed between the two organelles, although the possibility remains that endosome morphology may be affected by activated ARF without accompanying physical lysis.

Helms et al. (1993) have shown that in addition to non-saturable binding to membranes, activated ARF associates with a saturable class of membrane sites. More recent data by Zhu et al. (1998) further indicate that hydrolysis of GTP by ARF is extremely rapid and occurs during a priming step for AP-1 recruitment. These findings suggest that GTPγS-bound ARF may associate with specific membrane components to arrest events of endosome fusion. Further investigation is required to identify the endosomal factors that we envision to be the target of activated ARF and how they may regulate the fusion process.

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