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Intravesicular acidification correlates with binding of ADP-ribosylation factor to microsomal membranes

(low molecular weight GTP-binding protein/vacuolar-type H^+-ATPase/bafilomycin)

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ABSTRACT **The ADP-ribosylation factor (ARF), a highly conserved low molecular weight GTP-binding protein, has been implicated to function in intracellular protein transport to and within the Golgi complex. In pancreatic acinar cells the ARF is confined to the cytoplasmic faces of trans-Golgi stack membranes, a compartment known to maintain a low intravesicular pH, which is established by a chloride-dependent MgATP-driven proton pump. The present study shows that MgATP (2 mM), but neither adenosine 5′-[γ-thio]triphosphate in the presence of Mg^{2+} nor ATP in the absence of Mg^{2+}, increases transfer of ARF from the surrounding medium into the vesicle membranes. The specific vacuolar-type proton pump inhibitor bafilomycin B_1 (10 nM), the protonophore carbonylcyanide *m*-chlorophenylhydrazone (10 μM), and replacement of chloride in the incubation buffer by acetate or nitrate resulted in an almost complete inhibition of the MgATP-dependent association of ARF to the vesicle membranes. The results demonstrate that redistribution of ARF to the vesicle membrane correlates with the intravesicular pH established by a vacuolar-type H^+-ATPase. The intravesicular pH appears to be one mechanism by which certain low molecular weight GTP-binding proteins become relocated from the cytosol to their specific membrane vesicles.**

Evidence suggests a role for certain low molecular weight GTP-binding proteins in intracellular membrane trafficking and vesicle fusion (1–3). According to a model proposed by Bourne (4) these GTP-binding proteins in an active, GTP-bound state direct vesicles to specific target organelles. GTP hydrolysis would be required to trigger a conformational change of the protein, and the inactive, GDP-bound form of the molecule would then return to the cytosol to target another vesicle in a cyclic process. However, the physiological mechanism(s) by which low molecular weight GTP-binding proteins become relocated from the cytosol to the specific vesicles is unknown. The ADP-ribosylation factor (ARF), a highly conserved low molecular weight GTP-binding protein, has been located in the Golgi complex and implicated to function in intracellular protein transport from the endoplasmic reticulum to and within the Golgi complex (5, 6). A monoclonal anti-ARF antibody has been used to detect the ARF protein in a vesicle preparation of rat pancreatic acinar cells containing membranes of the endoplasmic reticulum and the Golgi complex (7, 8). By immunoelectron microscopy with the same antibody, ARF staining in pancreatic acinar cells was shown to be confined to the cytoplasmic faces of trans-Golgi stack membranes. These trans compartments of the Golgi complex have been shown to maintain a low intravesicular pH, which is established by a MgATP-driven vacuolar-type proton pump (9–11). This vacuolar-type H^+-ATPase is present in membrane vesicles from the pancreatic acinar cell preparation (8). Since both ARF (5) and the vacuolar-type H^+-ATPase (11) have been suggested to play a key role in intravesicular membrane traffic, we have investigated a possible link between H^+ uptake and ARF membrane binding. Our data show that intravesicular acidification due to MgATP-driven H^+ uptake by the vacuolar-type H^+ pump into pancreatic membrane vesicles leads to a transfer of ARF from the surrounding medium into the vesicular membranes.

MATERIALS AND METHODS

Materials. Bafilomycin B_1 was a gift from Bayer AG (Wuppertal, F.R.G.). Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was obtained from Boehringer Mannheim. Nigericin was from Calbiochem and acridine orange from Merck.

Preparation and Incubation of Pancreatic Microsomal Vesicles. Pancreatic microsomal vesicles were prepared from rats (200–240 g) that had fasted overnight. Acinar cells were isolated by collagenase digestion and homogenized in 280 mM mannitol/5 mM Hepes/10 mM KCl/1 mM $MgCl_2$/1 mM benzamidine/1 μM leupeptin/0.2 mM phenylmethanesulfonyl fluoride with trypsin inhibitor (20 μg/ml), adjusted to pH 7.0 with Tris. A membrane fraction enriched in endoplasmic reticulum and Golgi complex was prepared as described (7, 8). Freshly prepared microsomal vesicles were first kept on ice for 40 min to allow dissociation of ARF protein from the vesicle membrane into the medium. Subsequently, the vesicle suspension was incubated at 24°C (final protein concentration, 1 mg/ml) in 155 mM KCl/20 mM Hepes/0.2 mM EDTA/0.01 mM oligomycin/1 mM benzamidine/1 μM leupeptin/0.2 mM phenylmethanesulfonyl fluoride/trypsin inhibitor (20 μg/ml), pH 7.0 (Tris), containing 1 mM free Mg^{2+} and 0.002 mM free Ca^{2+} ("incubation buffer") in the presence or absence of 2 mM MgATP. The incubation was terminated by centrifugation at 200,000 × *g* and 4°C. Proteins in the supernatant were precipitated by methanol/chloroform/water, 4:1:3 (vol/vol).

Postembedding Immunogold Staining of Pancreatic Acinar Cells with Anti-ARF Antibody. Rat pancreatic tissue was fixed by immersion in 1% glutardialdehyde in phosphate-buffered saline (PBS), dehydrated, and embedded in LR white resin (Plano; Marburg, F.R.G.). Thin sections on Formvar-coated nickel grids were treated first with sodium metaperiodate, then with 2% glycine in PBS, and afterwards

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Abbreviations: ARF, ADP-ribosylation factor; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; ATP[γS], adenosine 5′-[γ-thio]triphosphate.

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with PBS containing 1% bovine serum albumin, 0.2% Tween 20, and 0.1% Triton X-100. Incubation with the monoclonal anti-ARF antibody 1D9 (which was raised in mouse cells against the complete recombinant ARF protein) was performed overnight at 4°C and repeated with a fresh solution for 1 hr at room temperature. Antibody binding sites were visualized with a second antibody coupled to gold particles. Sections were viewed in a Philips EM 300 electron microscope. Specificity was controlled by incubating the sections without the anti-ARF antibody but with label-coupled secondary antibodies and by applying nonimmune serum instead of the primary antibody.

SDS/PAGE and Immunoblotting. Proteins were subsequently resolved by SDS/12.5% polyacrylamide gel electrophoresis (12) and then electrophoretically transferred to nitrocellulose paper (13, 14). Transferred proteins were revealed with Ponceau S. Incubation of the nitrocellulose blot with monoclonal anti-ARF antibody 1D9 (1 μg/ml) was followed by a second (goat anti-mouse) and third (rabbit anti-goat) antibody and finally ^{125}I-labeled protein A. Bound radioactivity was detected by autoradiography and films were subsequently scanned by laser densitometry.

Measurements of H^+-Gradient Formation. The pH-sensitive dye acridine orange was used to visualize the formation and dissipation of the H^+ gradient. H^+ uptake was measured as described (8). The difference between the absorbance at 493 and that at 540 nm was recorded in an Aminco DW2 UV/visible spectrophotometer. Microsomal vesicles were preincubated in the absence or presence of the indicated substances. When the absorbance signal had reached a stable baseline, H^+ transport was initiated by addition of 2 mM MgATP or other adenine nucleotides as indicated. After a steady-state H^+ gradient had been obtained, the protonophore nigericin (1 μM) was added to dissipate the H^+ gradient over the vesicle membrane.

RESULTS AND DISCUSSION

Postembedding immunogold staining of rat pancreatic acinar cells with a monoclonal anti-ARF antibody showed that ARF binding was confined to the cytoplasmic faces of trans-Golgi membranes (Fig. 1). These trans compartments of the Golgi complex have been shown to maintain a low intravesicular pH, which is established by a vacuolar-type H^+-ATPase (9–11). ARF and the intravesicular acidification of Golgi-derived vesicles have been suggested to be involved in vesicular transport and protein sorting (5, 11). In the following experiments we investigated the possible link between H^+ uptake and ARF membrane binding.

Freshly prepared microsomal vesicles were first kept on ice in the homogenization buffer (7) to allow dissociation of ARF protein from the vesicle membrane into the medium. After 40 min 68 ± 4% of the ARF was found in the medium, while 32 ± 4% (n = 5) remained membrane-associated. The vesicle suspension was transferred into a 155 mM KCl buffer (15) (final protein concentration, 1 mg/ml) and incubated at 24°C in the absence or presence of MgATP (2 mM). In the presence of MgATP, association of ARF with the membrane had increased by 105 ± 5% after 30 min (n = 5), while in the supernatant the soluble form of ARF decreased at the same time (Fig. 2). Sixty ± 4% of ARF found in the supernatant following dissociation at 4°C re-bound to the vesicle membrane when incubated at 24°C in the presence of MgATP (2 mM). Recovery of immunoreactive ARF in the supernatant and the pellet at different times of incubation was >90%. Immediate incubation of freshly prepared microsomal vesicles in the incubation buffer in the presence of MgATP (2 mM) prevented ARF dissociation from the vesicles (data not shown).

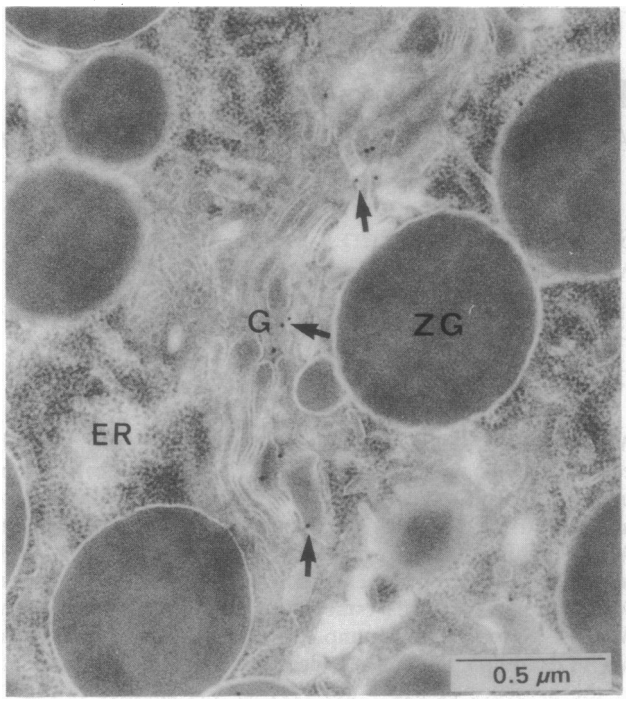


FIG. 1. Postembedding immunogold staining of rat pancreatic acinar cells with a monoclonal anti-ARF antibody, indicating that ARF is confined to the cytoplasmic faces of trans-Golgi membranes (see arrows). Rat pancreatic tissue was fixed, dehydrated, and embedded (see *Materials and Methods*). Incubation with the monoclonal anti-ARF antibody (1D9) was performed overnight in the cold and repeated with a fresh solution for 1 hr at room temperature. Antibody binding sites were visualized with a second antibody coupled to gold particles. In control experiments using only secondary gold-coupled antibodies or gold-coupled antibodies in the presence of nonimmune serum (1:100), no significant labelling to subcellular structures was observed. ER, endoplasmic reticulum; G, Golgi complex; ZG, zymogen granule.

The pH-sensitive dye acridine orange (8) was used to measure the activity of the vacuolar-type H^+-ATPase in the presence of various nucleotides. When MgATP was added to vesicles, H^+ uptake took place and reached a steady state after 30 min. The H^+ gradient was dissipated by addition of the electroneutral K^+/H^+ ionophore nigericin (Fig. 3 *Upper*, trace 2). H^+ uptake did not occur when ATP was added in the absence of Mg^{2+} or when MgATP had been exchanged for MgATP[γS] or for MgADP (Fig. 3 *Upper*, traces 3–5). The amount of membrane-associated ARF increased by about 100% in the presence of MgATP (2 mM) (Fig. 2 *Lower*; Fig. 3 *Lower*, lane 2 compared with lane 1). MgATP[γS], which can be utilized by protein kinases but not by ATPases, had no effect on ARF relocation from the medium to the membranes (Fig. 3 *Lower*, lane 4). Also, ATP in the absence of free Mg^{2+} or MgADP had no effect on the redistribution of ARF (Fig. 3 *Lower*, lanes 3 and 5). Subfractionation of the vesicle preparation by Percoll gradient centrifugation (8) revealed that H^+-pump activity cofractionated with the ARF protein (data not shown).

To investigate whether this MgATP-dependent ARF redistribution was indeed due to the activity of a MgATP-driven vacuolar-type H^+ pump, we incubated vesicles in the absence or presence of MgATP with bafilomycin B_1, a potent and highly specific inhibitor of vacuolar-type H^+-ATPases (15, 16) or with the electrogenic protonophore CCCP. In the presence of bafilomycin B_1 (10 nM) or CCCP (10 μM), the MgATP-dependent H^+ uptake was abolished (Fig. 4 *Upper*, traces 3 and 4, respectively) and the MgATP-induced relocation of ARF from the medium to the membrane was almost

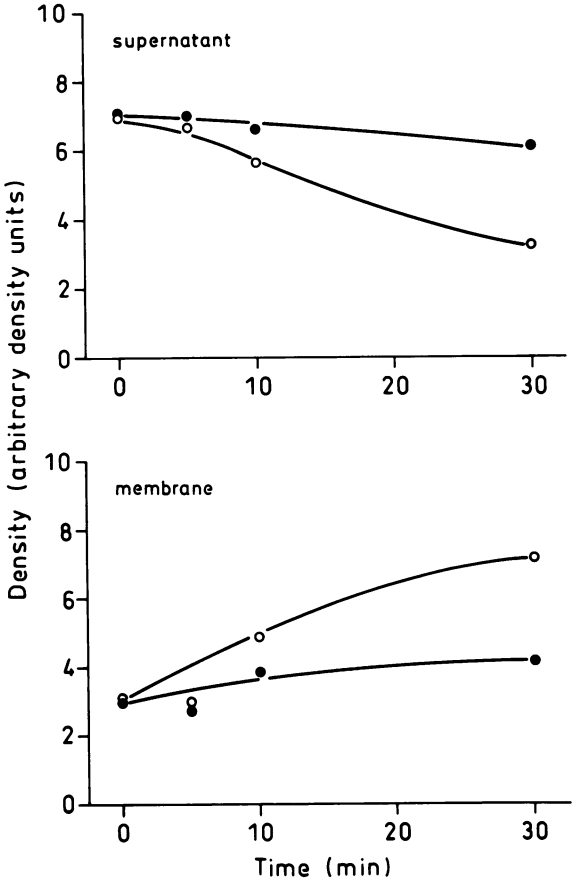


FIG. 2. Time-dependent MgATP effect on vesicle membrane binding of ARF. Pancreatic microsomal vesicles were incubated for 0, 5, 10 or 30 min at 24°C with (○) or without (●) MgATP (2 mM). The incubation was stopped by centrifugation for 15 min at 200,000 × *g* at 4°C. ARF protein was detected by immunoblotting techniques in the supernatant (*Upper*) and in the membrane pellet (*Lower*). Immunoblot bands were quantified by densitometric scanning of autoradiograms. In the presence of MgATP the amount of membrane-associated ARF increased with time while ARF decreased in the supernatant. The experiment shown is representative of five separate experiments.

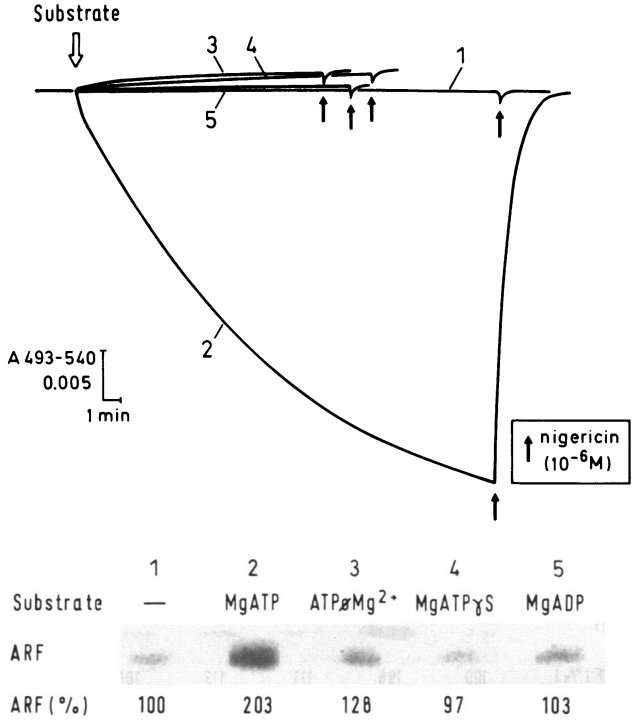


FIG. 3. Effect of MgATP, ATP in the absence of Mg^{2+}, Mg with adenosine 5'-[γ-thio]triphosphate (MgATP[γS]), and MgADP on H^+ uptake and on binding of the ARF protein in pancreatic membrane vesicles. (*Upper*) The pH-sensitive dye acridine orange was used to visualize the formation and dissipation of the H^+ gradient (8). Vesicles were preincubated with acridine orange (6 μM) at 24°C for 15 min in the 155 mM KCl incubation buffer (see *Materials and Methods*). The reaction was started by addition of 2 mM MgATP (trace 2), ATP without Mg^{2+} (buffer with 1 mM EDTA, 0.002 mM free Ca^{2+}, and <1 nM free Mg^{2+}) (trace 3), MgATP[γS] (trace 4), or MgADP (trace 5). The generated H^+ gradient was dissipated by nigericin (1 μM) as indicated. (*Lower*) Effect of various adenine nucleotides on binding of ARF protein to pancreatic microsomes was monitored by immunoblotting using a monoclonal anti-ARF antibody and ^{125}I-protein A. Microsomal vesicles (1 mg of protein per ml) were incubated for 30 min at 24°C without addition (lane 1) or with 2 mM MgATP (lane 2), with 2 mM ATP in the absence of Mg^{2+} (lane 3), with 2 mM MgATP[γS] (lane 4), or with MgADP (lane 5). Membrane-associated proteins were resolved by SDS/PAGE and transferred to nitrocellulose sheets. ARF protein was detected by immunoblotting, immunoreactive bands were scanned by laser densitometry, and the densities are expressed as percent of control. The experiment shown is representative of seven separate experiments.

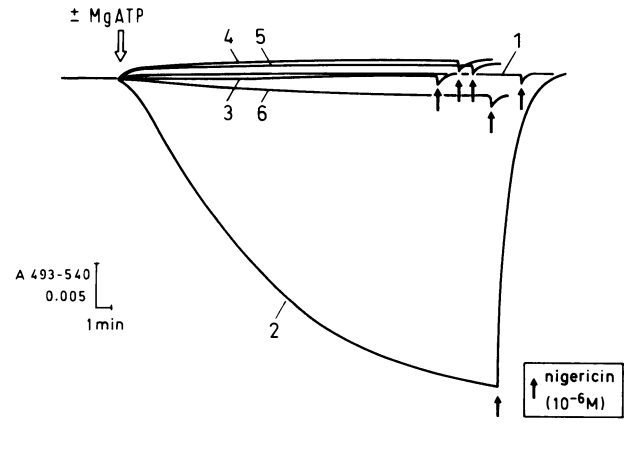
completely inhibited (Fig. 4 *Lower*, lanes 3 and 4, respectively). Parallel incubation in the absence of MgATP revealed that bafilomycin B_1 and CCCP themselves had no effect on the ARF protein redistribution (data not shown).

A feature common to the H^+ transport by all vacuolar-type H^+-ATPases is the requirement for a permeant anion, typically Cl^- (11). No H^+-uptake was observed when Cl^- was isoosmotically replaced by NO_3^- or CH_3COO^- in the incubation buffer (Fig. 4 *Upper*, traces 5 and 6). Correspondingly, MgATP did not affect ARF redistribution under these conditions (Fig. 4 *Lower*, lanes 5 and 6). These results strongly support the involvement of MgATP-dependent acidification due to the vacuolar-type H^+ pump in the regulation of vesicle membrane binding of the ARF protein.

We then addressed the question whether the actual intravesicular pH or the H^+ gradient over the vesicle membrane was crucial for relocation of ARF from the medium to the membrane. We therefore incubated the membrane vesicles for 30 min at 24°C in the presence of the K^+/H^+ exchanger nigericin (5 μM) to make sure that the intravesicular pH was completely clamped to the pH of the extravesicular medium (ranging from pH 4.5 to 7.0). An increase in the H^+ concentration facilitated relocation of the ARF protein to the vesicular membrane (Fig. 5). Nigericin by itself did not influence binding of ARF to the membrane. Since the vesicle membrane was not completely impermeant to H^+, the effect of a neutral vesicle inside pH and varying acidic outside pH values on ARF binding could not be tested.

The data shown in Fig. 5 together with those shown in Figs. 2–4 indicate that ARF redistribution is dependent on the intravesicular pH and not the H^+ gradient over the membrane. Use of "zero-point titration" techniques (8) has shown that the vacuolar-type H^+ pump establishes an intravesicular pH of about 5.0. This is well in accordance with the data of this study showing that in the presence of MgATP at an outside pH of 7.0, ARF redistribution (open circle in Fig. 5) correlated with a clamped intravesicular pH of about 5.5.

Using the same monoclonal antibody (1D9) raised against recombinant mammalian ARF protein, Serafini *et al.* (17) detected two ARF bands in vesicular stomatitis virus-infected CHO 15B cells (17). These two ARF bands were shown to be distinct proteins, even if they were immunologically very similar, since carbonate extraction released most of the faster migrating but not the more slowly migrating ARF species into a soluble form. Serafini *et al.* (17) speculated that this might reflect differences in the amino-terminal myris-



	1	2	3	4	5	6
MgATP	-	+	+	+	+	+
Anion	Cl^-	Cl^-	Cl^-	Cl^-	NO_3^-	CH_3COO^-
Bafilomycin B_1	-	-	+	-	-	-
CCCP	-	-	-	+	-	-
ARF						
ARF (%)	100	198	111	113	106	102

FIG. 4. Effect of the specific vacuolar-type H^+-ATPase inhibitor bafilomycin B_1, the protonophore CCCP, and various anions on MgATP-driven H^+-pump activity and on binding of the ARF-protein to pancreatic membrane vesicles. (*Upper*) After preincubation of membrane vesicles with the highly specific vacuolar-type H^+-ATPase inhibitor bafilomycin B_1 (10 nM) or with the electrogenic protonophore CCCP (10 μM), ATP-driven H^+ accumulation in pancreatic microsomal vesicles was abolished (traces 1–4). Furthermore, no MgATP-driven H^+ transport was observed in vesicles when Cl^- in the incubation buffer was replaced by NO_3^- or CH_3COO^- (traces 5 and 6). (*Lower*) Effect of bafilomycin B_1, CCCP, and various anions on ARF protein binding to microsomal vesicles. ARF was detected by immunoblotting with mouse monoclonal anti-ARF antibody. Immunoreactive bands were scanned by laser densitometry, and the densities are expressed as percent of control. The experiment shown is representative of six separate experiments.

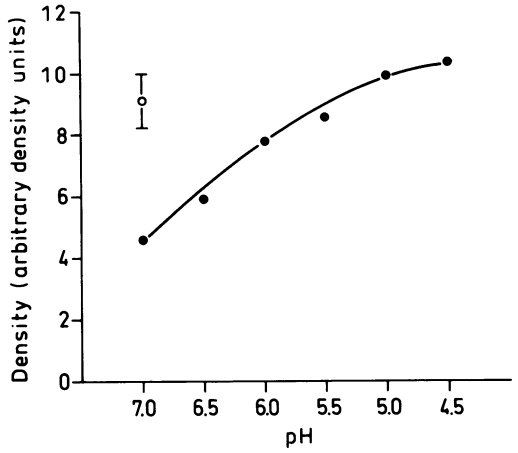


FIG. 5. Effect of pH on the binding of ARF protein to pancreatic microsomal vesicles. Microsomal vesicles prepared in 300 mM mannitol/5 mM Hepes, pH 7.0/40 mM KCl were incubated without MgATP for 30 min at 24°C in incubation buffer with 50 mM Hepes (pH 7.0 and 6.5) or 50 mM Mes (pH 6.0, 5.5, 5.0, and 4.5). Intravesicular pH was clamped to the pH of the extravesicular medium by nigericin (5 μM). The incubation was terminated by centrifugation at 4°C. Membrane-associated proteins were separated by SDS/PAGE and transferred to nitrocellulose for detection of the ARF protein by Western blot analysis. Immunoreactive bands were scanned by laser densitometry, and results (●) are expressed as percent of control (no MgATP, pH 7.0). Effect of MgATP-dependent H^+-uptake on the binding of ARF protein to the vesicle membrane at pH 7.0 is also shown (○, mean ± SD, n = 5).

toylation or some other posttranslational modification. In microsomal membranes and the cytosol of rat pancreatic acinar cells, we detected only one ARF-band with this monoclonal anti-ARF antibody. Like Serafini *et al.*, we used SDS/12.5% polyacrylamide gels for optimal resolution of the low molecular weight proteins. Thus, we obtained no evidence for the existence of different ARF proteins in the cytosol and the Golgi complex of pancreatic acinar cells. Incubation of microsomal vesicles from which ARF had been dissociated in the presence of MgATP and increasing amounts of cytosol (120–500 μg/ml) also showed an increase of ARF membrane binding with increasing amounts of cytosol (data not shown), indicating that not only membrane-dissociated but also cytosolic ARF proteins bind to the vesicle membrane.

Using an [α-^{32}P]GTP overlay technique, we recently obtained evidence that binding of a 19-kDa and a 21-kDa GTP-binding protein to vesicle membranes is regulated by the intravesicular pH (18). The data support the conclusion of the present study, which now identifies the 19-kDa protein as the ARF. From our data we cannot conclude that intravesicular acidification has a direct effect on the ARF protein. Likewise the activity of a regulatory protein of ARF in the vesicular membrane, such as a GTP/GDP exchange protein, could be influenced by the intravesicular pH. Trans-Golgi compartments are involved in vesicular protein transport and sorting mechanisms and have clearly been identified as a site of intravesicular acidification (9–11). ARF proteins, which have been shown to be involved in vesicular transport processes, may regulate the transport from an acidic donor compartment to a neutral acceptor compartment. Binding of the ARF proteins is facilitated to the compartment with a low intravesicular pH, and ARF dissociation occurs after pH neutralization, allowing the ARF protein to recycle.

ARF binding to microsomal vesicles has also been shown to be facilitated by guanosine 5′-[γ-thio]triphosphate (GTP[γS]) (19). Incubation of pancreatic microsomal vesicles with MgATP (2 mM), GTP[γS] (10 M), and MgATP plus GTP[γS] showed that the effects of MgATP and GTP[γS] were additive (data not shown).

Low molecular weight GTP-binding proteins have been suggested to target vesicles between different compartments. All cellular compartments containing vacuolar-type H^+ pumps are involved in membrane traffic pathways (9–11). In this study we have shown that redistribution of ARF to the vesicle membrane correlates with the intravesicular pH established by a vacuolar-type H^+-ATPase. The intravesicular pH might be one mechanism by which certain low molecular weight GTP-binding proteins become relocated from the cytosol to their specific membrane vesicles.

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