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Association of a 19- and a 21-kDa GTP-Binding Protein to Pancreatic Microsomal Vesicles is Regulated by the Intravesicular pH Established by a Vacuolar-Type H^+-ATPase

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Summary. Evidence suggests that certain *ras*-related small molecular weight GTP-binding proteins (smg-proteins) are involved in intracellular membrane trafficking and vesicle fusion. We have previously shown that intravesicular acidification due to a vacuolar-type H^+-ATPase, which is Cl^- dependent and highly sensitive to the specific inhibitor bafilomycin, enhances GTP-induced fusion of pancreatic microsomal vesicles (Hampe, W., Zimmermann, P., Schulz, I. 1990. *FEBS Lett.* **271**:62–66). This process may involve function of smg-proteins. The present study shows that MgATP (2 mM), but neither MgATPγS nor ATP in the absence of Mg^{2+}, increases association of 19- and 21-kDa smg-proteins to the vesicle membrane as monitored by their [α-^{32}P]GTP binding. The affinity of smg-proteins for [α-^{32}P]GTP was not altered by MgATP. Bafilomycin B_1 (10^{-8} M), the protonophore CCCP (10^{-5} M), and replacement of Cl^- in the incubation buffer by CH_3COO^- or NO_3^- resulted in an almost complete inhibition of the MgATP-dependent association of the 19- and 21-kDa smg-proteins to the vesicle membranes. Furthermore, the MgATP effect on both smg-proteins was found to be due to the intravesicular pH and not to the H^+ gradient over the vesicle membrane. We conclude that association of a 19-kDa (immunologically identified as the ADP-ribosylation factor, arf) and a yet unidentified 21-kDa GTP-binding protein to vesicle membranes is regulated by the intravesicular pH established by a vacuolar-type H^+-ATPase.

Key Words *ras*-related GTP-binding proteins · ADP-ribosylation factor · proton pump · bafilomycin · GTP-binding blots

Introduction

In addition to the family of heterotrimeric GTP-binding proteins (G-proteins) involved in signal transduction from receptors in the plasma membrane to certain effectors such as the adenylcyclase system, several phospholipases and ion channels [4], a large family of monomeric GTP-binding proteins with molecular masses of 18–30 kDa has been discovered. These proteins, designated as small molecular weight GTP-binding proteins (smg-proteins), are related to the *ras* proto-oncogene products sharing 30–85% sequence homologies [3, 6]. The family of *ras*-related small molecular weight GTP-binding proteins comprises some 30 distinct mammalian proteins including the products of *ras, ral, rac, rho, ram, rap* and *rab* genes and their close homologs in yeast including the *ypt* 1 and *sec* 4 genes (for reviews *see* ref. [2, 3, 6, 14]).

Several studies strongly suggest that small molecular weight GTP-binding proteins mediate vesicle trafficking and interorganelle transfer between several compartments of the cell by a process requiring GTP hydrolysis [2, 5]. Smg-proteins have been localized to specific exocytic and endocytic subcompartments in mammalian cells [9, 10, 21]. In *Saccharomyces cerevisiae* the products of the *ypt* 1 and *sec* 4 genes are involved in the processing and secretion of newly synthesized proteins from the endoplasmic reticulum (ER) to the plasma membrane [13, 23, 25]. In several cell-free transport systems the nonhydrolyzable GTP analog GTPγS has been shown to have striking inhibitory effects on intracellular transport, thus supporting a role of smg-proteins in the secretion pathway (reviewed in ref. [1]). Furthermore, GTP has been demonstrated to induce Ca^{2+} translocation between distinct intracellular Ca^{2+} pools, which may reflect function of smg-proteins in mediating interorganelle transfer [12].

Using light-scatter and fluorescence-dequenching techniques we recently demonstrated that in the presence of PEG intravesicular acidification enhances GTP-induced fusion of pancreatic microsomes [15].

The present study shows that association of a 19- and a 21-kDa GTP-binding protein to microsomal vesicle membranes is regulated by intravesicular acidification established by a MgATP-dependent vacuolar-type H^+ pump. The data give a possible explanation for the mechanism of MgATP-dependent GTP-induced vesicle fusion [15] and suggest that one or both 19- and 21-kDa smg-proteins might be involved in this process.

Materials and Methods

Materials

[α-^{32}P]GTP (3000 Ci/mmol) and anti-*ras* p21 antibodies were from New England Nuclear DuPont (Dreieich, FRG). All nucleotides, NBD-Cl, PMSF, Ponceau S and SDS-7 protein standards were obtained from Sigma (München, FRG). Bafilomycin B_1 was a gift from Bayer AG (Wuppertal, FRG). CCCP and trypsin inhibitor were obtained from Boehringer (Mannheim, FRG). Collagenase Type III, 132 U/mg from *Clostridium histolyticum* was from Worthington (Freehold, NJ). Nigericin was from Calbiochem (Frankfurt, FRG), acridine orange from Merck (Darmstadt, FRG) and Percoll (23%, density 1.129 g/ml) from Pharmacia (Freiburg, FRG). Benzamidine, leupeptin, NEM and oligomycin were purchased from Serva (Heidelberg, FRG). Nitrocellulose (0, 2 μm) was from Schleicher and Schüll (Dassel, FRG).

Preparation of Pancreatic Microsomal Vesicles

Pancreatic vesicles enriched in endoplasmic reticulum were prepared from rats (200–250 g), which had been fasted overnight. Acinar cells were isolated by collagenase digestion and homogenized in a buffer containing (in mmol/liter): mannitol 280, HEPES 5, KCl 10, $MgCl_2$ 1, benzamidine 1, leupeptin 0.001, PMSF 0.2, trypsin inhibitor 20 μg/ml adjusted to pH 7.0 with Tris. The homogenate was centrifuged at 1,000 × *g* for 12 min, and the supernatant subsequently centrifuged at 11,000 × *g* for 15 min at 4°C in a Beckman JA 20 rotor. The fluffy layer on top of the 11,000 × *g* pellet, which contains membranes of the endoplasmic reticulum and the Golgi complex [29], was carefully removed and resuspended in the same buffer at a concentration of 30 mg protein/ml. Protein concentration was measured according to Bradford [8] using bovine serum albumin as standard.

Fractionation of Microsomal Vesicles by Percoll Gradient Centrifugation

Pancreatic microsomal vesicles were prepared as described and subsequently fractionated by centrifugation on a Percoll gradient [30]. For this purpose 1 ml of the microsomal vesicle suspension was layered on top of 9 ml of a solution containing 11% (wt/vol) Percoll and (in mmol/liter) mannitol 280, HEPES 5, KCl 10, $MgCl_2$ 1, benzamidine 1, leupeptin 0.001, PMSF 0.2, trypsin inhibitor 20 μg/ml, pH 7.0 with HCl. The resulting density was 1.035 g/cm^{-3}. A gradient of densities from 1.020 to 1.141 g/cm^3 was generated by spinning the tubes at 41,000 × *g* for 40 min at 4°C in a Beckman Ti 60 rotor. Five 2-ml fractions (P_1 to P_5) were collected from the top to the bottom of the gradient. To separate vesicles from the Percoll, the fractions collected were spun down at 200,000 × *g* for 60 min in a Beckman Ti 60 rotor. The vesicle fractions were subsequently resuspended at a concentration of 20 mg/ml.

Incubation of Microsomal Vesicles

Freshly prepared microsomal vesicles (1 mg protein/ml) were incubated at 24°C for 30 min in a buffer containing (in mmol/liter): KCl 155, HEPES 20, $MgCl_2$ 3, $CaCl_2$ 0.038, EDTA 0.2, oligomycin 0.01, benzamidine 1, leupeptin 0.001, PMSF 0.2, trypsin inhibitor 20 μg/ml, pH 7.0 (Tris) unless stated otherwise. Adenosine nucleotides were added from a 200-mM stock solution adjusted to pH 7.0 (Tris) to a final concentration of 2 mM. The incubation was terminated by centrifugation at 200,000 × *g* and 4°C. The pellet was resuspended in a mannitol buffer (pH 7.0); proteins of the supernatant were precipitated with 800 μl of methanol/chloroform/water (4 : 1 : 3, vol/vol).

Solubilization of Microsomal Proteins

Microsomal vesicles were suspended in a mannitol buffer (pH 7.0) containing 1% Triton X-100. After incubation on ice for 60 min, the sample was centrifuged at 200,000 × *g* for 60 min at 4°C in a SW 50 Beckman rotor. Protein concentration was measured as described. The solubilized proteins were incubated for 30 min at a concentration of 1 mg/ml with various reagents as indicated. The final Triton X-100 concentration was below 0.05%. After incubation the proteins were precipitated as described.

SDS/PAGE and Blotting of Proteins

Microsomal proteins were resolved by SDS/polyacrylamide gel electrophoresis (12.5% gels) [17] followed by an electrophoretical transfer to nitrocellulose paper (0.2 μm) using a Bio-Rad Transblot cell [24, 31]. For apparent molecular mass determination protein standards were electrophoresed in parallel. Transferred proteins were revealed with Ponceau S.

[α-^{32}P]GTP Binding Assay

The nitrocellulose blots were incubated in a buffer containing 50 mM Tris/HCl, pH 7.5, 1 mM EGTA, 5 mM $MgCl_2$, 0.3% Tween 20 and 1 μCi of [α-^{32}P]GTP/ml [19]. To decrease nonspecific binding the incubation buffer was supplemented with 1 mM K_2HPO_4/KH_2PO_4, pH 7.5. After incubation for 60 min at 24°C, the nitrocellulose sheets were washed for 45 min with multiple (5–7) washes. The blots were air dried, and bound radioactivity was detected by autoradiography (24–96 hr at −70°C) using Kodak X-OMAT AR films and intensifying screens. The autoradiographs were scanned by a computerized laserdensitometer (Ultrascan LKB). The area of the bands is expressed in arbitrary density units.

Measurements of the Proton-Gradient-Formation

The pH-sensitive dye acridine orange was used to visualize the formation and dissipation of the H^+ gradient. Measurement of H^+ uptake was performed as described previously [30]. The difference in absorbance at 493–540 nm was recorded in an Aminco DW2 UV/vis spectrophotometer (Silverspring, MD). 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. After a steady-state H^+ gradient had been obtained, the protonophore nigericin (10^{-6} M) was added to dissipate the H^+ gradient over the vesicle membrane.

Statistical Analysis

Results are presented as mean ± SD. Statistical analysis was performed using Student's paired *t*-test.

Abbreviations

smg-protein: small molecular weight GTP-binding protein
arf: ADP-ribosylation factor
IP_3: inositol-1,4,5-trisphosphate,
HEPES: N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid
MES: morpholinoethane sulfonic acid
EDTA: ethylenediaminetetraacetic acid
EGTA: ethyleneglycol-bis-(β-aminoethylether)-N,N,N',N'-tetraacetic acid
PMSF: phenylmethylsulfonylfluoride
ATPγS: adenosine-5'-O-(3-thiotriphosphate)
GTPγS: guanosine-5'-O-(3-thiotriphosphate)
CCCP: carbonylcyanide-*m*-chlorophenylhydrazone
NBD-Cl: 7-chloro-4-nitrobenz-2-oxa-1,3-diazole
NEM: N-ethylmaleimide
SDS/PAGE: sodium dodecyl sulfate/polyacrylamide gel electrophoresis

Results

Effect of Adenosine Nucleotides on [α-^{32}P]GTP Binding to Small Molecular Weight GTP-Binding Proteins

As shown in Fig. 1 several smg-proteins with molecular masses between 18 and 27 kDa were detected by the [α-^{32}P]GTP binding assay in microsomal membrane vesicles of rat pancreatic acinar cells. In the presence of MgATP (2 mM) [α-^{32}P]GTP binding of a 19- and a 21-kDa smg-protein was increased by 84 ± 12% (n = 5) and by 94 ± 17% (n = 5), respectively (Fig. 1, lanes *1a* and *2a* and lanes *1b* and *2b*, respectively, and Fig. 10). No effect was observed during incubation with either MgADP (2 mM) or the nonhydrolyzable ATP analog MgATPγS (2mM) (Fig. 1, lanes *4* and *5*). Furthermore, no ATP effect was found when vesicles were incubated in a nominally Mg^{2+}-free buffer (Fig. 1, lane *3*). Incubation of microsomal proteins, which where solubilized with Triton X-100 as described in Materials and Methods, with MgATP or other adenosine nucleotides did not result in any change of the [α-^{32}P]GTP binding to smg-proteins (*data not shown*).

Effect of Bafilomycin B_1 and of CCCP on [α-^{32}P]GTP Binding to smg-Proteins and on the Microsomal H^+ATPase

Bafilomycins are extremely potent inhibitors of vacuolar-type H^+ ATPases [7]. Complete inhibition of vacuolar proton ATPases by bafilomycin occurs at

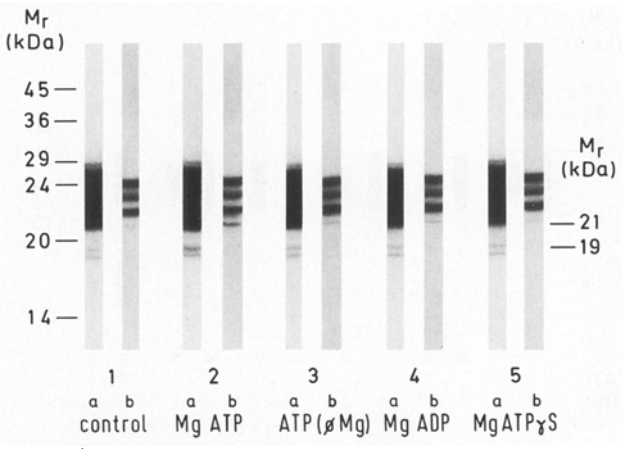

Fig. 1. Effect of different adenosine nucleotides on [α-^{32}P]GTP binding to low molecular weight GTP-binding proteins in pancreatic microsomes. Microsomal vesicles (1 mg protein/ml) were incubated for 30 min at 24°C without (lanes *1a* and *1b*) and with 2 mM MgATP (lanes *2a* and *2b*), with 2 mM ATP in a Mg^{2+}-free buffer (lanes *3a* and *3b*), with 2 mM MgADP (lanes *4a* and *4b*) or with MgATPγS (lanes *5a* and *5b*). Proteins were resolved by SDS/PAGE, transferred to nitrocellulose sheets, which were subsequently incubated with [α-^{32}P]GTP. Bound radioactivity was detected by autoradiography. Films were exposed for 72 (*a*) and 24 hr (*b*) to improve discrimination of different smg-proteins. The experiment shown is representative of five similar experiments.

concentrations between 10^{-9} and 10^{-8} M [7, 15]. Since we found that GTP-induced fusion of pancreatic microsomal vesicles was increased by intravesicular acidification and that vesicular H^+ uptake increased GTP-induced connection of different Ca^{2+} pools, we have investigated the effect of bafilomycin B_1 on the MgATP enhanced [α-^{32}P]GTP binding to smg-proteins. As shown in Fig. 2 bafilomycin B_1 (10^{-8} M) almost completely inhibited the MgATP-induced enhancement of the [α-^{32}P]GTP binding to the 19- and the 21-kDa smg-proteins. The same effect was observed when microsomal vesicles were incubated in the presence of MgATP (2 mM) and the electrogenic protonophore CCCP (10^{-5} M). No effect, neither of bafilomycin B_1 nor of CCCP, was found in control vesicles which had been incubated without MgATP.

The microsomal MgATP-driven H^+ uptake was measured in vesicles of the same preparation. As shown in Fig. 3, addition of MgATP (2 mM) to microsomal vesicles incubating in a high KCl medium with acridine orange (6 μM), resulted in a decrease of absorbance due to uptake of acridine orange. This uptake was due to intravesicular acidification which was demonstrated by the rapid discharge of accumulated protons in the presence of the electroneutral K^+/H^+ ionophore nigericin. In vesicles pretreated with bafilomycin B_1 (10^{-8} M) or CCCP (10^{-5} M) no intravesicular acidification was observed.

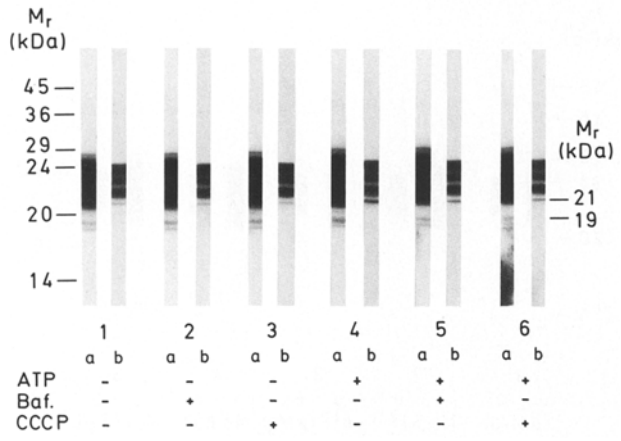

Fig. 2. Effect of bafilomycin B_1 and of CCCP on [α-^{32}P]GTP binding to low molecular weight GTP-binding proteins in microsomal vesicles. Rat pancreatic microsomal vesicles (1 mg protein/ml) were incubated at 24°C for 30 min without (lanes *1–3*) or with MgATP (lanes *4–6*), 10^{-8} M bafilomycin B_1 (lanes *2* and *5*) and 10^{-5} M CCCP (lanes *3* and *6*). Proteins were subjected to SDS/PAGE and blotted onto nitrocellulose. Subsequently, the [α-^{32}P]GTP binding assay and autoradiography were performed as described in Materials and Methods. Films were exposed for 72 (*a*) and 24 hr(*b*) to improve discrimination of different smg-proteins. Similar results were obtained in four additional experiments.

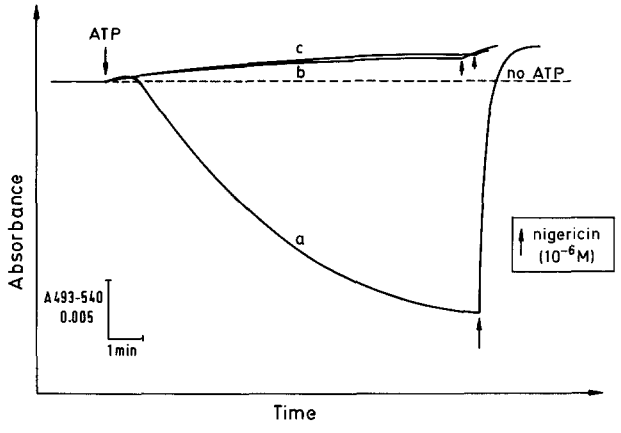

Fig. 3. MgATP-driven H^+ uptake into pancreatic microsomal vesicles. The pH-sensitive dye acridine orange was used to visualize the formation and dissipation of the H^+ gradient as described previously [15]. Vesicles of the same preparation used for the experiments shown in Fig. 2 were preincubated at 24°C for 15 min in a 155-mM KCl incubation medium with acridine orange (6 μM) as a control (*a*) or in the same medium containing 10^{-8} M bafilomycin B_1 (*b*) or 10^{-5} M CCCP (*c*). H^+ uptake was initiated by addition of 2 mM MgATP. The generated H^+ gradient was dissipated by nigericin (10^{-6} M) as indicated.

These results indicate that the MgATP effect observed on the 19- and the 21-kDa smg-proteins is due to intravesicular acidification of the pancreatic microsomal vesicles.

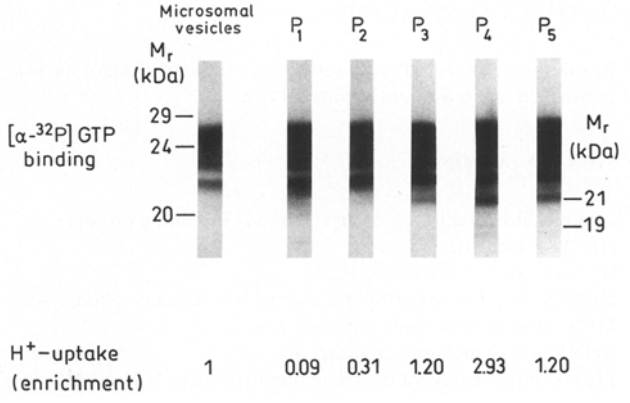

Fig. 4. [α-^{32}P]GTP binding to smg-proteins and H^+ ATPase activity in microsomal vesicles subfractionated by Percoll gradient centrifugation. Pancreatic microsomal vesicles were further separated by centrifugation on a Percoll gradient. Five fractions were collected from the top to the bottom of the gradient (P_1 to P_5). *Upper panel:* Proteins of each fraction were resolved by SDS/PAGE and transferred to nitrocellulose. The [α-^{32}P]GTP binding assay and autoradiography were performed as described in Materials and Methods. The experiment shown is representative of three similar experiments. *Lower panel:* MgATP-dependent H^+ uptake was determined in each microsomal fraction (P_1–P_5) and expressed as enrichment of H^+ uptake compared to the starting material [30].

In order to substantiate this point we have fractionated pancreatic microsomal vesicles by Percoll gradient centrifugation and have compared the distribution of the 19- and 21-kDa smg-proteins with that of H^+ uptake activity. As shown previously [30] maximal enrichment of H^+ transport (2.93-fold as compared to the starting material) is found in the fourth fraction from the top of the Percoll gradient. [α-^{32}P]GTP binding to smg-proteins in the different Percoll fractions is shown in Fig. 4. Both, the 19- and the 21-kDa smg-proteins were found predominantly present in fraction P_4, thus cofractionating with H^+ uptake activity.

To test whether intravesicular acidification in the presence of MgATP could change the affinity of the small molecular weight GTP-binding proteins for [α-^{32}P]GTP, competitive inhibition of the [α-^{32}P]GTP binding by native GTP was investigated. Vesicles were incubated for 30 min without or with MgATP (2 mM). Subsequently, microsomal proteins were resolved by SDS/PAGE and transferred to nitrocellulose. Single nitrocellulose lanes were incubated with [α-^{32}P]GTP and increasing concentrations of GTP as described in Materials and Methods for the [α-^{32}P]GTP binding assay. As shown in Fig. 5 for the 19-kDa smg-protein, half-maximal displacement of tracer occurred at 2.4×10^{-8} M GTP (control) and at 1.6×10^{-8} M GTP (MgATP stimulated). The respective values for the 21-kDa smg-protein were

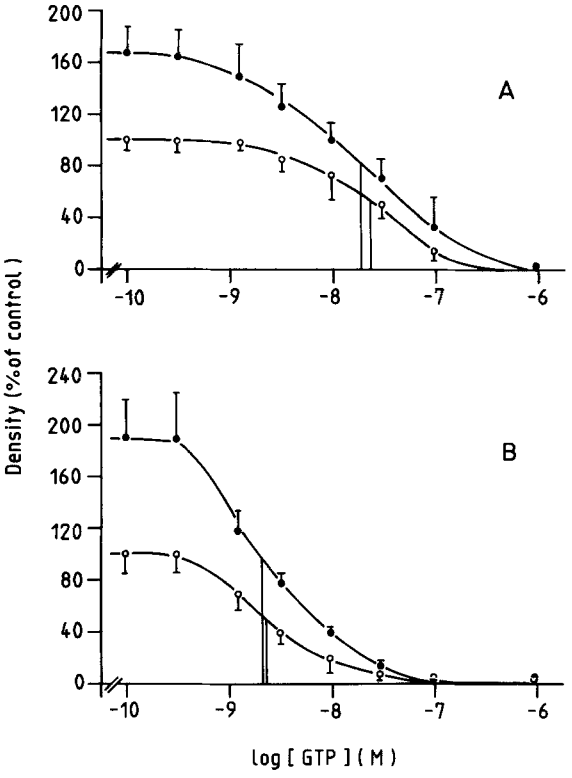

Fig. 5. Competitive inhibition of [α-^{32}P]GTP binding by unlabeled native GTP. Pancreatic microsomal vesicles were incubated for 30 min without (○——○) or with MgATP (2 mM) (●——●). The microsomal proteins were resolved by SDS/PAGE and transferred to nitrocellulose. Single nitrocellulose lanes were incubated with [α-^{32}P]GTP without and with increasing concentrations of native unlabeled GTP. Bound radioactivity was detected by autoradiography and films were subsequently scanned by laser densitometry. The results for the 19-kDa (*A*) and the 21-kDa smg-protein (*B*) are expressed as mean ± SD (n = 4).

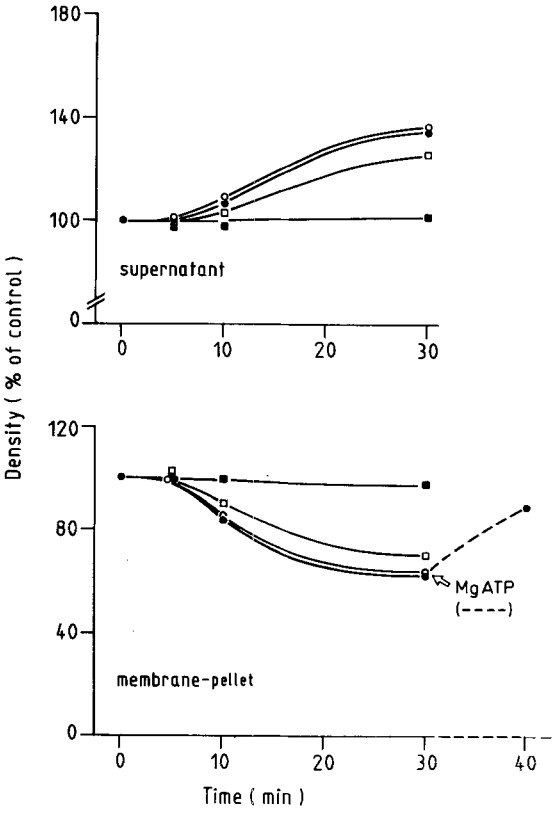

Fig. 6. [α-^{32}P]GTP binding to the 21-kDa smg-protein in the supernatant and in the membrane-associated fraction. Vesicles were incubated for 0, 5, 10, or 30 min at 24°C without MgATP in the absence (●——●) or presence of bafilomycin B_1 (○——○) or with MgATP in the absence (■——■) or presence of bafilomycin B_1 (□——□). The incubation was stopped by centrifugation at 200,000 × g and 4°C. Subsequently, [α-^{32}P]GTP binding to the 21-kDa smg-protein in the pellet and the supernatant was assessed as described in Materials and Methods. Addition of MgATP to vesicles which had been incubated for 30 min at 24°C in the absence of MgATP (●——●), showed reassociation of the 21-kDa smg-protein to the membrane (●----●). [α-^{32}P]GTP binding to the protein at time zero was expressed as 100%. The experiment shown is representative of four similar experiments.

2.4×10^{-9} M GTP (control) and 2.2×10^{-9} M GTP (MgATP stimulated). Thus, no apparent changes of the [α-^{32}P]GTP binding by MgATP for both the 19- and the 21-kDa smg-proteins were observed.

To investigate whether a translocation process of the 19- and 21-kDa smg-proteins occurs in the presence of MgATP, we incubated pancreatic microsomal vesicles without or with MgATP at 24°C. The reaction was terminated by centrifugation at 200,000 × g, and subsequently, the [α-^{32}P]GTP binding to smg-proteins was measured in the pellet and in the supernatant (soluble fraction). The same smg-proteins of both fractions showed no apparent difference in their electrophoretic mobility in SDS/PAGE. We assumed that any translocation process should increase [α-^{32}P]GTP binding in one fraction while concurrently decreasing binding in the other fraction. Figure 6 shows [α-^{32}P]GTP binding to the 21-kDa protein (pellet and supernatant) after incubating vesicles for 0, 5, 10 or 30 min without or with MgATP and bafilomycin B_1, respectively. In membrane vesicles which had been incubated without MgATP at 24°C a maximal decrease of the [α-^{32}P]GTP binding to the 21-kDa smg-protein by 40% was observed after 30 min, which did not change with longer incubation periods. This decline was temperature dependent and could not be observed at 4°C (*data not shown*). The decrease of the membrane-associated 21-kDa smg-protein was reflected in a proportional increase of this protein in the supernatant, indicating translocation of the 21-kDa protein from the membrane to the soluble fraction. In vesicles which had been incubated with MgATP the 21-kDa smg-protein

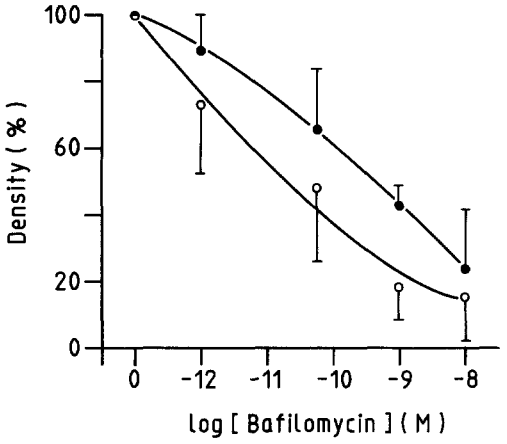

Fig. 7. Dose-response relationship for bafilomycin B_1 and the MgATP-dependent binding of the 19- (○——○) and the 21-kDa (●——●) smg-protein to microsomal vesicles. Microsomal vesicles were incubated for 30 min at 24°C with 2 mM MgATP and increasing concentrations of bafilomycin B_1. Proteins were separated by SDS/PAGE, transferred to nitrocellulose and a [α-^{32}P]GTP binding assay was performed. After autoradiography the films were scanned by laser densitometry. MgATP-dependent [α-^{32}P]GTP binding to each smg-protein in the absence of bafilomycin B_1 is expressed as 100%. Results are presented as mean ± SD (n = 3).

remained associated with the vesicle membranes; no change over time was observed in both the membrane-associated and the soluble fraction. Addition of MgATP to vesicles, which had been incubated for 30 min at 24°C in the absence of MgATP, showed reassociation of the 21-kDa smg-protein to the membrane (Fig. 6).

A different behavior was observed for the 19-kDa protein. In a 45-min incubation period about 70% of the 19-kDa smg-protein dissociated from the vesicle membrane into the surrounding medium at 4°C. Longer incubation periods did not further increase the degree of dissociation. Subsequent incubation for 30 min at 24°C in the presence of MgATP increased reassociation of the 19-kDa smg-protein by about 100% of the amount of the 19-kDa protein present in the membrane before MgATP addition. In the supernatant the soluble form of the protein decreased at the same time (*data not shown*).

Figure 7 shows the dose-response curve of bafilomycin B_1 on the MgATP-dependent binding of the 19- and the 21-kDa smg-proteins to the microsomal vesicles. We have previously shown that in microsomal vesicles both the MgATP-dependent H^+ uptake and the MgATP-dependent enhancement of GTP-induced vesicle fusion were completely inhibited by 10^{-9} M bafilomycin B_1; 50% inhibition of both effects occurred at 5×10^{-11} M bafilomycin B_1 [15]. As shown in Fig. 7 maximal inhibition of the MgATP-dependent binding of smg-proteins to microsomes was observed at 10^{-8} M bafilomycin B_1. Half-maximal inhibition occurred at 1.2×10^{-11} M and 8.0×10^{-11} M of bafilomycin B_1 for the 19- and 21-kDa smg-proteins, respectively.

Anion Dependence of the smg-Protein Binding to Microsomal Vesicles

The vacuolar-type H^+ ATPase located in microsomes from rat exocrine pancreas is known to be anion dependent in the sequence $Cl^- > Br^- >$ gluconate$^-$; in the presence of CH_3COO^- or NO_3^- no H^+ transport is observed [30]. Incubating membrane vesicles with 2 mM MgATP in a buffer containing 155 mM KCl a typical increase of binding of the 19- and 21-kDa smg-proteins to the microsomes was observed. When KCl was isosmotically replaced in the incubation medium by K^+ acetate or K^+ nitrate, binding of the 19- and the 21-kDa smg-proteins to the vesicle membrane was reduced (Fig. 8). [α-^{32}P]GTP binding to these two proteins in the K^+ acetate or K^+ nitrate buffer was indistinguishable from the [α-^{32}P]GTP binding to 19- and 21-kDa smg-proteins of vesicles treated with MgATP and bafilomycin B_1 in the presence of Cl^-.

Dependence of smg-Protein Binding to Pancreatic Microsomal Vesicles on pH

Incubating microsomal vesicles in a buffer containing 50 mM MES, pH 5.0, acidifies the intravesicular pH since the H^+ gradient cannot be maintained over the vesicle membrane (*data not shown*). At pH 5.0 binding of the 19- and the 21-kDa smg-proteins to the vesicle membrane is increased as compared to smg-proteins of vesicles which were incubated at pH 7.0 (Fig. 9). When membrane vesicles were incubated without MgATP for 30 min at 24°C in the presence of the K^+/H^+ exchanger nigericin (5×10^{-6} M) to assure that the intravesicular pH was completely clamped to the pH of the extravesicular medium, similar results as without nigericin were obtained (*data not shown*). This indicates that nigericin itself has no effect on GTP binding. [α-^{32}P]GTP binding to the other smg-proteins was not affected by pH. When vesicles were incubated with 2 mM MgATP in a buffer at pH 5.0 no MgATP effect was observed (Fig. 9). Addition of bafilomycin B_1 (10^{-8} M) to vesicles incubated with MgATP at pH 5.0 also had no effect on the [α-^{32}P]GTP binding to smg-proteins (Fig. 9).

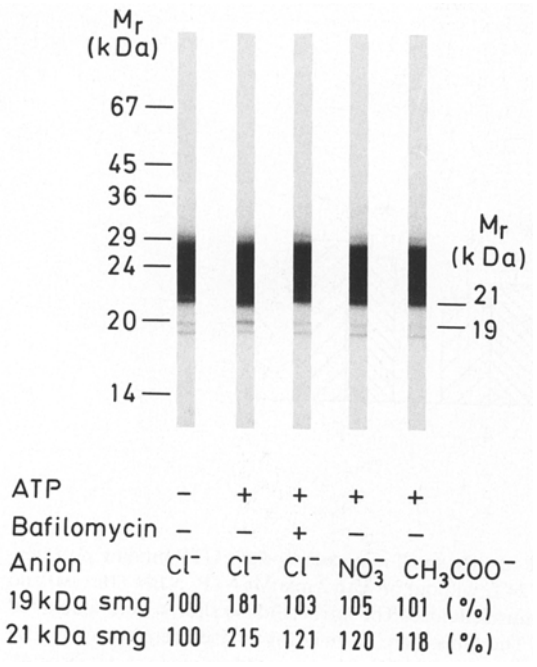

Fig. 8. Anion dependence of the binding of low molecular weight GTP-binding proteins to microsomal vesicles. Microsomal vesicles were incubated for 30 min at 24°C in a 155-mM KCl-buffer without (lane *1*), with 2 mM MgATP (lane *2*), or with 2 mM MgATP and 10^{-8} M bafilomycin B_1 (lane *3*). For lanes *4* and *5* vesicles were incubated with 2 mM MgATP in a buffer where Cl^- had been replaced by NO_3^- or CH_3COO^-, respectively. Proteins were subjected to SDS/PAGE, blotted to nitrocellulose and incubated with [α-^{32}P]GTP. After autoradiography the 19- and the 21-kDa smg-proteins were quantified by laser densitometry. Results are expressed as percent of control (=100%). The results shown are typical for two separate experiments.

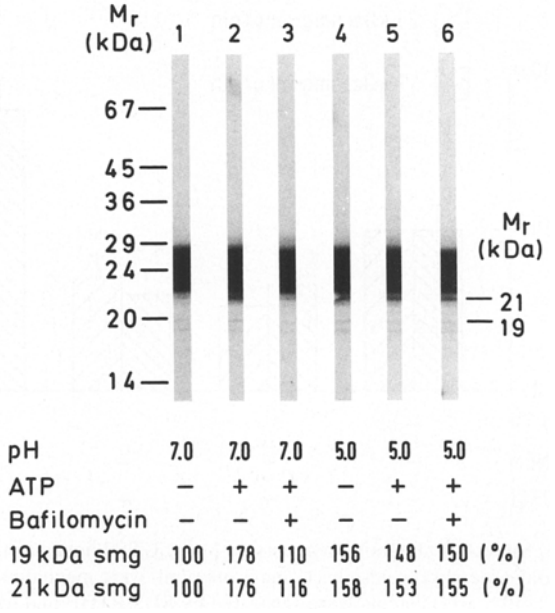

Fig. 9. Effect of pH on binding of smg-proteins to microsomal vesicles. Pancreatic microsomal vesicles (1 mg protein/ml) were incubated either in a buffer containing 50 mM Hepes, pH 7.0 (lanes *1–3*) or containing 50 mM MES, pH 5.0 (lanes *4–6*). Incubating the vesicles in the latter buffer acidifies the intravesicular pH since the vesicles cannot maintain the H^+ gradient over the membrane. MgATP (2 mM) (lanes *2*, *3*, *5* and *6*) and bafilomycin B_1 (10^{-8} M) (lanes *3* and *6*) were added as indicated. Proteins were separated by SDS/PAGE, transferred to nitrocellulose and incubated with [α-^{32}P]GTP for 60 min. After autoradiography the protein bands of the 19- and 21-kDa smg-proteins were scanned by laser densitometry. Results are expressed in percent of control (=100%). The experiment shown is representative for two similar experiments.

Effect of Alkylating Reagents on [α-^{32}P]GTP Binding to smg-Proteins

Other substances that potently inhibit vacuolar H^+ ATPases are the SH-alkylating reagent NEM and the tyrosine-, lysine- and SH-alkylating reagent NBD-Cl. Previous results from our laboratory have shown that the pancreatic microsomal H^+-ATPase can be completely inhibited at 10^{-4} M NEM and 10^{-5} M NBD-Cl [30]. In light-scatter and fluorescence-dequenching experiments preincubation of microsomal vesicles with Cl (10^{-5} M) or NEM (10^{-4} M) abolished GTP-induced fusion [15]. In the present study we have preincubated pancreatic microsomal vesicles with NBD-Cl (10^{-5} M) or NEM (10^{-4} M) in the absence or presence of MgATP (2 mM). As shown in Fig. 10 both reagents inhibited the MgATP-dependent binding of the 19- and the 21-kDa smg-proteins to the microsomal membrane. In contrast to bafilomycin B_1 and CCCP, both alkylating substances also significantly inhibited the [α-^{32}P]GTP binding to the 19- and 21-kDa smg-proteins in control vesicles incubated without MgATP ($P < 0.05$ and $P < 0.05$ in the presence of NEM, $P < 0.01$ and $P < 0.05$ in the presence of NBD-Cl for the 19- and 21-kDa smg-proteins, respectively). NEM (10^{-4} M) and NBD-Cl (10^{-5} M) also inhibited the [α-^{32}P]GTP binding to the solubilized 19- and 21-kDa smg-proteins (*data not shown*). Furthermore, [α-^{32}P]GTP binding to a 23-kDa microsomal smg-protein was inhibited by NBD-Cl (*data not shown*). These results indicate a direct effect of SH-alkylating reagents on [α-^{32}P]GTP binding to smg-proteins.

Effect of Salts and Detergents on Extraction of the 19- and 21-kDa Small Molecular Weight GTP-Binding Proteins from Microsomal Vesicles

Pancreatic microsomal vesicles were treated without (control) or with 1.0 M NaCl, 0.6 M KBr, 6 M

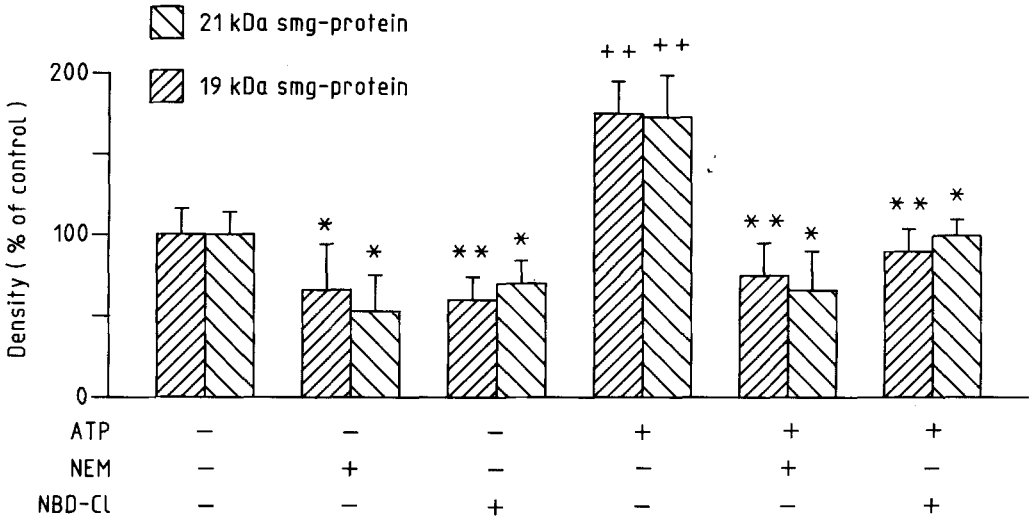

Fig. 10. Effects of alkylating reagents on [α-^{32}P]GTP binding to the 19- and the 21-kDa small molecular weight GTP-binding proteins. Microsomal membrane vesicles (1 mg protein/ml) were incubated for 30 min at 24°C without or with 2 mM MgATP, NEM (10^{-4} M) and NBD-Cl (10^{-5} M). Proteins were separated by SDS/PAGE and transferred to nitrocellulose. The nitrocellulose sheets were incubated with [α-^{32}P]GTP, and bound radioactivity was detected by autoradiography. The films were scanned by a laser densitometer. The results for the 19- and the 21-kDa smg-proteins are calculated in percent of control (=100%) and expressed as mean ± SD (n = 4). Asterisks (*) indicate the level of significance between control vesicles treated without or with MgATP and vesicles treated with NEM or NBD-Cl, respectively. Crosses (+) indicate the level of significance as compared to the bound radioactivity in the absence of MgATP. *$P < 0.05$; **$P < 0.01$; ††$P < 0.01$.

urea or 1% Triton X-100 for 60 min at 4°C, followed by centrifugation at 200,000 × g for 60 min. The 21-kDa smg-protein was not extracted by treatment with 1.0 M NaCl or 0.6 M KBr, partially extracted by 6 M urea and almost completely extracted by treatment with 1% Triton X-100 (Table). This indicates that this protein is tightly bound to the vesicle membrane.

About 70% of the 19-kDa smg-protein spontaneously dissociated from the vesicle membrane at 4°C, while 30% remained membrane associated. The remaining membrane-associated fraction was not extracted by the treatment with 1.0 M NaCl or 0.6 M KBr partially extracted by 6 M urea and completely extracted by 1% Triton X-100 (Table).

Discussion

Evidence suggests a role of certain *ras*-related low molecular weight GTP-binding proteins in intracellular membrane trafficking and vesicle fusion [2,5]. According to a model proposed by Bourne [5] smg-proteins in a GTP-bound active state direct vesicles to specific target organelles. GTP hydrolysis would be required to trigger a conformational change of the smg-protein, and the inactive GDP form of the protein would then return to the cytosol to target another vesicle in a cyclic process.

In this study we found evidence that MgATP regulates membrane association of a 19- and a 21-kDa smg-protein in pancreatic microsomal vesicles. MgATP increased association of both smg-proteins to the membrane-bound fraction while decreasing the amount of respective proteins in the supernatant. Neither effects of MgATP on the affinity of the 19- and 21-kDa smg-proteins for [α-^{32}P]GTP nor any effects of MgATP on other smg-proteins were observed. Both, the 19- and the 21-kDa smg-proteins migrated in the membrane-bound and soluble state without significant change in their electrophoretic mobility in SDS/PAGE. This suggests that no proteolytic cleavage of the proteins or other covalent modification such as phosphorylation [18], coincides with the translocation process, although small changes in the molecular mass would not be detectable by this method.

Incubation with bafilomycin B_1, a potent and specific inhibitor of vacuolar-type H^+ ATPases, or with the electrogenic protonophore CCCP, which completely blocks the formation of a H^+ gradient in the microsomal vesicles, abolished the MgATP-dependent association of both smg-proteins to the vesicle membrane almost completely. Furthermore, separation of pancreatic microsomal vesicles by Percoll gradient centrifugation showed that both smg-proteins cofractionate with the H^+ ATPase activity. Taken together the results suggest that MgATP regu-

Table. Extraction of the 19- and the 21-kDa smg-protein from microsomal vesicles by various reagents

	19-kDa smg-protein (% extracted)	21-kDa smg-protein
NaCl (1.0 M)	14 ± 7	15 ± 6
KBr (0.6 M)	5 ± 3	4 ± 3
Urea (6.0 M)	24 ± 9	31 ± 4
Triton X-100 (1%)	85 ± 8	80 ± 5

Freshly prepared microsomal vesicles containing 600 μg of protein were suspended in a mannitol buffer (control) supplemented with 1 M NaCl, 0.6 M KBr, 6 M urea or 1% Triton X-100. After incubation on ice for 60 min, the samples were centrifuged at 200,000 × *g* for 60 min at 4°C. Proteins in the supernatant and the pellet were dissolved in SDS sample buffer, run on a 12.5% SDS/PAGE gel and transferred to nitrocellulose. After the [α-^{32}P]GTP binding assay and subsequent autoradiography, laser densitometry was performed. In the control buffer only 30% of the 19-kDa but almost 100% of the 21-kDa smg-protein remained membrane associated after incubation on ice for 60 min. These values did not change during the subsequent 60-min centrifugation at 4°C. The results are expressed in percent extracted as compared to control after a 60-min incubation (mean ± SD, n = 3).

lates the association of the 19- and 21-kDa smg-proteins to the membranes of their specific vesicle population in a reversible process by intravesicular acidification. Inhibition of the MgATP-dependent translocation of both smg-proteins by bafilomycin B_1 was dose dependent with similar half-maximal inhibitory concentrations (1.2×10^{-11} M and 8×10^{-11} M for the 19- and 21-kDa smg-proteins, respectively) as previously reported for the inhibition of the vacuolar-type H^+ ATPase in pancreatic microsomal vesicles (5×10^{-11} M) [15].

A feature common to the acidification of virtually all vacuolar compartments is the requirement for a permeant anion, typically Cl^- [11]. We have previously shown that in the absence of Cl^-, MgATP-dependent acidification of pancreatic microsomes is completely blocked [30]. In the present study we showed that no MgATP effect on both smg-proteins was observed, when Cl^- was isosmotically replaced in the incubation buffer by CH_3COO^- or NO_3^-. These results strongly support the involvement of a vacuolar-type proton pump in the regulation of vesicle membrane association of the 19- and 21-kDa smg-proteins. Incubation at an intra- and extravesicular pH of 5.0 showed an increased association of the 19- and 21-kDa smg-protein to the microsomes. In experiments where the intravesicular pH was clamped to the extravesicular pH by nigericin similar results were obtained. Nigericin itself did not influence association of smg-proteins to the microsomes. This implies that the intravesicular pH and not the H^+ gradient over the vesicle membrane is crucial for the observed MgATP effect on both smg-proteins.

Both NEM and NBD-Cl are known to be potent inhibitors of vacuolar-type H^+ ATPases [30]. However, as shown in this study both reagents reduced the [α-^{32}P]GTP binding to the 19- and the 21-kDa smg-proteins in the absence and presence of MgATP. This is similar to recent observations showing that both NEM and NBD-Cl abolish GTP-induced fusion (i.e., in both the absence and presence of MgATP) of isolated pancreatic microsomal vesicles [15]. Furthermore, both alkylating substances reduced the [α-^{32}P]GTP binding to solubilized 19- and 21-kDa smg-proteins. This suggests that inhibition of GTP-induced vesicle fusion by NEM and NBD-Cl might be due to direct alkylation of one or both smg-proteins, thereby affecting GTP-binding and protein function.

Both, the 19- and 21-kDa smg-proteins were still present in microsomal membrane vesicles after osmotic lysis, which indicates that they are membrane bound. The 21-kDa smg-protein was not extracted by 1 M NaCl or by 0.6 M KBr, a cytoskeleton-depolymerizing agent, but was partly extracted by chaotropic 6 M urea, suggesting that the protein might be associated noncovalently with a membranous protein. About 70% of the 19-kDa smg-protein dissociated spontaneously from the vesicle membrane when incubated at 4°C. However, about 30% of the proteins remained associated with the membrane and were partially extracted by 6 M urea but not by 1 M NaCl or by 0.6 M KBr.

Using an alkaline phosphatase immunoassay we were able to identify the 19-kDa smg-protein in pancreatic acinar cells as the ADP-ribosylation factor (arf). A strong reaction of the polyclonal arf-antibodies was visible in the cytosolic fraction, and a weaker reaction was observed in microsomal membranes. (S. Zeuzem, P. Zimmermann, R.A. Kahn & I. Schulz, *unpublished data*).

We were not yet able to identify the 21-kDa smg-protein. Using specific antibodies and ADP-ribosylation with *Clostridium botulinum* ADP-ribosyltransferase C_3 we can exclude a *rab* 1 (*ypt* 1), *rab* 3, *rap*, *ras*, *rho* and *rac* gene product as the 21-kDa smg-protein (S. Zeuzem, P. Zimmermann & I. Schulz, *unpublished results*).

The ADP-ribosylation factor is localized in the cytosolic surface of Golgi membranes [28]. In vitro binding of the arf-protein to Golgi transport vesicles has recently been reported [26]. It therefore appears possible that the effect on the 19-kDa smg-protein (arf) as described in this study might be localized to condensing vacuoles derived from the Golgi complex. These vesicles have been shown to possess a vacuolar-type H^+ ATPase [11, 20, 22].

Cysteine residues in the C-terminal sequence as described for most *ras*-related smg-proteins are essential for membrane localization [14]. The arf-sequence, however, lacks a cysteine residue near the carboxyl terminus, but contains a consensus sequence for amino terminal myristylation [27]. This type of acylation has been shown to be functionally important for the transforming protein kinase $p60^{src}$ [16], although the specific role of the fatty acid in cellular localization or activity has not been demonstrated directly.

All cellular compartments containing vacuolar-type H^+ ATPases are involved in membrane traffic pathways [11]. Smg-proteins have been shown to target vesicles between different compartments. To our knowledge the data of this study indicate for the first time that there is a link between intravesicular pH and the association of certain low molecular weight GTP-binding proteins to the vesicle membrane. Further studies are necessary to clarify the regulatory process that links the intravesicular pH to membrane binding of smg-proteins.

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