Redistribution of ADP-ribosylation factor during stimulation of permeabilized cells with GTP analogues

Romano REGAZZI,* Susanne ULLRICH,* Richard A. KAHN† and Claes B. WOLLHEIM*

*Division de Biochimie Clinique, Department of Medicine, University of Geneva, 9 avenue de Champel, CH-1211 Geneva 4, Switzerland, and †Laboratory of Biological Chemistry, Division of Cancer Treatment, Building 37, Room 5D-02, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, U.S.A.

Low-molecular-mass GTP-binding proteins of the *ras* family were analysed by [³²P]GTP binding after PAGE and transfer to nitrocellulose membranes. By this technique, several GTP-binding proteins in the 20–30 kDa range were detected in both cytosolic and microsomal fractions of RINm5F cells. One of these, displaying an apparent molecular mass of about 20 kDa and a pI of 6.7, was mainly cytosolic and was shown to be the ADP-ribosylation factor (ARF) by using specific antibodies. When permeabilized RINm5F cells were incubated with the stable GTP analogues guanosine 5′-[γ-thio]triphosphate (GTP[S]) and guanosine 5′-[βγ-imido]triphosphate (p[NH]ppG) the amount of ARF increased in a fraction containing both Golgi and plasma-membrane markers, but not in the fraction containing secretory granules, mitochondria and lysosomes. GTP, GDP and its β-thio analogue did not enhance ARF binding to membranes. *smg25/rab3* and *rho*, as well as all the other small GTP-binding proteins detected by the [³²P]GTP binding, did not redistribute under these conditions. As GTP[S] stimulates insulin secretion in these cells, we also examined the relationship between ARF translocation and insulin secretion. Both phenomena were elicited by GTP[S] with an EC₅₀ (median effective concentration) of about 10 μM. p[NH]ppG was equipotent with GTP[S] in inducing insulin secretion (EC₅₀ about 10 μM), but higher concentrations (about 500 μM) were required to achieve the same maximal ARF redistribution. These results suggest that: (1) ARF is subject to cycling between a membrane-associated and a free/loosely attached form, determined by the species of bound guanine nucleotide; (2) ARF alone does not seem to regulate exocytosis in insulin-secreting cells.

INTRODUCTION

A growing number of proteins able to bind and hydrolyse GTP have been described [1,2]. They can be subdivided into five different families: (i) initiation and elongation factors controlling protein synthesis; (ii) tubulin subunits; (iii) trimeric G-proteins; (iv) *ras*-related low-molecular-mass G-proteins; (v) ADP-ribosylation factor (ARF) [2]. The functions of the two last groups have been studied in yeast by taking advantage of a genetic approach. Some of the *ras*-related proteins, including YPT1, SEC4 and SAR1, have been implicated in secretory processes in *Saccharomyces cerevisiae* [3–5]. ARF, initially described as a cofactor in cholera-toxin-catalysed ADP-ribosylation [6], has recently been allocated a role in yeast secretion [7]. YPT1, SAR1 and ARF appear to be regulating vesicular transport between endoplasmic reticulum and Golgi complex as well as intra-Golgi transport [3,5,7], whereas SEC4 controls the more distal steps [4]. It has been proposed, by using site-directed mutagenesis of SEC4, that the actions of these proteins necessitate cycling between a membrane-bound and a soluble form, depending on the liganded nucleotide [8]. In mammalian cells, the functions of the low-molecular-mass GTP-binding proteins remain obscure, despite the purification and cloning of several of these proteins [2]. *ras*-like proteins have been suggested to participate in various cellular processes, including vectorial transport of vesicles and phospholipase C activation [2]. ARF has now been localized by immuno-electron microscopy to the Golgi complex also in mammalian cells, suggesting a more generalized role of this GTP-binding protein in vesicular transport and secretion [7]. At present it is not known whether the mechanism of action proposed for SEC4 is applicable to other GTP-binding proteins, including those found in mammalian cells.

A member of the *ras* family was reported to redistribute from

the microsomal to the cytosolic compartment upon incubation of platelets with a prostacyclin agonist [9]. This protein was shown to be *smg21/rap1/krev1*, and it was suggested that its redistribution was mediated by a cyclic AMP-dependent phosphorylation [9,10]. Such a covalent modification could thus represent an alternative to the model proposed for SEC4 for the regulation of the function of these GTP-binding proteins. In the present study we have used permeabilized RINm5F cells to examine the effect of guanine nucleotides on the distribution of the low-molecular-mass GTP-binding proteins. Since this preparation secretes insulin in a regulated fashion it was also possible to probe for the function of these proteins in insulin secretion.

MATERIALS AND METHODS

Materials

[α-³²P]GTP, [³²P]NAD⁺ and ¹²⁵I-labelled anti-rabbit antibodies were from New England Nuclear. Proteins *rhoB* and *smg21*, purified from bovine brain membranes and human platelet membranes respectively, the polyclonal antibody against *smg21* and the monoclonal antibody against *smg25A* were kindly provided by Dr. Y. Takai (University of Kobe, Japan). The exoenzyme C3 from *Clostridium botulinum* was generously given by Dr. K. Aktories (University of Giessen, Germany). The peptide polyclonal antibody against ARF and recombinant ARF1 were prepared as described previously [11,12]. Sources of other materials were as previously described [13,14].

Cell permeabilization

RINm5F cells were cultured, electrically permeabilized and incubated in K⁺/glutamate (140 mM) buffer exactly as described previously [13,14]. The free Ca²⁺ concentration in the incubation medium was 100 nM and was obtained by using Ca²⁺/EGTA

Abbreviations used: GTP[S], guanosine 5′-[γ-thio]triphosphate; p[NH]ppG, guanosine 5′-[βγ-imido]triphosphate; GDP[S], guanosine 5′-[β-thio]diphosphate; p20, 20 kDa GTP-binding protein; ARF, ADP-ribosylation factor; EC₅₀, median effective concentration.

buffers [14]. In each experiment where GTP-binding assays were performed, insulin release into the supernatant before homogenization was measured by radioimmunoassay [13,14] and served as a control for guanine nucleotide efficacy.

Subcellular fractionation

At the end of the incubation period the cells (about 15×10^6) were centrifuged for 30 s at 13000 *g*. The resulting supernatant was collected for insulin measurements, and the pellet was resuspended in 0.5 ml of homogenization buffer (HB: 10 mM-Tris/HCl, pH 7.5, 5 mM-EDTA, 10 μ g of leupeptin/ml and 2 μ g of aprotinin/ml) and the cells were disrupted by brief sonication. The homogenate was centrifuged for 30 min at 13000 *g* and the resulting supernatant (cytosol) frozen immediately. The crude microsomal pellet was resuspended in 0.5 ml of HB by sonication and subsequently stored at -20°C until used. By this homogenization procedure, about two-thirds of the proteins were recovered in the supernatant and one-third in the crude membrane fraction.

In some experiments subcellular fractions were prepared by a previously described method [15]. In this case approx. 3×10^8 cells were permeabilized, incubated with or without GTP[S] and homogenized by nitrogen cavitation. After elimination of nuclei and cell debris, plasma-membrane and granule-enriched fractions were obtained by using a self-generating 15% (w/v) Percoll gradient [15]. 5'-Nucleotidase activity (plasma-membrane marker) and insulin immunoreactivity were enriched in the respective fractions by a factor of 6 over the homogenate. The fraction previously considered to be enriched in plasma-membrane marker [15] displayed a similar 6-fold increase in 5'-nucleotidase and galactosyltransferase (EC 2.4.1.151) (Golgi-membrane marker enzyme) activity over the homogenate.

GTP binding

Samples were dissolved in SDS buffer [10 mM-Tris/HCl, pH 7.9, 7 mg of dithiothreitol/ml, 1 mM-EDTA, 3% (w/v) SDS and 13% (w/v) glycerol], boiled for 2 min and loaded on 13% (w/v) polyacrylamide gels. For two-dimensional PAGE the samples were dissolved in urea buffer [9.0 M-urea, 4% (w/v) Nonidet P40, 21 mg of dithiothreitol/ml, 10% glycerol and 2% (w/v) Ampholines (pH 3.5–10)]. The first dimension consisted of an isoelectric-focusing gel containing 2% Ampholines (pH 3.5–10) and the second dimension was an SDS/13%-polyacrylamide electrophoresis gel. The resolved proteins were transferred to nitrocellulose membranes by the semi-dry blotting procedure [9]. Binding of [α - ^{32}P]GTP (1 $\mu\text{Ci}/\text{ml}$, final concn. about 1 nM) to the blotted proteins was carried out essentially as described previously [9,16]. The assay buffer contained 50 mM-Tris/HCl, pH 7.5, 0.3% (w/v) Tween-20, 12 μM - MgCl_2 and 0.3% (w/v) BSA. GTP binding was found to be linear between 20 and 300 μg of cytosol proteins, as assessed by cutting out and counting radioactivity of the appropriate region of the nitrocellulose membrane. Routinely a portion of 50–100 μg of protein was applied. GTP binding was also proportional to the amount of added pure ARF standard.

Western blotting

Immunoblotting was performed by exposing the blotted proteins to the various antibodies and detecting the immune reaction either with horseradish-peroxidase-labelled second antibody as described by Mizoguchi *et al.* [17] or with ^{125}I -labelled second antibody by the method of Kahn *et al.* [11].

ADP-ribosylation assay

ADP-ribosylation was carried out essentially as described in ref. [18]. The reaction mixture contained 50 mM-Tris/HCl,

pH 7.5, 10 mM-thymidine, 0.5 mM-ATP, 0.1 mM-GTP, 3 mM-EDTA, 1 μM -[^{32}P]NAD $^+$ (1 μCi), 1 μg of C3 exoenzyme/ml and 45 μg of the cell extracts in a total volume of 50 μl .

RESULTS

Many low-molecular-mass GTP-binding proteins retain the ability to bind GTP even after boiling in SDS, subsequent separation by PAGE and transfer to nitrocellulose membranes [9,16]. At least three different groups of such proteins were observed in the cytosolic fraction of RINm5F cells, displaying apparent molecular masses of 27, 25 and 20 kDa (Fig. 1a). An additional band at 23 kDa was also observed after longer exposure of the film (result not shown). All these proteins were soluble and not attached to light membranes, since they were not pelleted even after 60 min centrifugation at 100000 *g* (result not shown). In the crude membrane fraction, proteins of 27, 25 and 23 kDa are detected. The identity of these proteins remains to be established. The 20 kDa protein (referred to as p20) was also observed in the membrane extract, but was much less abundant, since it was only revealed after prolonged exposure of the film. Most of these proteins are extractable from the membranes in the presence of 1% Triton X-100 (Fig. 1a). The binding of labelled GTP could be completely prevented by simultaneous addition of unlabelled GTP[S] (10 μM) (Fig. 1b). In contrast, ATP at the same concentration had no effect (result not shown). When cytosolic proteins were resolved by two-dimensional PAGE, p20 could be shown to correspond to a single spot with pI of 6.7 ± 0.1 (mean $\pm$ S.E.M., $n = 6$) (Fig. 2a). Although less abundant, the same spot could be detected by two-dimensional gel electrophoresis of the crude membrane fraction (Fig. 2b). In contrast, both in cytosol and in crude membrane fraction the band migrating at about 25 kDa consisted of at least three different spots (Fig. 2).

Treatment of platelets with a prostacyclin analogue was shown

Fig. 1. GTP binding to cytosolic and crude membrane proteins of RINm5F cells

RINm5F cell extracts were prepared as described in the Materials and methods section. Triton extraction was performed by treatment of the 13000 *g* pellet with 1% (w/v) Triton X-100 and centrifugation at the same speed for 30 min. The proteins were resolved by PAGE and transferred to nitrocellulose membranes. (a) [^{32}P]GTP-binding experiments performed as indicated in the Materials and methods section. (b) Results obtained after inclusion of 10 μM -GTP[S] in the binding buffer. Equal amounts of protein were applied to each lane, and the same film was used for the two parts. Key: C, cytosol; TE, Triton X-100 extract; P, Triton X-100-resistant pellet.

Fig. 2. GTP binding to cytosolic and crude membrane proteins of RINm5F cells after separation by two-dimensional gel electrophoresis

The cytosolic proteins (a) and those of the crude membrane fraction (b) were resolved by isoelectric focusing (IEF) in the first dimension and by SDS/PAGE in the second dimension. After transfer to nitrocellulose membranes, the proteins were revealed by [32 P]GTP binding as described in the Materials and methods section.

Fig. 3. Effect of GTP analogues on subcellular distribution of small GTP-binding proteins in permeabilized RINm5F cells

Electrically permeabilized RINm5F cells were incubated for 15 min at 4 °C and 5 min at 37 °C in the presence of vehicle, 100 μ M-GTP[S] or 100 μ M-p[NH]ppG. Cell fractionation and [32 P]GTP binding to the blotted proteins were performed as described in the Materials and methods section.

to cause the redistribution from the microsomal to the cytosolic fraction of a *ras*-like protein [9]. In contrast, in intact RINm5F cells no changes in the subcellular distribution of any of the small GTP-binding proteins were detected when insulin secretion was stimulated with forskolin, phorbol esters, carbachol or glyceraldehyde, or inhibited by the neuropeptide galanin (results not shown). However, when electrically permeabilized RINm5F cells were incubated with the poorly hydrolysable GTP analogue GTP[S], the labelling of p20 associated with the crude membrane fraction increased (Fig. 3). There was no significant change in the labelling of p20 localized in the cytosol, nor in that of the other bands. The effect was seen both in the absence (< 0.1 nM) or presence (0.1 or 10 μ M) of free Ca^{2+} , and the cation alone was ineffective (results not shown). When used at equimolar concentration (100 μ M), another poorly hydrolysable GTP analogue, p[NH]ppG, caused a smaller increase in the labelling of membrane-associated p20 (Fig. 3). Since autoradiography does not allow for proper quantification of the labelling of p20, the p20 band was cut out and radioactivity was directly counted. The

Fig. 4. Subcellular distribution of p20 after stimulation of permeabilized RINm5F cells with GTP[S]

Permeabilized RINm5F cells were incubated in the absence (a–d) or in the presence (e–h) of 100 μ M-GTP[S] for 15 min at 4 °C and 5 min at 37 °C. Cytosol, plasma-membrane- and granule-enriched fractions were prepared by using a 15% (w/v) self-generating Percoll gradient [15]. The same amount of protein was applied to each lane. Electrophoresis, blotting and GTP binding were performed as described in Fig. 1. Lanes: a and e, homogenate; b and f, cytosol; c and g, plasma-membrane- and Golgi-enriched fraction; d and h, granule-enriched fraction.

maximal labelling of p20 in the membranes achieved in the presence of GTP[S] was about 2.6-fold over the control.

GTP, GDP and GDP[S] (100 μ M each) did not significantly alter the labelling of membrane-associated p20 ($94 \pm 6\%$ of control for GTP; $99 \pm 9\%$ of control for GDP; $103 \pm 22\%$ of control for GDP[S]; means $\pm$ S.E.M., $n = 3$). These results suggest that the increase in the labelling of p20 is not merely the result of a protective effect against degradation exerted by guanine nucleotides [19], since GTP, GDP and GDP[S] should also act in a similar manner. However, several experiments were performed in order to exclude the possibility that the effect of GTP[S] occurred during the preparation of the cell extracts. Thus the exposure of crude membranes (instead of permeabilized cells) to GTP[S] either for 30 min at 4 °C or for 15 min at 4 °C, followed by 5 min incubation at 37 °C, did not alter the degree of labelling during the GTP-binding assay (results not shown). In addition, the increase in p20 was not due to the trapping of cytosol in membrane vesicles, as resuspension by sonication of the microsomal pellet followed by a second pelleting of the membranes gave the same result (not shown).

In order to identify the cellular compartment where the increase in p20 takes place, we then analysed different subcellular fractions prepared from electrically permeabilized RINm5F cells exposed to GTP[S]. As shown in Fig. 4, the increased labelling was found in the fraction enriched in 5'-nucleotidase and galactosyl-transferase activity. The labelling of p20 upon GTP[S] treatment seems therefore to be enhanced in a plasma-membrane- and Golgi-enriched fraction (Fig. 4). It should be noted that the heavy fraction, enriched in secretory granules, mitochondria and lysosomes [15], displayed no significant labelling.

Several experiments were performed in order to identify p20. Because of its preponderant localization in the cytosol, N-*ras*, Ki-*ras* and Ha-*ras* proteins, which are mainly membrane-associated in most systems [20], were considered unlikely candidates. The *rho* proteins, on the other hand, are more abundant in the cytosol than in membranes [21]. The relationship to the latter proteins was examined either by performing ADP-ribosylation experiments with C3 exoenzyme from *Clostridium botulinum* [18,21] or by co-migration of the pure protein in our system. In both cases the bands were detected at 22 kDa, thus clearly distinct from the protein under study. In addition to the difference in mobility, exposure of the cells to GTP[S] did not alter the subcellular distribution of the ADP-ribosylated proteins (results not shown). *smg25/rab3* was identified by Western blotting of the subcellular fractions and displayed the expected mobility of

Fig. 5. Sequential immunoblotting with ARF antiserum and GTP binding to cytosolic and crude membrane proteins of RINm5F cells

Permeabilized cells were incubated in the absence or presence of 100 μ M-GTP[S] as described in Fig. 3. After cell fractionation, protein separation and their transfer to nitrocellulose membranes, immunoblotting with ARF antiserum (1:1000) was first performed. The immunocomplex was detected after addition of 125 I-labelled anti-rabbit antibody and exposing the film for 3 days. The same membrane was then washed extensively and [32 P]GTP binding was performed. In this case the exposure time was only 6 h. The same amount of protein was applied to each lane: a, cytosol of control cells; b, crude membrane from control cells; c, crude membrane from cells exposed to GTP[S].

Fig. 6. Effect of GTP analogues on subcellular distribution of ARF in permeabilized RINm5F cells

Permeabilized RINm5F cells were incubated in the absence or presence of either GTP[S] or p[NH]ppG (100 μ M each) as described in Fig. 3. Cell fractionation, PAGE and transfer of the proteins to the nitrocellulose membrane were performed as described in the Materials and methods section. The same amount of protein was applied to each lane. The nitrocellulose membrane was first incubated for 1 h with ARF antiserum (dilution 1:1000) and then revealed by autoradiography after labelling of the immunocomplex with 125 I-labelled anti-rabbit antibodies.

25 kDa (results not shown). This protein, in contrast with p20, did not exhibit redistribution after stimulation with GTP[S] or p[NH]ppG (result not shown). It also appeared that p20 was not *smg21/rap/1/krev-1* [22], since purified *smg21* had a higher relative molecular mass (22 kDa) in our system. Furthermore, the antibody against *smg21* did not react with the proteins extracted from RINm5F cells (result not shown).

It appears that ARF is similar to p20 in terms of molecular mass and subcellular distribution [11]. We therefore tested the identity between these two proteins. This was achieved by performing Western blotting with a polyclonal antibody against ARF and GTP-binding in sequence on the same nitrocellulose membrane after one- or two-dimensional PAGE. In both cases,

Fig. 7. Guanine-nucleotide-induced insulin secretion and membrane association of ARF in permeabilized RINm5F cells

Electrically permeabilized RINm5F cells were incubated with GTP[S] (O) or p[NH]ppG (Δ) for 15 min at 4 °C and 5 min at 37 °C. (a) Amount of insulin released into the supernatant during the 5 min incubation period, measured by radioimmunoassay as previously described [14]. The results are given as percentages of control (means $\pm$ S.E.M.) of three independent experiments. In (b), after cell fractionation and PAGE the proteins were blotted and [32 P]GTP labelling of ARF in the crude membrane fraction was measured by counting the radioactivity bound to the 20 kDa band after cutting out the appropriate region of the nitrocellulose membrane. The results are given as percentages of control of two to seven independent observations; S.E.M. is indicated where three or more independent experiments were performed. Control values ranged from 112 to 161 c.p.m.

the band or the spot, respectively recognized by the antibody and labelled by [32 P]GTP, coincided exactly. In addition, this dual approach confirmed that ARF and p20 displayed the same subcellular distribution and that GTP[S] treatment of the permeabilized cells increased the amount of p20/ARF in the crude membrane fraction (Fig. 5). These conclusions were confirmed by digital image analysis of the data shown in Fig. 5. In this experiment by Western blotting, taking into account the total amount of protein present in the given fraction, 88% and 12% of ARF was in the cytosol and in the membranes respectively, corresponding to 89% and 11% by GTP binding. In both cases GTP[S] treatment of the permeabilized cells increased the membrane-associated ARF by about 3-fold. Finally, in one experiment the amount of ARF was quantified by comparison with different concentrations of ARF standard by GTP binding and counting of the appropriate regions of the nitrocellulose membrane. Assuming that the recombinant ARF [12] displays the same binding characteristics as the RINm5F cell protein, ARF represented 467 and 50 ng/mg of total cellular protein in cytosol and crude membrane respectively under control conditions. After exposure to GTP[S] the corresponding values were 402 and 136. Analysis by Western blotting of cytosols and crude membranes of permeabilized cells exposed to the two GTP analogues confirmed that GTP[S] is more efficient in increasing ARF association with the crude membranes than is p[NH]ppG (Fig. 6).

Although ARF has been shown to be involved in protein secretion in yeast [7], its function in mammalian cells remains to be established. To probe for a possible role in secretion, insulin release and ARF binding to RINm5F cell membranes were

examined in parallel by using different concentrations of GTP[S] and p[NH]ppG. The EC_{50} for stimulation of insulin secretion was close to $10\ \mu\text{M}$ for each analogue (Fig. 7). In contrast, the EC_{50} for the increase in ARF binding to the membranes was about $10\ \mu\text{M}$ for GTP[S], compared with $100\ \mu\text{M}$ for p[NH]ppG (Fig. 7). Although both nucleotides promote association of ARF to membranes, the difference in affinity of the two processes makes it unlikely that ARF alone is the main GTP-binding protein regulating exocytosis.

DISCUSSION

Small GTP-binding proteins of the *ras* family have been suggested to change their subcellular location, depending on which form of guanine nucleotide is bound [8]. Such events can be investigated in permeabilized cells by the use of poorly hydrolysable GTP analogues. These analogues resulted in an increase in the association of a 20 kDa GTP-binding protein with a membrane compartment. This protein could be identified as ARF on Western blots using specific antibodies. Under our experimental conditions GTP binding and Western blotting were found to be valid quantitative assessments of the amount of protein and gave concordant results. Until recently, ARF was mainly considered to act as a cofactor in cholera-toxin-mediated ADP-ribosylation of the stimulatory regulatory subunit of adenylate cyclase, G_s [6]. Interestingly, in *Saccharomyces cerevisiae*, ARF appears to be implicated in the transport and processing of newly synthesized proteins from the endoplasmic reticulum to the Golgi complex [7], playing a similar role to that previously assigned to another *ras*-like GTP-binding protein, YPT1 [3]. The function of ARF in other cells is at present unknown. However, immunocytochemical studies have localized this protein to the cytoplasmic faces of Golgi stack membranes in fibroblasts, suggesting that ARF could play a role in vesicular transport and secretion also in mammalian cells [7]. In spite of these results, in RINm5F cells, as in other cell systems [11], about 90% of ARF is recovered in the soluble fraction after cell fractionation. Under control conditions, therefore, ARF is presumably loosely attached to the membranes, and cell fractionation causes the release of the protein into the supernatant (cytosol). Since ARF is known to be myristoylated at the *N*-terminus [11], this post-translational modification could facilitate a reversible attachment to cellular membranes. Our results suggest that the release of ARF into the cytosol is, at least in part, prevented by the incubation of permeabilized cells with poorly hydrolysable GTP analogues. Such treatment seems to stabilize the binding of the protein to membranes. Cell fractionation after GTP[S] treatment showed that ARF associates exclusively with plasma and/or Golgi membranes, indicating that this phenomenon is not simply due to an increased hydrophobicity of the protein.

In contrast with the non-hydrolysable GTP analogues, GTP, GDP and GDP[S] did not alter the amount of ARF in the membrane fraction. The simplest explanation for these results is that ARF displays different conformations depending on whether GDP or GTP is the ligand. In the GDP-liganded state the protein would be loosely associated with the membrane, whereas in the GTP-bound form ARF would become more firmly attached. It has indeed been shown that GDP and GTP promote different conformations of ARF [6]. Further, a membrane-associated GTPase-activating protein capable of markedly stimulating GTP hydrolysis by ARF has been found in yeast and bovine brain (C. Rulka & R. A. Kahn, unpublished work). Non-hydrolysable GTP analogues would fix ARF in the 'GTP' conformation and therefore cause a net increase in the amount of this GTP-binding protein in the membrane fraction. The GTP[S]-induced

association of ARF with membranes is in agreement with the model proposed previously for the activation of SEC4, another *ras*-like GTP-binding protein involved in secretion in yeast [8]. In intact cells, various agents that increase cytosolic Ca^{2+} or stimulate protein kinase C or cyclic-AMP-dependent protein kinase did not affect ARF distribution. A possible explanation for this finding is that in intact cells the cycling of ARF rather than the amount of the protein at the membrane is increased.

It should be noted that *rho* and the other non-identified GTP-binding proteins did not behave as ARF, indicating different mechanisms of cycling or, alternatively, its absence. With respect to *smg25/rab3*, it was recently reported that the purified protein associates with cellular membranes in a GTP-dependent manner, whereas the interaction of the GDP-liganded form of the protein with membranes is prevented by another protein, the GDP-dissociation inhibitor (GDI) [23]. Failure to detect redistribution of *smg25/rab3* in our system may be due to excess of GDI in intact cells, which counteracts the binding of GTP[S].

The property of ARF to associate with the crude membrane fraction in the GTP[S]-liganded state offers the possibility of testing whether the phenomenon is a prerequisite for the stimulation of insulin secretion in permeabilized RINm5F cells. The two GTP analogues used in this study, GTP[S] and p[NH]ppG, display similar EC_{50} values for insulin release (about $10\ \mu\text{M}$), in accord with previously published results [13,14]. The dose-response for the association of ARF with the membranes, in the presence of GTP[S], was found to match well the dose-response for insulin secretion. In contrast, such was not the case for p[NH]ppG, where the EC_{50} for membrane association was higher (about $100\ \mu\text{M}$). The difference observed between GTP[S] and p[NH]ppG probably reflects a different affinity of ARF for the two ligands and is presumably not the result of a less stable association of ARF with the membranes in the presence of p[NH]ppG, since the same maximal redistribution can be achieved with $500\ \mu\text{M}$ of this analogue. Interestingly, a similar difference in EC_{50} between these two GTP analogues has been described previously for the inhibition of cell-free transport of vesicular-stomatitis-virus-encoded glycoprotein between successive cisternae of Golgi stacks [24] and the budding of vesicles from the trans-Golgi network [25]. Other GTP-binding proteins, such as G_s [26] and transducin [27], also display a 5–10-fold higher affinity for GTP[S] than for p[NH]ppG. In addition, in permeabilized RINm5F cells the GTP-binding protein involved in inositol trisphosphate production was much less activated by p[NH]ppG than by GTP[S] [14]. Our findings would indicate that the association of ARF with the membrane is not a prerequisite for the guanine-nucleotide-stimulated insulin secretion in permeabilized cells. This conclusion is, however, based on the assumption that only the redistributed ARF is functional and does not preclude another type of activation not involving membrane association. Alternatively, ARF could act only in concert with other GTP-binding proteins. As the short-term assessment of insulin release reflects exocytosis of insulin stored in secretory granules which are ready to fuse with the plasma membrane, this conclusion is not in contradiction with the proposed role for ARF in the transfer of vesicles from the endoplasmic reticulum to the Golgi complex or intra-Golgi transfer [7]. In addition, both intra-Golgi transfer [24] and budding of vesicles (including immature secretory granules) [25] require GTP hydrolysis and are inhibited, not stimulated, by GTP analogues. It is therefore possible that exocytosis, the last step in the secretory pathway, is regulated in a different manner from the preceding steps.

We are indebted to Dr. Y. Takai for his generous supply of the purified *rhoB*, *smg21* and *smg25A* proteins as well as for the antibodies against *smg21* and *smg25A*. We also thank Dr. K. Aktories for providing

the *Clostridium botulinum* C3 exoenzyme. We express our gratitude to Ms. A.-S. Annen for preparing the cultured cells and to Ms. C. Bartley for skilled technical assistance. We are grateful to Dr. J.-M. Theler for performing digital image analysis of the autoradiographs and to Dr. A. Spät for helpful discussions. This work was supported by Grant 32.25665.88 from the Swiss National Science Foundation.

REFERENCES

- Gilman, A. G. (1987) *Annu. Rev. Biochem.* **56**, 615–649
- Burgoyne, R. D. (1989) *Trends Biochem. Sci.* **14**, 394–396
- Segev, N., Mulholland, J. & Botstein, D. (1988) *Cell* **52**, 915–924
- Goud, B., Salminen, A., Walworth, N. C. & Novick, P. J. (1988) *Cell* **53**, 753–768
- Nakano, A. & Muramatsu, M. (1989) *J. Cell Biol.* **109**, 2677–2691
- Kahn, R. A. & Gilman, A. G. (1986) *J. Biol. Chem.* **261**, 7906–7911
- Stearns, T., Willingham, M. C., Botstein, D. & Kahn, R. A. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 1238–1242
- Walworth, N. C., Goud, B., Kabcenell, A. K. & Novick, P. J. (1989) *EMBO J.* **8**, 1685–1693
- Lapetina, E. G., Lacal, J. C., Reep, B. R. & Molina y Vedia, L. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 3131–3134
- White, T. E., Lacal, J.-C., Reep, B., Fischer, T. H., Lapetina, E. G. & White, G. C., II (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 758–762
- Kahn, R. A., Goddard, C. & Newkirk, M. (1988) *J. Biol. Chem.* **263**, 8282–8287
- Weiss, O., Holden, J., Rulka, C. & Kahn, R. A. (1989) *J. Biol. Chem.* **264**, 21066–21072
- Regazzi, R., Li, G., Ullrich, S., Jäggi, C. & Wollheim, C. B. (1989) *J. Biol. Chem.* **264**, 9939–9944
- Vallar, L., Biden, T. J. & Wollheim, C. B. (1987) *J. Biol. Chem.* **262**, 5049–5056
- Ullrich, S. & Wollheim, C. B. (1989) *Acta Endocrinol. (Copenhagen)* **121**, 525–532
- Bhullar, R. P. & Haslam, R. J. (1987) *Biochem. J.* **245**, 617–620
- Mizoguchi, A., Kim, S., Ueda, T. & Takai, Y. (1989) *Biochem. Biophys. Res. Commun.* **162**, 1438–1445
- Aktories, K., Rösener, S., Blaschke, U. & Chhatwal, G. S. (1988) *Eur. J. Biochem.* **172**, 445–450
- Hattori, S., Yamashita, T., Copeland, T. D., Oroszlan, S. & Shih, T. Y. (1986) *J. Biol. Chem.* **261**, 14582–14586
- Willingham, M. C., Pastan, I., Shih, T. Y. & Scolnick, E. M. (1980) *Cell* **19**, 1005–1014
- Rubin, E. J., Gill, D. M., Boquet, P. & Popoff, M. R. (1988) *Mol. Cell. Biol.* **8**, 418–426
- Kawata, M., Matsui, Y., Kondo, J., Hishida, T., Teranishi, Y. & Takai, Y. (1988) *J. Biol. Chem.* **263**, 18965–18971
- Araki, S., Kikuchi, A., Hata, Y., Isomura, M. & Takai, Y. (1990) *J. Biol. Chem.* **265**, 13007–13015
- Melançon, P., Glick, B. S., Malhotra, V., Weidman, P. J., Serafini, T., Gleason, M. L., Orci, L. & Rothman, J. E. (1987) *Cell* **51**, 1053–1062
- Tooze, S. A., Weiss, U. & Huttner, W. B. (1990) *Nature (London)* **347**, 207–208
- Northup, J. K., Smigel, M. D. & Gilman, A. G. (1982) *J. Biol. Chem.* **257**, 11416–11423
- Yamanaka, G., Eckstein, F. & Stryer, L. (1986) *Biochemistry* **25**, 6149–6153

Received 23 October 1990/28 December 1990; accepted 14 January 1991