Regulated Exocytosis in Chromaffin Cells

A POTENTIAL ROLE FOR A SECRETORY GRANULE-ASSOCIATED ARF6 PROTEIN

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The ADP-ribosylation factor (ARF) GTP-binding proteins are believed to function as regulators of vesicular budding and fusion along the secretory pathway. To investigate the role of ARF in regulated exocytosis, we have examined its intracellular distribution in cultured chromaffin cells by subcellular fractionation and immunochemical analysis. We found that ARF6 is specifically associated with the membrane of purified secretory chromaffin granules. Chemical cross-linking and immunoprecipitation experiments suggested that ARF6 may be part of a complex with BY subunits of trimeric G proteins. Stimulation of intact chromaffin cells or direct elevation of cytosolic calcium in permeabilized cells triggered the rapid dissociation of ARF6 from secretory granules. This effect could be inhibited by AlF4-, which selectively activates trimeric G proteins. Furthermore, a synthetic myristoylated peptide corresponding to the N-terminal domain of ARF6 strongly inhibited calcium-evoked secretion in streptolysin-O-permeabilized chromaffin cells. The possibility that ARF6 plays a role in the effector pathway by which trimeric G proteins control exocytosis in chromaffin cells is discussed.

Studies on diverse secretory cell types have highlighted the importance of GTP-binding proteins in Ca2+-regulated exocytosis. Proteins able to bind and hydrolyze GTP can be subdivided into different families including (i) trimeric G proteins, (ii) ras-related low molecular mass G proteins, and (iii) ADP-ribosylation factor (ARF) proteins. Trimeric G proteins have been found associated with the membrane of secretory granules in various secretory cells (1–3), suggesting a role in exocytosis. Indeed, the participation of a plasma membrane-bound Gq protein in the late stages of exocytosis in mast cells has been demonstrated (4). Direct control of exocytosis by Gi and Go proteins has also been described in insulin-secreting cells (5). Thus regulated exocytosis appears as a possible effector pathway for trimeric G proteins although the mechanisms by which this class of G proteins relates to the exocytic machinery remain to be elucidated.

Besides heterotrimeric G proteins, recent studies suggest that a subset of small GTPases of the ras superfamily may also participate in the control of calcium-dependent exocytosis. For example, rab3 isofoms which are specifically localized on the membrane of secretory vesicles (6, 8), have been proposed to serve as a regulator of exocytosis in chromaffin cells (9) and in anterior pituitary cells (10). Other investigations have postulated the participation of ARF in exocytosis (11, 12).

ARF is a monomeric GTP-binding protein that appears to control a wide range of vesicle transport and fusion steps along the secretory pathway. This may include budding, transport, and fusion steps in the Golgi complex (13, 14), in the endoplasmic reticulum (15), and in the endocytotic pathway (16). Initial evidence for the involvement of ARF in the secretory pathway came from genetic studies in yeast Saccharomyces cerevisiae where deletion of the ARF gene resulted in a secretory defect (17). In mammalian cells, ARF proteins are classified into three families according to their size and sequence homology (18). ARF1, ARF2, and ARF3 form class I, ARF4 and ARF5 form class II, and ARF6 forms class III. The best characterized ARF protein is ARF1. It is localized on the Golgi apparatus and is involved in the recruitment of cytosolic coat proteins to membranes during the formation of transport vesicles (19). ARF6 has been recently localized in the endocytotic pathway in transfected mammalian cells, where it appears to control the fusion of endosomes (16).

The aim of the present work is to assess the possible involvement of ARF in regulated exocytosis. We report that ARF6 is specifically associated to the membrane of secretory chromaffin granules, presumably through an interaction with Gβγ subunits. Stimulation of chromaffin cells triggered the dissociation of ARF6 from secretory granules. This dissociation can be blocked by aluminium fluoride, an activator of trimeric G proteins. We propose that ARF may be part of the effector pathway by which trimeric G proteins control the exocytotic pathway in chromaffin cells.

EXPERIMENTAL PROCEDURES

Culture of Chromaffin Cells and [3H]Noradrenaline Release Experiments—Bovine adrenal chromaffin cells were isolated and maintained in primary culture essentially as described previously (6, 20). Cells were loaded with [3H]noradrenaline (14.68 Ci/mmol, Dupont NEN) and washed with Locke’s solution (6). To trigger release from intact cells, chromaffin cells were stimulated for an indicated period of time with Locke’s solution containing 10 μM nicotine (6). The addition of nicotine to chromaffin cells was performed with streptolysin-O (SLO; Institut Pasteur, Paris, France) for 2 min in Ca2+-free KG medium (3). Cells were then stimulated for 10 min, in the presence of the compound to be tested, in KG medium containing 20 μM free Ca2+ and 1 mM free Mg2+ (3).

Subcellular Fractionation of Cultured Chromaffin Cells—Chromaffin granules were purified from cultured chromaffin cells on 1.6 M
The intracellular distribution of ARF proteins in chromaffin cells was estimated by immunoreplica analysis using a monoclinal anti-ARF antibody (1D9). As illustrated in Fig. 1A, ARF was detected in the cytosol and in the Golgi apparatus, displaying an apparent molecular mass of 20 kDa. ARF6 was also found associated to chromaffin granule membranes but with an apparent molecular mass of 18 kDa. The subtype of ARF proteins was further defined with several antibodies able to distinguish between the different classes of ARF. We found that the 20-kDa ARF protein detected in the cytosolic and Golgi fractions was specifically labeled with the affinity-purified anti-ARF1 antibody (Fig. 1A) and with the anti-aARF1I1 antisemur (data not shown) but it was not recognized by the anti-ARF6 antibody (Fig. 1A) or by antisera against ARF5 or ARF6 (data not shown). In contrast, the 18-kDa ARF protein found associated to chromaffin granule membranes was selectively labeled with the anti-ARF6 antibody (Fig. 1A) and with anti-ARF6 antiserm (data not shown). Accordingly, ARF6 migrates slightly faster than ARF1 on SDS-polyacrylamide gel electrophoresis (31).

The specific association of ARF6 with chromaffin granule membranes was further substantiated by immunoblotting analysis of fractions collected from a sucrose density gradient layered with the crude chromaffin membrane pellet. The specific association of ARF6 with chromaffin granule membranes (2.5 mg) was immunoprecipitated with 50 \( \mu \)l of anti-dopamine \( \beta \)-hydroxyase antisemur and 250 \( \mu \)l of ImmunoPure immobilized protein A (Pierce) (7). Proteins linked to the beads were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotting.

Cross-linking of Chromaffin Granule Membranes—Chromaffin granule membranes were cross-linked with 5 \( \mu \)mol disuccinimidyl suberate exactly as described previously (29). Membranes were collected by centrifugation at 100,000 \( \times g \) for 15 min, and proteins were analyzed by SDS-PAGE and immunoblotting.

Electrophoresis and Immunoblotting—One-dimensional SDS-PAGE was performed on 15% acrylamide gels in Tris-glycine buffer (30). Proteins were transferred to nitrocellulose sheets (30) at a constant current of 120 mA for 1 h. Blots were developed with secondary antibodies coupled to horseradish peroxidase (Amerham, Les Ulis, France), and immunoreactive bands were detected with the ECL system (Amersham). The conditions for separation of proteins by electrophoresis on two-dimensional gels were described as previously (30).

Peptides—The synthetic ARF(23–36) peptide CVGLDAAGKTWILYK was obtained from NeoSystem (Strasbourg, France). Myristoylated (myrARF6) and non-myristoylated (nmyrARF6) N-terminal ARF6 peptides (GVKLKSKIFGNKE), non-myristoylated (nmyrARF5) N-terminal ARF5 peptide (GNIFANLRFGL-FKKE) were synthesized in our laboratory (432A Peptide Synthesizer Synergy, Applied Biosystems, Warrington, UK). Purity was checked on high performance liquid chromatography. Sequence analysis was performed by Edman degradation on an automated gas phase protein sequencer (Applied Biosystems, Warrington, UK), and mass spectrometry was used to assess the structure of the final product.

**RESULTS**

**Association of ARF6 with Chromaffin Granule Membranes**—The intracellular distribution of ARF proteins in chromaffin cells was estimated by immunoreplica analysis using a monoclonal anti-ARF antibody (1D9). As illustrated in Fig. 1A, ARF was detected in the cytosol and in the Golgi apparatus, displaying an apparent molecular mass of 20 kDa. ARF6 was also found associated to chromaffin granule membranes but with an apparent molecular mass of 18 kDa. The subtype of ARF proteins was further defined with several antibodies able to distinguish between the different classes of ARF. We found that the 20-kDa ARF protein detected in the cytosolic and Golgi fractions was specifically labeled with the affinity-purified anti-ARF1 antibody (Fig. 1A) and with the anti-aARFII1 antisemur (data not shown) but it was not recognized by the anti-ARF6 antibody (Fig. 1A) or by antisera against ARF5 or ARF6 (data not shown). In contrast, the 18-kDa ARF protein found associated to chromaffin granule membranes was selectively labeled with the anti-ARF6 antibody (Fig. 1A) and with anti-ARF6 antisemur (data not shown). Accordingly, ARF6 migrates slightly faster than ARF1 on SDS-polyacrylamide gel electrophoresis (31).

The specific association of ARF6 with chromaffin granule membranes was further substantiated by immunoblotting analysis of fractions collected from a sucrose density gradient layered with a crude membrane preparation (Fig. 1B). The highest immunosignal for ARF1 was detected in fractions 2 and 3 containing the plasma membranes as estimated by SDS-PAGE with protein A-Sepharose (IP). Control experiments were performed with protein A-Sepharose in the absence of anti-dopamine \( \beta \)-hydroxylase antibodies (C). Precipitated proteins were separated by SDS-PAGE, transferred and analyzed with anti-ARF1 and anti-dopamine \( \beta \)-hydroxylase antibodies (D). Chromaffin granule membranes (1 mg) were separated by two-dimensional SDS-PAGE (15% acrylamide), transferred on nitrocellulose membranes, and probed with anti-ARF6 antibodies (total IgG fraction).
comigrate with chromaffin granules in the sucrose density gradients. Dopamine β-hydroxylase is a specific marker for chromaffin granules. Fig. 1C shows that preincubation of purified granule membranes with an anti-dopamine β-hydroxylase antibody followed by protein A- Sepharose addition resulted in the coadsorption of ARF6 and dopamine β-hydroxylase. Thus ARF6 is an associated component of the chromaffin granule membrane.

Two-dimensional gel electrophoresis of purified chromaffin granule membranes revealed the presence of two spots labeled with the anti-ARF6 antibody. These two spots displayed only a different isoelectric point value (respectively, 7.7 and 7.9), but no difference in the apparent molecular mass (Fig. 1D). Occasionally, the ARF-labeled band present in chromaffin granule membranes could also be resolved into two components on monodimensional gels (Fig. 1A). This observation may indicate some post-translational modification, such as phosphorylation or sulfation of the granule-associated ARF protein, although such modifications have not yet been reported for ARF proteins.

**ARF6 Interacts with the β Subunit of Trimeric G Proteins within Chromaffin Granule Membranes**—The presence of trimeric G proteins on the chromaffin granule membrane (1, 3), together with the recent in vitro data indicating an interaction between ARF and Gβγ subunits (32, 33), encouraged us to examine whether ARF6 may bind to Gβ in chromaffin granule membranes. For this purpose, sodium cholate extracts of granule membranes were incubated with protein A-Sepharose beads coated with affinity-purified antibodies raised against a synthetic peptide corresponding to the consensus ARF(23-36) sequence. As illustrated in Fig. 2A, immunoprecipitation with the polyclonal anti-ARF(23-36) antibody which recognized the chromaffin granule-associated ARF protein, resulted in the coprecipitation of an additional 34-kDa protein labeled with the anti-Gβ antibodies.

We performed protein cross-linking experiments to confirm the possible ARF6-Gβ interaction. Fig. 2B shows that cross-linking of chromaffin granule-associated proteins with disuccinimyl suberate resulted in the appearance of a 50-kDa adduct that reacted with antibodies against both ARF and Gβ. With the exception of bands corresponding approximately to the mass of ARF dimers, no additional adducts were observed (Fig. 2B). Therefore, it is likely that this additional 50-kDa band is due to the cross-linking of ARF6 (18 kDa) to Gβ (34 kDa), which is in agreement with the idea that ARF6 and Gβ may be bound to each other in the granule membrane.

**Effect of the N Terminus ARF6 Synthetic Peptides on Ca2+-evoked Secretion from SLO-permeabilized Chromaffin Cells**—In order to probe the role of ARF6 in the exocytotic pathway, we investigated, in SLO-permeabilized chromaffin cells, the effects of the myristoylated (myrARF6(2–13)) and the nonmyristoylated (ARF6(2–13)) synthetic peptides corresponding in sequence to residues 2–13 of ARF6. MyrARF6(2–13) produced a dose-dependent inhibition in Ca2+-dependent catecholamine secretion with a mean inhibitory dose, ID50, of approximately 40 μM (Fig. 3). The secretory response was almost completely abolished in the presence of 100 μM peptide. In contrast, ARF6(2–13), did not modify Ca2+-evoked secretion (Fig. 3). Neither myrARF6(2–13) nor ARF6(2–13) affected the basal catecholamine release measured in the absence of calcium, indicating that in the range of the tested concentrations, the peptides had no membrane-perturbing effects due to their amphipathic properties. We also examined the effect of a myristoylated synthetic peptide corresponding to the N-terminal region of ARF1 (myrARF1(2–17)). However, myrARF1(2–17) reduced only slightly the Ca2+-evoked secretory response in SLO-permeabilized cells, indicating that the strong inhibition of secretion observed in the presence of myrARF6(2–13) was not due to some unspecific effect of the myristoyl group. Thus,
ARF6 may represent an important component of the regulated exocytotic machinery in chromaffin cells.

**ARF6 Dissociates from Chromaffin Granule Membranes during Exocytosis**—In order to compare the intracellular distribution of ARF6 in resting and stimulated chromaffin cells, secretory granules were purified by sucrose density gradients from a crude membrane fraction obtained either from intact cells stimulated with 10 μM nicotine or from SLO-permeabilized cells challenged with 20 μM free Ca^{2+}. The amount of ARF6 associated to granule membranes was analyzed by immunodetection with the 1D9 anti-ARF antibody (Fig. 4). Stimulation of chromaffin cells strongly reduced the quantity of ARF6 associated with chromaffin granule membranes. In intact cells, nicotine reduced by 70% the amount of ARF6 bound to granules (Fig. 4). In permeabilized cells, calcium decreased the quantity of ARF6 recovered in the chromaffin granule membrane fraction by approximately 60% (Fig. 4). Parallel analysis with the anti-ARF6 antibody revealed a similar decrease in the amount of granule-associated ARF6 in stimulated cells (data not shown). Furthermore, the quantity of ARF6 detected with the 1D9 antibody in the crude membrane fraction remained unchanged in resting and nicotine-stimulated cells (data not shown), indicating that the reduction of ARF6 associated to the granule membrane fraction was not due to a possible degradation or a covalent modification of the protein in stimulated cells. These results suggest that the exocytotic process may be accompanied by the translocation of ARF6 from the secretory granule to some unknown subcellular compartment. Since ARF6 was not detected among the cytosolic proteins that escape through the pores created in the plasma membrane of permeabilized cells (data not shown), we assume that ARF6 translocates from the granule membrane to another membrane-bound compartment in stimulated cells.

We examined the time course of ARF6 translocation in nicotine-stimulated cells. As illustrated in Fig. 5, the dissociation of ARF6 from the chromaffin granules was rapid and almost complete within 1 min of stimulation. By comparison, maximal [^3H]noradrenaline release required 2–3 min of stimulation (Fig. 5), suggesting that the dissociation of ARF6 may represent an early event in the pathway of regulated exocytosis. Note that Gβ remained attached to the granule membrane during the entire stimulation period (Fig. 5).

The presence of Gβ₃ on the chromaffin granule membrane (3), which displays a high affinity for Gβγ and may thereby compete with ARF for the binding to Gβ, (32, 33), led us to investigate whether AlF₄⁻ may interfere with the translocation of ARF6 in stimulated cells. AlF₄⁻ is an activator of trimeric G proteins which prevents the association of the α subunit with βγ subunits (34). In the presence of AlF₄⁻, the amount of ARF6 detected on chromaffin granule membranes was comparable in resting and calcium-stimulated cells (Fig. 6), indicating that...
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ARF6 was able to inhibit the translocation of ARF6 induced by a rise of cytosolic calcium.

Studies with purified proteins indicate that ARF binds to Gβγ in its GDP-ligated form (32, 33). We examined the effect of the nonhydrolyzable GTP analogue, GTPγS, on the attachment of ARF6 to chromaffin granule membranes. As shown in Fig. 6, the presence of GTPγS in the incubation medium did not significantly affect the calcium-induced dissociation of ARF6 from secretory granules in permeabilized chromaffin cells.

DISCUSSION

The current general model of vesicular fusion suggests that the so-called SNARE complex functions in many fusion events throughout the secretory pathway, including transport at the endoplasmic reticulum and the Golgi apparatus, and exocytotic and endocytotic fusions (35). Cumulative evidence also implicates ARF as a molecular switch in the regulation of vesicular transport and fusion through early compartments of the secretory pathway (reviewed in Refs. 19, 36, and 37). These apparent similarities of the mechanisms involved in intracellular vesicular trafficking led us to investigate whether ARF may also be a component of the regulated exocytotic machinery. We report here the presence of an immunologically related ARF6 protein on the membrane of purified secretory chromaffin granules. Stimulation of chromaffin cells by a choleric agonist or a direct elevation of cytosolic calcium triggers a rapid decrease of the amount of ARF6 associated to granule membranes, suggesting that exocytotic fusion is accompanied by the release of ARF6 from secretory granules in chromaffin cells. It is noteworthy that a similar sequence of events has been described in the Golgi where the release of ARF from vesicles seems to be a prerequisite for fusion with the acceptor membrane (35).

ARF was originally discovered as a cofactor required for the ADP-riboseylation by cholera toxin of the heterotrimeric G protein Gα (39). Further studies confirmed the close relationships between trimeric G proteins and ARF. For example trimeric G proteins seem to regulate the association of ARF and coat components onto the Golgi membrane (23, 40, 41). A collaboration among trimeric G proteins and ARF has also been suggested in the control of endosomal fusion (32). Finally direct interactions between ARF and the Gβγ subunits have been described (32, 33). Indeed, ARF proteins have more in common with G protein α subunits than with other small GTPases when comparing GTP-binding consensus sequences and cotranslational modifications such as myristoylation of the N terminus (37). By cross-linking and communoprecipitation experiments, we observed here that ARF6 interacts with Gβ in chromaffin granule membranes. Since Gβ exists only in close association with Gγ (38), we propose that the Gβγ complex may represent the membrane receptor that could stabilize the interaction of ARF6 with secretory granules.

In chromaffin cells, trimeric G proteins control the ATP-dependent priming step and the late calcium-regulated fusion event of exocytosis (3, 6). However, their downstream effectors in the exocytotic machinery remain unknown. We found that ARF6 can prevent the calcium-induced uncoupling of ARF6-Gβγ on the secretory granule membrane in stimulated cells. Chromaffin granule membranes contain both Gα and Gβγ subunits (3). Thus, upon the activation by ARF6-Gα, Gα is likely to dissociate from Gβγ which may thereby provide a site of interaction for ARF6 and prevent its translocation in response to calcium stimulation. Conversely, calcium may trigger the dissociation of ARF6 from the granule membrane by allowing the reassociation of Gα with Gβγ which becomes then unable to retain ARF6. ARF6, as well as mastoparan, inhibit calcium-evoked catecholamine secretion in permeabilized chromaffin cells by a mechanism which can be selectively reversed by anti-Gα antibodies (6). This suggests that Gα blocks the exocytotic machinery when activated and dissociated from Gβγ. Thus, our data support a model in which exocytosis requires the inactivation of the granule-associated Gα. In stimulated cells, the activation of Gα may be impaired by a calcium-dependent process and Gα reassociates with Gβγ. This triggers the release of ARF6 from Gβγ, an event which may be necessary for the subsequent exocytotic steps to occur.

We investigated the possible function of ARF6 in the exocytotic machinery by using synthetic peptides corresponding in sequence to the N-terminal residues of ARF1 and ARF6. The N-terminal region of ARF was chosen because it has been implicated as the putative effector domain. Indeed, the N terminus of ARF seems to be critical for its function since deletion of this region results in a global reduction of ARF activities (42). Furthermore, N-terminal ARF peptides have proved to be potent inhibitors of ARF functions, including endoplasmic reticulum to Golgi transport, intra-Golgi transport and endocytotic vesicle fusion (15, 40, 42, 43). The strong inhibition of calcium-evoked catecholamine secretion by the myristoylated ARF6(2-13) peptide implicates ARF6 as a key component of the exocytotic pathway in chromaffin cells. Recent studies in HL60 cells have shown that ARF contributes to the restoration of secretion in permeabilized cytosol-depleted cells (44). Hence, the data presented here are convergent with the idea that ARF proteins may play a general role in the exocytotic pathway in endocrine and neuroendocrine cells.

In conclusion, based on subcellular fractionation techniques and immunological detection, the present results strongly suggest that ARF6 dissociates from secretory granules in stimulated chromaffin cells. Thus, an important future undertaking will be to confirm this observation by immunofluorescence and confocal microscopy since we cannot completely rule out some artefactual redistribution due to cell lysis. Nevertheless, the finding that ARF6 interacts with Gβ in the secretory granule membrane offers an attractive speculation: the dissociation of ARF6 from secretory granules may allow the interaction of ARF6 with its nucleotide exchange factor resulting in activation in the target membrane. In view of the current proposed functions for ARF proteins in vesicular transport, ARF6 is a good candidate to be involved in the assembly of a multisubunit complex required for fusion or to contribute to the generation of fusogenic lipids at the plasma membrane.

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