Identification of a Plasma Membrane-associated Guanine Nucleotide Exchange Factor for ARF6 in Chromaffin Cells

POSSIBLE ROLE IN THE REGULATED EXOCYTOTIC PATHWAY*

Anne-Sophie Caumont‡, Nicolas Vitale, Marc Gensse, Marie-Christine Galas, James E. Casanova§, and Marie-France Bader¶

From the INSERM, U-338 Biologie de la Communication Cellulaire, 5 rue Blaise Pascal, 67084 Strasbourg Cedex, France and the §Combined Program in Pediatric Gastroenterology and Nutrition, Massachusetts General Hospital East, Charlestown, Massachusetts 02129

ADP-ribosylation factors (ARFs) constitute a family of structurally related proteins that forms a subset of the Ras superfamily of regulatory GTP-binding proteins. Like other GTPases, activation of ARFs is facilitated by specific guanine nucleotide exchange factors (GEFs). In chromaffin cells, ARF6 is associated with the membrane of secretory granules. Stimulation of intact cells or direct elevation of cytosolic calcium in permeabilized cells triggers the rapid translocation of ARF6 to the plasma membrane and the concomitant activation of phospholipase D (PLD) in the plasma membrane. Both calcium-evoked PLD activation and catecholamine secretion in permeabilized cells are strongly inhibited by a synthetic peptide corresponding to the N-terminal domain of ARF6, suggesting that the ARF6-dependent PLD activation near the exocytotic sites represents a key event in the exocytotic reaction in chromaffin cells. In the present study, we demonstrate the occurrence of a brefeldin A-insensitive ARF6-GEF activity in the plasma membrane and in the cytosol of chromaffin cells. Furthermore, reverse transcriptase-polymerase chain reaction and immunoreplica analysis indicate that ARNO, a member of the brefeldin A-insensitive ARF-GEF family, is expressed and predominantly localized in the cytosol and in the plasma membrane of chromaffin cells. Using permeabilized chromaffin cells, we found that the introduction of anti-ARNO antibodies into the cytosol inhibits, in a dose-dependent manner, both PLD activation and catecholamine secretion in calcium-stimulated cells. Furthermore, co-expression in PC12 cells of a catalytically inactive ARNO mutant with human growth hormone as a marker of secretory granules in transfected cells resulted in a 50% inhibition of growth hormone secretion evoked by depolarization with high K⁺. The possibility that the plasma membrane-associated ARNO participates in the exocytotic pathway by activating ARF6 and downstream PLD is discussed.

ADP-ribosylation factors (ARFs) are a group of structurally related GTP-binding proteins that form a subset of the Ras superfamily of regulatory GTP-binding proteins. ARFs are ubiquitous in eukaryotic cells with an amino acid sequence that is highly conserved across diverse species, suggesting a fundamental role in cellular physiology. Initially discovered as cofactors of the cholera toxin-catalyzed ADP-ribosylation of Goα (1), ARFs appear to be critical to vesicular trafficking in various subcellular compartments of the cell (2). More recently, members of the ARF family have been shown to activate phospholipase D (PLD) in several cellular systems as well as in isolated membranes (3–5). The ARF family has been divided into three classes based on size, sequence homology, gene structure, and phylogenetic analysis. Class I ARFs (ARFs 1–3) were initially identified as components of vesicles that originate from the Golgi (6, 7) and the endoplasmic reticulum (8), whereas class III ARF6, which is the most structurally divergent member of the family, has more recently been implicated in the exocytotic and endocytotic pathways at the plasma membrane (9–12). Little is known about class II ARF4 and ARF5.

Like other G proteins, ARFs cycle between a GDP-bound and a GTP-bound conformation. The GTP-induced conformational change is the “on” signal that permits the ARF proteins to bind to and activate specific protein effectors. Isolated ARFs have little detectable GTPase activity and exchange bound nucleotide very slowly. In cells, their GTPase cycle requires an interaction with GTPase-activating proteins and guanine nucleotide exchange factor (GEFs) which catalyze the nucleotide exchange activity on ARF. The identification of ARF1 GEFs was facilitated by the discovery that the fungal metabolite brefeldin A (BFA) disrupts Golgi trafficking by inhibiting a Golgi-associated ARF1 exchange factor (13). Several GEF activities have been described, but the breakthrough toward the identification of GEFs acting on ARF proteins was the cloning of two related BFA-sensitive ARF1 GEFs-encoding genes in yeast Saccharomyces cerevisiae, Gea1 and Gea2 (14). This lead to the discovery of cytohesin-1 (15). ARNO (ARF nucleotide-binding-site opener, Ref. 16) and GRP1 (17) which promote guanine nucleotide exchange on ARF1 by a BFA-insensitive catalytic mechanism. Subsequent studies demonstrated that ARNO, GRP1, and cytohesin-1 can also promote GDP/GTP exchange on ARF6 in both cell free and intact cell assays.
(18–20). Recently, Franco and co-workers (21) isolated a novel protein, EFA6, which triggers rapid and efficient exchange on ARF6, and it was suggested that EFA6 represents the first identified ARF6-specific GEF. All known ARF GEFs share a conserved central region, the Sec7 domain, responsible for their exchange activity and displaying a high degree of homology with the yeast sec7p, a molecule required for membrane traffic from the yeast (22). Similarities between the ARNO-related GEFs comprise also a coiled coil N terminus and a C-terminal plekstrin homology domain required for phosphoinositide binding and phosphatidylinositol (4,5)P₂-dependent activation of ARNO exchange activity (16, 17).

We recently described the presence of a secretory granule-associated ARF6 protein in adrenal medullary chromaffin cells (11). Secretagogue-evoked stimulation of chromaffin cells triggers the rapid translocation of ARF6 from secretory granules to the plasma membrane and the concomitant activation of PLD in the plasma membrane (23). The observation that both calcium-evoked PLD activation and calcium-induced catecholamine secretion can be inhibited by a synthetic peptide corresponding to the N-terminal domain of myristoylated ARF6, led us to propose that ARF6 participates in the exocytotic reaction by controlling a plasma membrane-bound PLD and the generation of fusogenic lipids at the exocytic sites (23). Here, we examine the intracellular localization and function of the nucleotide exchange factor for ARF6 in chromaffin cells in order to identify the partners of ARF6 in calcium-evoked secretion. Our data reveal the presence of a BFA-insensitive ARNO-related GEF activity for ARF6 in both cytosolic and plasma membrane-bound fractions. Furthermore, the introduction of anti-ARNO antibodies into the cytosol of permeabilized chromaffin cells strongly inhibited calcium-evoked PLD activation and catecholamine secretion. In line with this observation, overexpression of a catalytically inactive ARNO mutant reduced to a similar extent stimulated exocytosis in PC12 cells. We propose that ARNO plays a critical role in the exocytotic pathway in chromaffin cells, by controlling the ARF6 GTPase cycle at the plasma membrane, thereby enabling the ARF6-dependent activation of PLD.

**EXPERIMENTAL PROCEDURES**

**[3H]Noradrenaline Release from Permeabilized Chromaffin Cells—** Chromaffin cells were isolated from fresh bovine adrenal glands and maintained in primary culture essentially as described previously (24). Cells were usually cultured as monolayers on either 24 or 24 multiple 16-mm Costar plates (Cambridge, MA) at a density of 2.5 × 10⁶ cells/well or 100-mm Costar plates at a density of 5 × 10⁶ cells/plate. Catecholamine stores were labeled by incubating 3–7-day-old cultured chromaffin cells according to the manufacturer’s instruction. After 5 h of incubation at 37 °C, 1 ml of culture medium with fetal bovine serum, horse serum, and antibiotics was added. Expression of GH, ARNO, or ARNO mutant proteins was assessed after 48 h by immunocytochemistry. Therefore, PC12 cells were fixed for 15 min in 4% paraformaldehyde in 0.12 M sodium phosphate, pH 7.5, and plasma membranes were recovered from fractions 2 and 3 containing the highest Na⁻/K² ATPase activity (25). The cytosol and the Golgi membranes were recovered from fractions 10 and 11 containing the highest DjH activity (25). The cytosol and the Golgi membranes were recovered from the 20,000 × g supernatant. After centrifugation at 100,000 × g for 45 min, the supernatant (cytosol) and the pellet (enriched in Golgi membranes) were saved and protein content was determined.

**Nucleotide Exchange Assays—** Nucleotide exchange assays were performed essentially as described (26, 27). Briefly, chromaffin cell subcellular fractions (20 μg) were incubated with 1 μM GTPγS (~5 × 10⁶ cpm) in 15 mM Tris/HCl, pH 7.2, 100 mM NaCl, 1 mM EDTA, 0.5 mM MgCl₂, 1 mM dithiothreitol, 50 μg/ml bovine serum albumin, and 30 μg/ml phosphatidylserine (final volume 50 μl). Reactions were initiated by adding recombinant myristoylated ARF6 (1 μM) at 37 °C. When indicated, the reaction was terminated by addition of 2 ml of ice-cold 20 mM Tris/HC1, pH 8, 100 mM NaCl, 25 mM MgCl₂.
Protein bound radioactivity was determined by nitrocellulose filter trapping (28). Nonspecific binding to nitrocellulose was estimated with 3 μCi [35S]GTP•S (5 × 10^5 cpm) and this value was subtracted from all determinations. Specific binding to ARF6 was determined by measuring, in a parallel set of samples, the binding in the absence of ARF6 and this value was subtracted from the total binding measured in the presence of ARF6. Results shown are representative of three independent experiments.

RNA Isolation and Amplification of cDNA—Total RNA was isolated from cultured chromaffin cells (1.3 × 10^6 cells/assay) according to Chomczynski and colleagues (29). Total RNA (1 μg) or purified poly(A) RNA (50 μg), prepared with the Micro-FastTrack kit from Invitrogen, were transcribed into cDNA with Superscript RTase H•Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.) and an adapter primer (see Table I) from Life Technologies, Inc. or 6-nucleotide random primers. An aliquot (2 μl) of the cDNA produced was used as a template for PCR (total volume of 50 μl) using Taq polymerase (MBI fermentas) and specific primers. Expression of ARNO cDNA was tested with primers arno, for and arno, rev (Table I). Expression of EFA6 cDNA was tested with primers efa, for1 and efa, rev1 or 2, and efa, for2 with efa, rev2 (Table I), according to Franco et al. (21). After 5 min of denaturation at 90 °C, 35 cycles of the following profile were done: 94 °C for 1 min, 45 °C or 60 °C for 1 min, and 72 °C for 2 min. Finally, an extension step of 10 min was added. The PCR products were purified using the CleanPrep Kit (Talent, cloned using the pMOSSBlue T-vector Kit (Amersham Pharmacia Biotech), and transformed into Escherichia coli. The plasmids were isolated with Qiagen columns and sequenced on an automated system (Applied Biosystems).

cDNA Cloning and Preparation—The human ARNO cDNA was cloned in the cytomegalovirus-based mammalian expression vector pCB7 as described earlier (19). The E156K mutation in ARNOpCB7 was protected with the ECL system (Amersham Pharmacia Biotech). and immunoreactive bands were detected with secondary antibodies coupled to horseradish peroxidase and developed with 3,3′-diaminobenzidine as a chromogen (Torrance, CA).

Antibodies and Recombinant Proteins—Polyclonal anti-ARNO antibody was raised in rabbits with His6-tagged recombinant human ARNO kit (Qiagen). Anti-ARNO Fab fragments were prepared by incubating 100 μg of IgG with 1 μg of papain in a buffer containing 100 mM sodium phosphate, pH 7.5, 2 mM EDTA, 10 mM cysteine for 20 h at 37 °C as described (30). Fab fragments were separated from Fc fragments and intact IgGs using protein A-Sepharose (30). Rabbit polyclonal antibodies against dopamine-β-hydroxylase (DbH) were prepared in our laboratory and their specificity demonstrated (31). Rabbit polyclonal anti-N-CAM antibodies were kindly provided by Dr. Philippe Chavrier (Institut Curie, Paris). Rabbit polyclonal anti-human ARNO antibodies were generously provided by Dr. A. F. Parlow and the NIDDKs National Hormone and Pituitary Program (Torrance, CA).

Recombinant myristoylated ARF6 protein (myrARF6) was produced in a bacterial expression system containing the N-myristoyl transferase in the plasmid pACYC177/T7T3d, and purified according to the procedure previously described (32). Recombinant ARNO was expressed as described (18). Recombinant ARNOSec7 domain was cloned in the plasmid pQE30, expressed, and purified as a His6 fusion protein as described (33).

Protein Determination, Electrophoresis, and Immunoblotting—Protein concentration was routinely determined using the Bradford procedure with Bio-Rad Dye reagent and bovine serum albumin as standard. One-dimensional SDS-gel electrophoresis was performed on 12% acrylamide gels in Tris glycine buffer. Proteins were transferred to nitrocellulose sheets at a constant current of 25 mA for 1 h. Blots were developed with secondary antibodies coupled to horseradish peroxidase (Amersham Pharmacia Biotech) and immunoreactive bands were detected with the ECL system (Amersham Pharmacia Biotech).

RESULTS

Subcellular Localization of the Nucleotide Exchange Activity for ARF6 in Chromaffin Cells—To determine the intracellular localization of the ARF6 GEF activity in chromaffin cells, we measured the ability of various subcellular fractions to catalyze the binding of guanosine 5′-γ-thiotriphosphate ([35S]GTP•S) on recombinant myristoylated ARF6 (myrARF6). The common characteristic of the ARF exchange factors is the centrally located Sec7 domain essential for GEF activity. Fig. 1A illustrates the time course for nucleotide exchange on myrARF6 catalyzed by the recombinant ARNOSec7 domain. At a physiological concentration of Mg2+ (5 mM), ARNOSec7 promoted a rapid binding of [35S]GTP•S to myrARF6 which reached a maximal rate within 10 min. Using these experimental conditions, we measured the nucleotide exchange activity present in the cytosol or associated with the plasma membrane or secretory granule membrane prepared by subcellular fractionation of cultured chromaffin cells. As shown in Fig. 1B, chromaffin granule membranes were unable to catalyze nucleotide exchange on myrARF6. In contrast, substantial GEF activity was found in both cytosolic and plasma membrane-bound fractions (Fig. 1B). Note that boiling completely abolished the ARF6 GEF activity detected in these two fractions (data not shown). These results suggest that the GDP-GTP exchange factor for ARF6 is localized both on the plasma membrane and in the cytosol of chromaffin cells.

Exchange factors for ARF proteins can be divided into two classes depending on their size and susceptibility to brefeldin A of their Sec7 domain: the large ~200-kDa GEFs (Sec7, Gae1, and Gae2) are inhibited by brefeldin A, whereas those of the cytohesin family (~55 kDa) are brefeldin A-insensitive (34, 35). To further characterize the type of ARF6 GEF activity present on the plasma membrane and in the cytosol in chromaffin cells, we examined the effect of brefeldin A. We found that the nucleotide exchange on myrARF6 catalyzed by either the plasma membrane or the cytosol was not significantly affected by the addition of 300 μM brefeldin A (Fig. 1B). This observation indicates that the ARF6 GEF activity present in chromaffin cells is likely to belong to the cytohesin family.

Presence and Distribution of ARNO in Chromaffin Cells—The brefeldin A-insensitive GEFs include ARNO, cytohesin-1, GRP1/ARNO3, and the more recently identified, EFA6. Since both ARNO and EFA6 have been reported to catalyze nucleotide exchange on ARF6, we investigated the presence of their
mRNA in chromaffin cells using RT-PCR. cDNA from bovine chromaffin cells were prepared from total RNA using random primers or the oligo(dT) adapter primer (Table I). Expression of ARNO and EFA6 was then tested by PCR with the specific primers described in Table I. An intense band of 1200 bp, corresponding to the size of the ARNO cDNA (Fig. 2A) was detected with an annealing temperature of 60 °C in both cDNA preparations. Sequencing of this band confirmed that it corresponded to ARNO and not to cytohesin-1 or GRP1/ARNO3 (data not shown). The bovine ARNO cDNA sequence presents 96% identity with the published human ARNO sequence (16, 18), indicating a high degree of conservation across species of the ARNO gene. In contrast, we were not able to detect any product corresponding to the entire EFA6 cDNA (expected size of 1970 bp) or to EFA6 fragments (5'-terminal 1250-bp or 3'-terminal 720-bp fragments) with the PCR conditions described above or when the annealing temperature was decreased to 40 °C. Moreover, expression of EFA6 mRNA was not detected by RT-PCR when purified poly(A)+ RNA were used.

The intracellular localization of ARNO in various subcellular fractions of chromaffin cells was analyzed by immunodetection on nitrocellulose sheets (Fig. 2). We found that ARNO was predominantly localized in the cytosol and in the upper fractions of a sucrose density gradient layered with a crude membrane preparation. The presence of the peak of ATPase Na/K activity (25) and the cell surface adhesion protein N-CAM (Fig. 2D) indicated that these fractions were enriched in plasma membranes. Note that ARNO was not detected in the fractions containing secretory granules identified by the presence of dopamine-β-hydroxylase (Fig. 2D). To evaluate the portion of ARNO present in the cytosol, cultured chromaffin cells were collected, and the content of ARNO was estimated in three fractions defined as the total homogenate, the cytosol and the upper fractions defined as the total homogenate, the cytosol and the upper fractions defined as the total homogenate, the cytosol and the upper fraction (25 ng) from cytosol, plasma membranes, and chromaffin granule membranes were separated by SDS-gel electrophoresis and analyzed by immunoblotting with anti-EFA6 antibodies. Recombinant EFA6 protein (20 μg) was loaded in an adjacent lane. D, fractions 3–12 (100 μg of protein/fraction) collected from a continuous sucrose density gradient layered with the crude chromaffin membrane pellet were subjected to gel electrophoresis and immunodetection on nitrocellulose sheets using either anti-N-CAM antibodies to detect plasma membranes, or anti-DJ1 antibodies to detect chromaffin granules or anti-ARNO antibodies. Recombinant His-ARNO (25 ng) was loaded in an adjacent lane for comparison. Note that ARNO is exclusively detected in the fractions containing plasma membranes.

Ca2+-evoked PLD activation and catecholamine secretion in permeabilized chromaffin cells. In the range of concentrations tested (0–50 μM), breFredolin A modified neither the basal nor the calcium-evoked [3H]noradrenaline release (data not shown). It also had no effect on either basal or calcium-stimulated PLD activity as estimated by the formation of [3H]PEt (data not shown). Thus, the exchange factor required in the exocytotic machinery for ARF6 activation and subsequent PLD stimulation is likely to belong to the brefredolin A-insensitive cytohesin family. This finding correlates well with the presence of ARNO at the plasma membrane in chromaffin cells.
To further integrate ARNO in the exocytotic pathway, we investigated the effect of anti-ARNO antibodies on catecholamine secretion in chromaffin cells. When introduced into the cytosol of permeabilized cells, anti-ARNO antibodies induced a dose-dependent inhibition of the Ca\(^{2+}\)-evoked secretory response without modifying basal noradrenaline release, estimated in the absence of calcium (Fig. 3A). Neither preimmune serum nor anti-ARNO antibodies preincubated with an excess of recombinant ARNO, significantly affected calcium-dependent catecholamine secretion (Fig. 3B). It is also interesting to note that the presence of anti-EFA6 antibodies in the cytosol did not reduce the calcium-stimulated secretory activity in streptolysin-O-permeabilized cells (Fig. 3B). To exclude a possible effect resulting from the bivalence of native immunoglobulins, Fab fragments were tested for their effect on secretory activity. As illustrated in Fig. 3C, the incubation of permeabilized chromaffin cells with increasing concentrations of anti-ARNO Fab fragments also resulted in inhibition of calcium-evoked catecholamine secretion, reaching a value comparable to that obtained with native immunoglobulins.

We then examined the effect of the anti-ARNO and anti-EFA6 antibodies on PLD activity in stimulated chromaffin cells. We found a close correlation between their effects on PLD activation and their ability to inhibit catecholamine secretion in stimulated cells. Indeed, only anti-ARNO antibodies were able to inhibit the formation of \(^{3}H\)PEt and \(^{3}H\)PA in permeabilized cells stimulated with 20 \(\mu\)M free calcium (Fig. 4). In contrast, preimmune serum or anti-EFA6 antibodies had no significant effect on calcium-stimulated PLD activation. These results suggest that ARNO seems to be implicated in regulated secretion in chromaffin cells most likely by activating a pathway leading to the stimulation of PLD. Since both ARNO, ARF6, and the activated PLD are located at the plasma membrane following stimulation (Ref. 23; and the present report), it is tempting to propose that ARNO is the upstream regulator of the ARF6-dependent PLD required for exocytosis in chromaffin cells.

Co-expression of ARNO or a Catalytically Inactive ARNO Mutant with Human GH in PC12 Cells—To further probe the implication of ARNO in the molecular machinery underlying dense-core granule exocytosis, we performed transient transfection studies in PC12 cells. Co-transfection with a plasmid encoding GH has been used by several investigators to study proteins involved in regulated exocytosis (36–38). GH following PC12 cell transfection is targeted to dense-core granules (Fig. 5A) and it has been established that secretagogue-evoked GH release shows all the characteristics expected for dense-core granule exocytosis (39). In addition, the assay offers the advantage to analyze secretion in only those cells that take up the plasmids. In the present experiments, we estimated by immunocytochemistry with anti-GH antibodies, that 5 to 15% of cells were transfected. Previous studies established that more than 90% of the cells expressing GH also express the second protein (36, 37). We have confirmed this high efficiency of co-expression with a plasmid encoding the green-fluorescent protein. Essen-

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**Fig. 3.** Effect of anti-ARNO antibodies on Ca\(^{2+}\)-evoked catecholamine secretion in streptolysin-O-permeabilized chromaffin cells. A, chromaffin cells were permeabilized with streptolysin-O for 4 min in the presence of the indicated concentrations of anti-ARNO antibodies. Cells were then incubated for 10 min with Ca\(^{2+}\)-free KG medium (open circles) or stimulated for 10 min with KG medium containing 20 \(\mu\)M free Ca\(^{2+}\) (closed circles). B, cells were permeabilized with streptolysin-O in the absence (Control) or presence of 0.2 \(\mu\)g/ml of anti-ARNO antibodies (Anti-ARNO), anti-ARNO antibodies preincubated with an excess of recombinant ARNO (Preabsorbed anti-ARNO), preimmune serum (Preimmune), or anti-EFA6 antibodies (Anti-EFA6). Subsequently, cells were stimulated with KG medium containing 20 \(\mu\)M free calcium. Basal release estimated in the absence of calcium was not modified by the antibodies and was subtracted. Results are expressed relative to the net \(^{[3]}H\)noradrenaline release obtained in control cells. Preabsorbed anti-ARNO was prepared by incubating the antiserum with human recombinant ARNO transferred on nitrocellulose strips until no immunoreactivity against ARNO could be detected in the serum. C, cells were permeabilized with streptolysin-O for 4 min in the presence of the indicated concentrations of anti-ARNO Fab fragments (squares) or protein A-bound material containing Fc fragments and the remaining IgGs (circles). Cells were subsequently stimulated for 10 min with Ca\(^{2+}\)-free KG medium (open symbols) or KG medium containing 20 \(\mu\)M free Ca\(^{2+}\) (closed symbols).

**Fig. 4.** Effect of anti-ARNO antibodies on Ca\(^{2+}\)-evoked PLD activation in streptolysin-O-permeabilized chromaffin cells. Chromaffin cells labeled with \(^{[3]}H\)myristic acid were permeabilized with streptolysin-O in the absence (Control) or presence of anti-ARNO antibodies, preimmune serum, or anti-EFA6 antibodies at a final concentration of 0.2 \(\mu\)g/ml. Cells were then stimulated for 10 min with KG medium containing 20 \(\mu\)M free Ca\(^{2+}\) and 1% ethanol. Phospholipids were subsequently extracted from cells and analyzed by thin layer chromatography. The formation of \(^{[3]}H\)PEt or \(^{[3]}H\)PA is expressed as a percentage of total counts recovered in extracted lipids. The net PLD activity was obtained by subtracting the basal unstimulated PLD activity formed in the absence of calcium (\(^{[3]}H\)PEt: 0.04 \(\pm\) 0.002% of total counts; \(^{[3]}H\)PA: 0.07 \(\pm\) 0.01% of total counts). Data are expressed as the mean \(\pm\) S.E. (n = 3).
ARNO in the Exocytotic Pathway

Effect of the overexpression of wild-type ARNO or catalytically inactive ARNO(E156K) mutant on secretion of co-expressed GH from PC12 cells. PC12 cells were transfected (4 μg/well of each plasmid) with pCB7 (Control), pCB7-ARNO, or pCB7-ARNO (E156K) plasmids along with plasmid (4 μg/well) encoding GH. 48 h after transfection, cells were washed and subsequently stimulated for 10 min with a depolarizing concentration of K+. Basal release was estimated by incubating cells for 10 min with Locke’s solution. A, intracellular distribution of GH in transfected PC12 cells. Immunofluorescence confocal micrographs with anti-GH antibodies (diluted 1:100) detected with cyanine 2-conjugated anti-rabbit antibodies (diluted 1:2000). Optical sections were taken through the center of the nucleus. B, total GH content per well estimated by radioimmunoassay (n = 6). C, GH secreted into the extracellular medium and expressed as a percentage of total GH present in each well before stimulation. The K+-evoked secretory response was obtained by subtracting the basal release from the release measured in the presence of 50 mM K+. Control, ARNO-, and ARNO (E156K)-transfected PC12 cells incubated in Locke’s solution released within 10 min 13.6 ± 0.4, 13.3 ± 0.3, and 13.2 ± 0.3% of the total GH, respectively. Data are given as the mean values ± S.E. (n = 6). Similar results were obtained in three independent experiments done with different cell cultures. *p < 0.001 when tested by Student’s test.

Data are given as the mean values ± S.E. (n = 6). Similar results were obtained in three independent experiments done with different cell cultures. *p < 0.001 when tested by Student’s test.

Discussion

ARF proteins have traditionally been thought to play a role in the regulation of intracellular membrane trafficking (40). ARF1, which is the best characterized of the six mammalian ARFs, is recruited from the cytosol to the Golgi complex where it mediates the binding of coat proteins and adaptins to Golgi membranes (2, 12, 41). It is clear from recent work that ARF6 serves distinct functions in eukaryotic cells. In contrast to ARF1 which is generally associated with the Golgi complex, ARF6 has been localized at the plasma membrane where it is likely to modulate some aspects of the vesicular trafficking to and from the cell surface (9, 10, 12, 43). Several lines of evidence also support a unique role for ARF6 in regulating cortical actin structure and function (44, 45). In view of the convergence in molecules and mechanisms underlying the multiple steps of intracellular vesicular transport, we previously investigated the possible function of members of the ARF family in calcium-regulated exocytosis (11, 23). Our findings suggested that a granule-bound ARF6 plays a direct role in the late stages of exocytosis, most likely by stimulating a PLD activity located at the plasma membrane.

Important to our understanding of ARF6 function in the exocytotic pathway will be the identification of the factors which catalyze nucleotide exchange (GEFs) and GTP hydrolysis (GTPase-activating proteins). In the present study, we report the presence of a GEF activity for ARF6 in the cytosol and at the plasma membrane of chromaffin cells. It is worth noting that no GEF activity could be detected on purified secretory granules. This observation suggests that ARF6 is in its inactive GDP-bound conformation when associated with the chromaffin granule membrane. Previous results obtained by chemical cross-linking and immunoprecipitation indicated that the membrane receptor that stabilizes the interaction of ARF6 with chromaffin granules is likely to be the Gβγ subunits of trimeric G protein (11). In line with these results, it has been previously reported that the interaction between ARF1 and Gβγ is favored when ARF1 is in its GDP-ligated form (46, 47). Because ARF6 is apparently not cytosolic in chromaffin cells (11), it may be predicted that ARF6 undergoes its GTPase cycle and becomes activated at the plasma membrane where its GEF appears to be located. This idea correlates well with our previous observations showing the activation of an ARF6-regulated PLD at the plasma membrane in secretagogue-stimulated cells (23). Taken together, our results support a model in which docking of secretory granules to the exocytic sites at the plasma membrane upon cell stimulation allows ARF6 to transiently switch from the granular Gβγ complex to the plasma membrane-associated GEF. Formation of GTP-bound ARF6 subsequently activates downstream effectors (s) in the exocytic machinery, in particular the ARF6-regulated PLD present at the plasma membrane (23). In view of the strong inhibition induced by the synthetic N-terminal domain of ARF6 on both PLD activation and catecholamine secretion in permeabilized chromaffin cells (11, 23), ARF6-dependent activation of the plasma membrane-associated PLD is likely to represent a key event in the exocytic pathway.

We report here that the brefeldin A-insensitive GEF, ARNO, colocalizes with ARF6 at the plasma membrane in stimulated
ARNO in the Exocytotic Pathway

15643

chromaffin cells. Indeed, both PLD activation and catecholamine secretion in permeabilized chromaffin cells are insensitive to brefeldin A. In agreement, recent work indicates that brefeldin A disrupts the Golgi membranes, but does not affect the pattern of catecholamine release monitored by carbon fiber amperometry nor does it modify the calcium sensitivity, granule mobilization, or initial rate of exocytosis in single rat chromaffin cells (48). ARNO-related proteins, first characterized as efficient exchange factors for ARF1 (16, 49) have subsequently been shown to regulate ARF6 activity and functions in intact cells (19, 20). In the present study, we examined whether an exchange factor specific for ARF6 might be expressed in chromaffin cells. EFA6, a protein mainly expressed in brain (50), promotes nucleotide exchange preferentially on ARF6 (21). EFA6 was an attractive candidate to play the role of an ARF6-specific GEF in the exocytotic machinery since studies in transfected HeLa cells indicate that the protein is preferentially localized in the subplasmalemmal region where it seems to regulate membrane trafficking and the organization of peripheral actin filaments (21). However, EFA6 was not detectable in chromaffin cells both in RT-PCR and Western blot expression studies. Furthermore, the introduction of anti-ARNO antibodies, but not anti-EFA6 antibodies, into the cytosol of permeabilized chromaffin cells, inhibited to a similar extent PLD activation and catecholamine secretion in calcium-stimulated cells. Consistent with this result, overexpression of a catalytically inactive ARNO mutant reduced the secretory activity in transfected PC12 cells. Collectively, these findings strongly suggest that the plasma membrane-associated ARNO described here represents the endogenous GEF for ARF6 in the exocytotic pathway in chromaffin cells.

In contrast to a recent report describing ARNO mostly as a cytosolic protein in HeLa cells (51), we found here that a large portion of ARNO is associated with the plasma membrane in chromaffin cells. This localization of ARNO was apparently not modified in secretagogue-stimulated cells. An obvious candidate for targeting ARNO to the plasma membrane is its C-terminal plekstrin homology domain (16). Plekstrin homology domains are generally thought to mediate recruitment of proteins to membrane surfaces through their interaction with phosphoinositides (52). Plekstrin homology domains have been subdivided into classes based on their affinity for different polyphosphoinositide species (53). Interestingly, recent work indicates that ARNO exhibits a much higher affinity for phosphatidylinositol 3,4,5-trisphosphate than for either phosphatidylinositol 4,5-bisphosphate or phosphatidylinositol 3,4-bisphosphate in vitro (54, 55). In adipocytes, insulin-dependent translocation of ARNO to the plasma membrane requires phosphatidylinositol 3-kinase activity (55). These observations fit well with our previous findings that a phosphatidylinositol 3-kinase present in the subplasmalemmal region in chromaffin cells plays a critical function in the pathway leading to catecholamine secretion (56). Thus, it will be quite interesting to determine whether there is regulatory cross-talk between ARNO and the generation of 3-phosphorylated inositides in the plasma membrane during exocytosis.

The present observations that ARNO antibodies inhibited in parallel PLD activation and catecholamine secretion in permeabilized chromaffin cells are consistent with the predicted importance of the ARNO/ARF6/PLD cascade in the exocytotic machinery in neuroendocrine cells (23). PLD hydrolyzes phosphatidylcholine to generate phosphatidic acid and choline. The conversion of phosphatidylcholine to phosphatidic acid in the plasma membrane replaces a nonfusogenic phospholipid with a fusogenic one, a potentially positive lipid modification that may allow the membrane fusion machinery to function. On the other hand, the fact that an ARF6-dependent PLD activation accompanies the exocytotic reaction does not necessarily preclude the occurrence of some other downstream effector(s) for ARNO/ARF6 in the regulated secretory pathway. ARF6 is unique in its ability to regulate the structure of the cortical actin cytoskeleton (44, 45). Furthermore, overexpression of ARNO in HeLa cells promotes a protein kinase C-dependent remodeling of the cortical actin cytoskeleton, similar to those observed in cells expressing GTP-bound ARF6 (19). In secretory cells, actin filaments form a cortical barrier that excludes the majority of secretory granules from the subplasmalemmal zone. Secretagogue-evoked stimulation promotes a specific reorganization of the actin network, which is a prerequisite for exocytosis enabling docking and fusion of secretory granules with the plasma membrane (31, 57, 58). Interestingly, activation of protein kinase C by phorbol ester treatment disrupts cortical F-actin and increases both the number of granules within the subplasmalemmal zone and the initial rate of stimulated catecholamine secretion in chromaffin cells (58). In view of their actin remodeling properties, ARNO/ARF6 represent attractive candidates capable of mediating actin rearrangements underlying membrane trafficking at the site of exocytosis. One molecule that has been shown to play a role in the ARF6-induced cytoskeletal reorganization is the Rac1-interacting protein POR1 (44). The occurrence of POR1 in secretory cells is currently unknown, but would represent an interesting subject of investigation in view of the coordinated control apparently exerted by ARF6 and Rho-related GTPases on regulated exocytosis (42, 59–61).

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REFERENCES

1. Kahn, R. A., and Gilman, A. G. (1986) J. Biol. Chem. 261, 7906–7911
2. Moss, J., and Vaughan, M. (1995) J. Biol. Chem. 270, 12327–12330
3. Brown, H. A., Gutekunst, S., Moonaw, C. R., Slaughter, C., and Sternweis, P. C. (1993) Cell 75, 1137–1144
4. Cockcroft, S., Thomas, G. M. H., Fensome, A., Geny, B., Cunningham, E., Gout, I., Hille, I., Totty, N. F., Tsoung, O., and Hsuam, J. J. (1994) Science 263, 525–526
5. Exton, J. H. (1997) Physiol. Rev. 77, 303–320
6. Strearrna, T., Willingham, M. C., Botstein, D., and Kahn, R. A. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 1238–1242
7. Serafini, T., Orci, L., Anberhled, M., Brunner, M., Kahn, R. A., and Rothman, J. E. (1991) Cell 67, 239–253
8. Dascher, C., and Balch, W. E. (1994) J. Biol. Chem. 269, 1437–1448
9. D’Inost-Schorey, C., Li, G., Colombo, M. I., and Stahl, P. D. (1995) Science 267, 1175–1178
10. Peters, P. J., Hsu, W. W., Osi, C. E., Finazzi, D., Teal, S. B., Dorschat, V., Donaldson, J. G., and Klausner, R. D. (1995) J. Cell Biol. 128, 1053–1017
11. Galas, M. C., Helms, J. B., Vitale, N., Thierse, D., Aunis, D., and Bader, M. F. (1997) J. Biol. Chem. 272, 2788–2793
12. Reckerskrowna, H., and Donaldson, G. J. (1997) J. Cell Biol. 139, 49–61
13. Donaldson, J. G., Finazzi, D., and Kraussler, R. D. (1992) Nature 360, 350–352
14. Peyroche, A., Paris, S., and Jackson, C. L. (1996) Nature 384, 479–481
15. Maree, E., Teale, S. C., Adamik, R., Moss, J., and Vaughan, M. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 1745–1748
16. Chardin, P., Paris, S., Antonny, B., Robineau, S., Bouraud-Dufour, S., Jackson, C. L., and Chabre, M. (1996) Nature 384, 481–484
17. Klarlund, J. K., Guilberme, A., Hollik, J. J., Virbasius, J. V., Chawla, A., and Czech, M. P. (1997) Science 275, 1927–1930
18. Frank, S., Uppendri, S., Hansen, S. H., and Casanova, J. E. (1998) J. Biol. Chem. 273, 23–27
19. Frank, S. K., Hatfield, J. C., and Casanova, J. E. (1998) Mol. Biol. Cell 9, 3133–3146
20. Langlies, S. E., Patki, V., Klarlund, J. K., Buxton, J. M., Hollik, J. J., Chawla, A., Cervers, S., and Czech, M. P. (1999) J. Biol. Chem. 274, 27099–27104
21. Franco, M. P., P. J., Boreto, J. van Donessa, E., Neri, A., D’Osuza-Schorey, C., and Chavrier P. (1999) EMBO J. 18, 1489–1491
22. Achtert, T., Franzussoy, A., Field, C., and Schekman, R. (1988) J. Biol. Chem. 263, 11711–11717
23. Caumont, A. S., Galas, M. C., Vitale, N., Aunis, D., and Bader, M. F. (1998) J. Biol. Chem. 273, 1373–1378
24. Vitale, N., Mukai, H., Roux, B., Thiers, D., Aunis, D., and Bader, M. F. (1993) J. Biol. Chem. 268, 14715–14723
25. Vitale, N., Genese, M. Chassserot-Golaz, S., Aunis, D., and Bader, M. F. (1996) Eur. J. Neurosci. 8, 1275–1285
ARNO in the Exocytotic Pathway

26. Terui, T., Kahn, R. A., and Randazzo, P. A. (1994) J. Biol. Chem. 269, 28130–28135
27. Franco, M., Chardin, P., Chabre, M., and Paris, S. (1995) J. Biol. Chem. 270, 1337–1341
28. Northup, J. K., Smigel, M. D., and Gilman, A. G. (1982) J. Biol. Chem. 257, 11416–11423
29. Chomczynski, P., and Sacchi, N. (1987) Anal. Biochem. 162, 156–159
30. Perrin, D., Langley, O. K., and Aunis, D. (1987) Nature 326, 498–501
31. Sontag, J. M., Aunis, D., and Bader, M. F. (1988) Eur. J. Cell Biol. 46, 316–326
32. Haun, R. S., Tsai, S. C., Adamik, R., Moss, J., and Vaughan, M. (1993) J. Biol. Chem. 268, 7064–7068
33. Pacheco-Rodriguez, G., Meacci, E., Vitale, N., Moss, J., and Vaughan, M. (1998) J. Biol. Chem. 273, 26543–26548
34. Togawa, A., Morinaga, N., Ogasawara, M., Moss, J., and Vaughan, M. (1999) J. Biol. Chem. 274, 12308–12315
35. Peyroche, A., Antonny, B., Robineau, S., Acker, J., Cherfils, J., and Jackson, C. L. (1999) Mol. Cell. 3, 275–285
36. Chung, S. H., Takai, Y., and Holz, R. W. (1995) J. Biol. Chem. 270, 16714–16718
37. Orita, S., Sasaki, T., Komuro, R., Sakaguchi, G., Maeda, M., Igarashi, H., and Takai, Y. (1996) J. Biol. Chem. 271, 7257–7260
38. Xu, J., and Tse, F. W. (1999) J. Biol. Chem. 274, 22059–22066
39. Klarlund, J. K., Rameh, L. E., Cantley, L. C., Buxton, J. M., Holik, J. J., Sakelis, C., Parki, V., Corvera, S., and Czech, M. P. (1998) J. Biol. Chem. 273, 1859–1862
40. Venkateswarlu, K., Oatey, P. B., Tavare, J. M., and Cullen, P. J. (1998) Curr. Biol. 8, 463–466
41. Chasserot-Golaz, S., Hubert, P., Thiersé, D., Dirrig, S., Vlahos, C. J., Aunis, D., and Bader, M. F. (1998) J. Neurochem. 70, 2347–2356
42. Linstedt, A. D., and Kelly, R. B. (1987) Trends Neurosci. 10, 446–448
43. Vitale, M. L., Seward, E. P., and Trifaro, J. M. (1995) Biochem. Pharmacol. 54, 1097–1108
44. Brown, A. M., O’Sullivan, A. J., and Gomperts, B. D. (1998) Mol. Biol. Cell 9, 1053–1063
45. Song, J., Khachikian, Z., Radhakrishna, H., and Donaldson, J. G. (1999) J. Cell Sci. 111, 2257–2267
46. Franco, M., Paris, S., and Chabre, M. (1995) FEBS Lett. 362, 286–290
47. Colombo, M. I., Inglese, J., D’Souza-Schorey, C., Berr, W., and Stahl, P. D. (1995) J. Biol. Chem. 270, 24564–24571
48. Xu, J., and Tse, F. W. (1999) J. Biol. Chem. 274, 19095–19102
49. Franco, M., Boretto, J., Robineau, S., Monier, S., Goud, B., Chardin, P., and Chavrier, P. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 9926–9931
50. Perez, M., Talarico, D., Trecha, D., Rechett, D., Fracchioni, N. S., Maiolo, A. T., and Neri, A. (1997) Genomics 46, 215–259
51. Monier, S., Chardin, P., Robineau, S., and Goud, B. (1998) J. Cell Sci. 111, 3427–3436
52. Lemmon, M. A., Falcone, K. M., and Klausner, R. D. (1997) Trends Cell Biol. 7, 237–242
53. Rameh, L. E., Arvidsson, A. K., Carraway, K. L., III, Couvillon, A. D., Rathbun, G., Crompton, A., Vanfleteren, B., Czech, M. P., Ravichandran, K. S., Burakoff, S. J., Wang, D. S., Chen, C. S., and Cantley, L. C. (1997) J. Biol. Chem. 272, 22059–22066
54. Chasserot-Golaz, S., Hubert, P., Thiersé, D., Dirrig, S., Vlahos, C. J., Aunis, D., and Bader, M. F. (1998) J. Biol. Chem. 274, 17619–17625
55. Millar, C. A., Powell, K. A., Hickson, G. X. R., Bader, M. F., and Gould, G. W. (1999) J. Biol. Chem. 274, 17619–17625
56. Gasman, S., Chasserot-Golaz, S., Hubert, P., Thiersé, D., Dirrig, S., Vlahos, C. J., Aunis, D., and Bader, M. F. (1998) J. Neurochem. 70, 2347–2356
57. Linstedt, A. D., and Kelly, R. B. (1987) Trends Neurosci. 10, 446–448
58. Vitale, M. L., Seward, E. P., and Trifaro, J. M. (1995) Biochem. Pharmacol. 54, 1097–1108
59. Brown, A. M., O’Sullivan, A. J., and Gomperts, B. D. (1998) Mol. Biol. Cell 9, 1053–1063
60. Gasman, S., Chasserot-Golaz, S., Hubert, P., Aunis, D., and Bader, M. F. (1998) J. Biol. Chem. 273, 16913–16920