Regulation of Neuroendocrine Exocytosis by the ARF6 GTPase-activating Protein GIT1*

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Neuroendocrine cells release hormones and neuropeptides by exocytosis, a highly regulated process in which secretory granules fuse with the plasma membrane to release their contents in response to a calcium trigger. Using chromaffin and PC12 cells, we have recently described that the granule-associated GTPase ARF6 plays a crucial role in exocytosis by activating phospholipase D1 at the plasma membrane and, presumably, promoting the fusion reaction between the two membrane bilayers. ARF6 is activated by the nucleotide exchange factor ARNO following docking of granules to the plasma membrane. We show here that GIT1, a GTPase-activating protein stimulating GTP hydrolysis on ARF6, is the second molecular partner that turns over the GDP/GTP cycle of ARF6 during cell stimulation. Western blot and immunofluorescence experiments indicated that GIT1 is cytosolic in resting cells but is recruited to the plasma membrane in stimulated cells, where it colocalizes with ARF6 at the granule docking sites. Over-expression of wild-type GIT1 inhibits growth hormone secretion from PC12 cells; this inhibitory effect was not observed in cells expressing a GIT1 mutant impaired in its ARF-GTPase-activating protein (GAP) activity or in cells expressing other ARF6-GAPs. Conversely reduction of GIT1 by RNA interference increased the exocytotic activity. Using a real time assay for individual chromaffin cells, we found that microinjection of GIT1 strongly reduced the number of exocytotic events. These results provide the first evidence that GIT1 plays a function in calcium-regulated exocytosis in neuroendocrine cells. We propose that GIT1 represents part of the pathway that inactivates ARF6-dependent reactions and thereby negatively regulates and/or terminates exocytotic release.

The exocytotic mechanism, which has been the topic of intense investigation for decades, has been recognized as a multi-step process. After recruitment and docking at the plasma membrane, secretory granules enter a priming process, which is ultimately followed by the fusion process. Many molecular players that orchestrate the targeting, docking, and fusion of secretory granules with the plasma membrane have been identified (1). Among them, SNARE (soluble NSF attachment complex proteins) are the only subgroups that are active on ARF6, the most divergent protein of the ARF family (7). GIT1 was originally isolated as a G protein-coupled receptor kinase interactor (8) and subsequently isolated by other groups through the interaction with various other proteins (7, 9). The GIT family of proteins contains two subgroups that are active on ARF6, the most divergent protein of the ARF family (7). GIT1 was originally isolated as a G protein-coupled receptor kinase interactor (8) and subsequently isolated by other groups through the interaction with various other proteins (7, 9). The GIT family of proteins contains two

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3 The abbreviations used are: PLD1, phospholipase D1; PA, phosphatidic acid; PIP, phosphatidylinositol 4-phosphate 5-phosphate 5-kinase type I γ; PIP5K, phosphatidylinositol 4-phosphate 5-kinase type I γ; GAP, GTPase-activating protein; GI, growth hormone; ARF, ADP-ribosylation factor; GIT, G protein-coupled receptor kinase interactor; HA, hemagglutinin; shRNA, small hairpin RNA; siRNA, small interfering RNA; PIPES, 1,4-piperazinediethanesulphonic acid; GST, glutathione S-transferase; MT-2, metallothionein-2.
known members, GIT1/Cat-1/p95 APP1 and GIT2/Cat-2/PKL/p95-APP2/KIAA0148. Both proteins contain an amino-terminal GAP domain that stimulates GTP hydrolysis by all members of the ARF family including ARF6 (10). In addition, GIT proteins contain various domains for protein interactions, including ankyrin repeats, a SpaII PIX, possibly involved in the organization of the cytoskeletal matrix assembled at active zones, a specialized presynaptic plasma membrane region where synaptic vesicles dock and fuse (13, 19). However, the role of GIT proteins in neurotransmitter release and regulated exocytosis remains unknown, despite their widespread tissue expression, including in the brain.

Here we report that GIT1 is present in neuroendocrine chromaffin and PC12 cells. In secretagogue-activated cells, GIT1 is recruited to the plasma membrane where it co-localizes with ARF6 and is responsible for GTP hydrolysis and ARF6 inactivation. Amperometric analysis of catecholamine release performed on individual chromaffin cells shows that GIT1 reduces the secretory activity. Our data suggest that GIT1 is a component of the exocytotic machinery that inactivates ARF6-dependent reactions and thereby negatively regulates and/or terminates exocytotic release.

MATERIALS AND METHODS

Antibodies, DNA Constructs, and Proteins—The antibodies used include rabbit polyclonal anti-ARF6 (6), rabbit polyclonal anti-human growth hormone (GH) (kindly provided by Dr. A. F. Parlow and the NIDDK, National Institutes of Health, National Hormone and Pituitary Program, Torrance, CA), polyclonal anti-SNAP25 (Chemicon Int., Temecula, CA), monoclonal anti-HA (BabCO, Richmond, CA), and monoclonal anti-FLAG (Novacstra Laboratories). GIT1/2 antibodies were described previously (8). Alexa 488 and Alexa 555 secondary antibodies were obtained from Molecular Probes.

PC12 cells mRNA were prepared as described previously (20). The ARF, ARNO, ARF-GAP1, GIT1, and Scribble constructs were described previously (6, 7, 20, 21, 22). ACAP1 and ACAP2 were cloned in the pBK plasmid with the HA tag. GIT1(R39A) was generated by site-directed mutagenesis with a QuikChange mutagenesis kit from Stratagene. The plasmid with the HA tag. GIT1(R39A) was generated by site-directed mutagenesis with a QuikChange mutagenesis kit from Stratagene.

Electrochemical Measurement of Catecholamine Secretion from Single Chromaffin Cells—Chromaffin cells were isolated from fresh bovine adrenal glands and maintained in primary culture essentially as described previously (24). Cells cultured on 35-mm Costar plates (Cambridge, MA) at a density of 7.5 × 10⁴ cells/plate were washed with ascorbate-free Locke’s solution and placed on the stage of an inverted microscope. Microinjection of GIT1, (Δ45)GIT1, and ARF-GAP1 in 10 mM PIPES, 50 mM potassium glutamate, pH 7.4, was performed with the

GH, a cassette containing the H1-RNA promoter and the silencing sequence was amplified by PCR as described previously (21) and subcloned within the HindIII and XbaI sites of pXGH5 (Nichols Institute, San Juan Capistrano, CA). To estimate the silencing effect, the GIT1 shRNA plasmids were electroporated in 10⁵ PC12 cells using an AMAXA electroporator (protocol U029), and 72 h post-transfection, cells were used for Western blot and immunofluorescence experiments. The transfection efficiency under these conditions was measured by counting GH-positive cells and was found to be nearly 50% in all conditions tested.

Recombinant ARF-GAP1-His₆, GIT1-His₆, and (Δ45)GIT1-His₆ proteins were expressed and purified as described previously (10). GST-MT-2 fusion protein was expressed in Escherichia coli and conjugated to glutathione beads as described (23).

Culture, Transfection, and Assay of GH Release from PC12 Cells—PC12 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with glucose (4500 mg/liter) and containing 30 mM NaHCO₃, 5% fetal bovine serum, 10% horse serum, and 100 units/ml penicillin/streptomycin. Mammalian expression vectors were introduced into PC12 cells together with the GH plasmid pXGH5 (6-well dishes, 80% confluent, 4 μg of each plasmid/well) using GenePorter (Gene Therapy Systems) according to the manufacturer’s instructions. After 5 h of incubation at 37 °C, 2 ml of culture medium containing fetal bovine serum, horse serum, and antibiotics was added.

GH release experiments were performed 48 h after transfection. PC12 cells were washed twice with Locke’s solution and then incubated for 10 min with calcium-free Locke’s solution (basal release) or stimulated for 10 min with an elevated K⁺ solution (Locke’s containing 59 mM KCl and 85 mM NaCl). The supernatant was collected and the cells harvested by scraping in 10 mM phosphate-buffered saline. The amounts of GH secreted into the medium and retained in the cells were measured using a radioimmunoassay kit (Nichols Institute). The amount of GH secretion is expressed as a percentage of total GH present in the cells before stimulation.

Subcellular Fractionation—PC12 cells were washed twice with Locke’s solution and then incubated for 10 min with Locke’s solution (resting) or stimulated for 10 min with an elevated K⁺ solution. Medium was removed and cells immediately scraped in 1 ml of sucrose 0.32M (20 mM Tris, pH 8.0). Cells were broken in a Dounce homogenizer and centrifuged at 800 × g for 15 min. The supernatant was further centrifuged at 20,000 × g for 20 min. The resulting supernatant was further centrifuged for 60 min at 100,000 × g to obtain the cytosol (supernatant) and microsomes (pellet enriched in endosomes). The 20,000 × g pellet containing the crude membrane fraction was resuspended in sucrose 0.32 M (20 mM Tris, pH 8.0), layered on a cushion sucrose density gradient (sucrose 1–1.6 M, 20 mM Tris, pH 8.0) and centrifuged for 90 min at 100,000 × g to separate the plasma membranes from the secretory granules (20). The upper fractions containing SNAP25 (plasma membrane marker) and the pellet containing GH and dopamine-β-hydroxylase (secretory granule markers) were collected and resuspended in buffer (20 mM Tris, pH 8.0, 100 mM NaCl, 1 mM MgCl₂) before protein quantification by Bradford measurements.

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Eppendorf injection system (Eppendorf, Hamburg, Germany) using commercial glass microcapillaries (Femtotips, Eppendorf). Electropho-chemical measurements of catecholamine secretion were performed 15–30 min after microinjection as described previously (5). Intracellular changes in calcium concentration during stimulation were monitored as described previously (25).

Pull-down Assay for Activated ARF6 in PC12 Cells—PC12 cells grown in 100-mm plates were transfected with various plasmids (for each 10 μg/plate) using GenePorter. 48 h after transfection, cells were washed twice with Locke’s solution and then incubated for 15 min with Locke’s solution (basal release) or stimulated with an elevated K⁺ solution. Medium was removed and cells immediately scraped in 1 ml of ice-cold lysis buffer (20 mM Tris, pH 8.0, 100 mM NaCl, 1 mM MgCl₂, 1% Triton X-100, 0.05% cholate, 0.01% SDS, 1 mM dithiothreitol) with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 1 mM NaF, 1 mM vanadate). Cell lysates were clarified by centrifugation at 14,000 rpm for 10 min at 4 °C with rock-ing. The beads were collected and washed five times with 1 ml of phosphate-buffered saline containing 2 mM ZnCl₂ and protease inhibitors. Bound proteins were eluted at 100 °C for 10 min in 100 μl of SDS loading buffer, 25-μl aliquots were subjected to SDS-PAGE. To compare ARF6 levels precipitated from extracts, Western blots were carried out with anti-HA antibodies.

Immunoblotting, Immunofluorescence, and Confocal Microscopy—One-dimensional SDS gel electrophoresis was performed on 10% acrylamide gels in Tris-glycine buffer. The proteins were transferred to nitrocellulose sheets, and the immunoreactive bands were detected using the ECL system (Amersham Biosciences).

For immunocytochemistry, PC12 cells grown on poly-D-lysine-coated glass coverslips were maintained in Locke’s solution or stimulated with elevated K⁺. The cells were then fixed for 20 min in 4% paraformaldehyde in 0.12 M sodium phosphate, pH 7.0, and for a further 10 min in fixative containing 0.1% Triton X-100. Immunostaining was performed as described previously (6), and stained cells were visualized using a Zeiss LSM 510 confocal microscope. Quantification was performed using the Zeiss CSLM instrument 3.2 software.

RESULTS

Expression and Subcellular Distribution of GIT1 in Chromaffin and PC12 Cells—Reverse transcription-PCR and immunoblot analysis using specific antibodies against GIT1 and GIT2 revealed that GIT1 is the predominant form expressed in chromaffin and PC12 cells (data not shown). Subcellular fractionation experiments indicated that GIT1 is primarily present in the cytosol (Fig. 1A), in agreement with the pre-dominant soluble distribution of GIT1 found in other cell types (12–16). We compared the distribution of endogenous GIT1 in subcellular fractions prepared from resting and secretagogue-stimulated PC12 cells. As illustrated in Fig. 1, A and B, stimulation with elevated K⁺ for 10 min, which initiates calcium-dependent exocytosis in PC12 cells, increased the amount of GIT1 detected in the plasma membrane fraction at the expense of the cytosolic fraction, revealing the translocation of GIT1 from the cytosol to the plasma membrane upon cell stimulation. The intracellular distribution of GIT1 was also examined by immunofluo-rescence in transfected PC12 cells expressing GIT1-FLAG because we
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FIGURE 2. Scribble is involved in the stimulation-induced GIT1 translocation to the plasma membrane. PC12 cells co-expressing GIT1-FLAG and either GFP-Scribble wild type (Scribble WT) or GFP-Scribble<sup>PL</sup> were incubated for 10 min in Locke’s solution (Resting) or in high potassium solution (Stimulated). Cells were then stained with anti-FLAG antibodies (GIT1). Scale bar = 5 μm. Similar observations were made with four different cell preparations. An asterisk represents a cell transfected only with the GIT1-FLAG expression plasmid. In this case, GIT1 is normally recruited at the plasma membrane upon stimulation of exocytosis.

could not detect endogenous GIT1. In resting cells, GIT1-FLAG staining appeared diffuse throughout the cell (Fig. 1C). In contrast, after membrane depolarization by high K<sup>+</sup>, a clear redistribution of GIT1 to the cell periphery was observed (Fig. 1C). Double labeling experiments revealed that in stimulated cells, GIT1 partially co-localizes with the plasma membrane marker protein SNAP25 (Fig. 1C). A semiquantitative analysis revealed that 11 ± 1% (n = 17 cells) of GIT1 co-localized with SNAP25 in resting cells, whereas in stimulated cells the co-localization level increased to 35.6 ± 2.7% (n = 15 cells). Taken together, these data indicate that secretagogue-evoked stimulation triggers the partial recruitment of cytosolic GIT1 to the cell periphery where exocytosis occurs in secreting cells.

Scribble Retains GIT1 at the Plasma Membrane in Stimulated Cells—Scribble is a PDZ domain-containing protein present at the plasma membrane in PC12 cells. PDZ domains are protein-protein interaction modules devoted to the scaffolding of proteins in large multimolecular complexes. Using anti-Scribble antibodies to precipitate the protein from various mammalian cell extracts, we have recently identified the βPIX-GIT1 complex as a major component of the Scribble network (22). Because in PC12 cells Scribble recruits βPIX to the plasma membrane in response to high K<sup>+</sup> stimulation (22), we investigated whether Scribble might also be implicated in the anchoring of GIT1. In agreement with our previous study, Scribble is present at the plasma membrane in resting and stimulated PC12 cells (Fig. 2). Anchoring of Scribble at the plasma membrane

FIGURE 3. Over-expression of GIT1 alters secretion of co-expressed GH from PC12 cells. A, PC12 cells were transfected (4 μg/well for each plasmid) with either pBK (control), pBK-GIT1, pBK-(Δ45)GIT1, pBK-GIT1(R39A), pBK-ARF-GAP1, pBK-ACAP1, or pBK-ACAP2 plasmids along with the plasmid (4 μg/well) encoding GH. 48 h after transfection, cells were incubated for 10 min in calcium-free Locke’s solution (R, resting) or stimulated for 10 min with a depolarizing concentration of K<sup>+</sup> (S, stimulated). Cells were stained as described in Fig. 1 with anti-FLAG and anti-HA antibodies for GIT1 and ACAP proteins, respectively. B, extracellular fluids were then collected, and GH present in solutions and in cells was estimated by immunocytochemistry. Approximately 5-10% of the cells were successfully transfected with the GH-expressing vector as estimated by immunocytochemistry, and of these, more than 90% became co-transfected with the second plasmid. Data are given as the mean values ± S.E. (n = 3). Similar results were obtained in three independent experiments performed with different cell cultures. Scale bar = 5 μm.

GIT1 Is a Functional Element of the Exocytotic Machinery in PC12 and Chromaffin Cells—To establish whether GIT1 plays a role in exocytosis, we expressed various GIT1 proteins (GIT1 wild type, (Δ45)GIT1 in which the amino-terminal zinc finger motif has been deleted, and GIT1(R39A) having its GAP activity on ARF impaired) in PC12 cells. The effect of these proteins on PC12 cell secretion was examined using ectopically expressed GH as a secretory reporter (5). Fig. 3A illustrates the intracellular distribution of these various GIT1 proteins. All of them were cytosolic in resting cells and retained the ability to be recruited to the plasma membrane in stimulated cells. Moreover, we verified that over-expression of these GIT1 variants modified neither the level of total GH in PC12 cells nor its distribution in secretory granules (data not shown). Expression of GIT1 strongly inhibited GH secretion evoked by high K<sup>+</sup> (Fig. 3B). In contrast, neither (Δ45)GIT1 nor GIT1(R39A) modified GH secretion (Fig. 3B). Expression of ARF-GAP1, an ARF-GAP inactive on ARF6 (10) and associated with the Golgi apparatus in PC12 cells (data not shown), did not affect the level of GH release (Fig. 3B). We also examined the effect of the
expression of two other GAPs for ARF6, namely ACAP1 and ACAP2 (7). Both ACAP1 and ACAP2 were found in the cytosol in resting PC12 cells. However, they failed to be recruited at the cell periphery after cell stimulation (Fig. 3A), and they did not affect the level of GH release (Fig. 3B). Taken together these results argue for a negative role of GIT1 in regulated exocytosis. In addition, they reveal that the GAP activity of GIT1 is critically important for its function in the exocytotic pathway.

To further address the role of GIT1 in PC12 cell exocytosis, we selectively reduced the endogenous levels of GIT1 using an RNA interference strategy (21). Therefore, we engineered a plasmid to express simultaneously reduced the endogenous levels of GIT1 using an RNA interfer-

FIGURE 4. Selective decrease of GIT1 expression by RNA interference increases GH secretion from PC12 cells. PC12 cells were transiently transfected with the empty shRNA vector (Control) or with the GIT1-shRNA plasmids (siRNA1 and siRNA2) for 72 h. A, cells were lysed, and aliquots (25 μg of proteins) were used for electrophoresis and Western blot analysis using anti-actin and anti-GIT1 antibodies. B, densitometry scanning of the films from three independent experiments was performed. After normalization to the transfection efficiency, the protein levels of GIT1 (open columns) and actin (filled columns) were calculated. C, PC12 cells transfected with the empty shRNA vector (Control) or the GIT1-shRNA plasmids for 72 h were washed and subsequently incubated with 1 ml of calcium-free Locke’s solution (basal release) or 59 mM potassium solution. 200-μl aliquots were collected at the indicated periods of time. Basal GH release was unchanged and was subtracted from the GH release evoked by 59 mM KCl to obtain the net secretory response. Data are given as the mean values ± S.E. (n = 6). Similar results were obtained in two independent experiments performed with different cell cultures. Student’s t test was used with the corresponding control values; *, p < 0.05.

Long-term expression of GIT1 may affect various steps in the exocytotic pathway. To probe the involvement of GIT1 in the final exocytotic event, we expressed and purified recombinant GIT1, (Δ45)GIT1, and ARF-GAP1 proteins and microinjected these recombinant proteins into primary adrenal chromaffin cells. Cells were stimulated 15–30 min after microinjection by a local application of nicotine, and the exocytotic release of catecholamines was monitored by amperometry using a carbon fiber electrode. Microinjection of GIT1 (Fig. 5A), (Δ45)GIT1, or ARF-GAP1 (data not shown) did not modify the nicotine-evoked rise in cytosolic calcium monitored with the calcium probe Indo-1. However, GIT1 produced a strong inhibition of catecholamine secretion in response to nicotine-evoked (Fig. 5B) or high K+ -evoked stimulation (data not shown). The extent of catecholamine secretion, quantified by integrating the surface area below the amperometric current curve, was inhibited by 67% in cells injected with 1 μM GIT1 (Fig. 5C). In line

FIGURE 5. Effect of GIT1 on nicotine-evoked catecholamine secretion from single chromaffin cells. A, Indo-1 loaded chromaffin cells were microinjected with buffer, incubated for 10 min in Locke’s solution, and then stimulated by a local application of 100 μM nicotine (Control). The same cell was then allowed to recover for 30 min in Locke’s solution, microinjected with 1 μM GIT1, incubated for 10 min, and stimulated for a second time with 100 μM nicotine. Variations in cytosolic [Ca2+]i after the first and the second stimuli were recorded on-line as a voltage signal (F340/F405 ratio). Representative traces of [Ca2+]i transients in response to a 5-s nicotine pulse (arrowhead) are shown. B, catecholamine secretion was recorded in parallel by amperometry using a carbon fiber electrode. Microinjection of 1 μM GIT1 strongly inhibited the exocytotic release of catecholamines as revealed by the reduced amperometric response. C, cells were microinjected with buffer (n = 25), 1 μM GIT1 (n = 33), 1 μM (Δ45)GIT1 (n = 33), or 1 μM ARF-GAP1 (n = 33) and after 20 min were stimulated with 100 μM nicotine. The amperometric response was integrated to obtain the total catecholamine secretion expressed in pA/s ± S.E. D, number of release spikes per cell in buffer- and GIT1-injected cells. Identical results were obtained in two different cell preparations. Student’s t test was used with the corresponding control values; *, p < 0.001.
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FIGURE 6. GIT1 regulates the GDP/GTP cycling of ARF6 in PC12 cells. A, PC12 cells were co-transfected with pXS-ARF6-HA along with an empty vector (Control), pBK-GIT1, pBK-GIT1(R39A), pCB7-ARNO, or pCB7-ARNO(E156K). Approximately 5–10% of the cells were successfully transfected with the HA-expressing vector as estimated by immunocytochemistry, and of these, more than 90% became co-transfected with the second plasmid. Cell lysates prepared from resting (R) or stimulated (S) cells were incubated with 20 μg of GST-MT-2 fusion protein conjugated with glutathione beads. 10% of the lysates were probed with anti-HA antibodies (top gel) revealing equal levels of recombinant ARF6 expression. After washings, bound proteins were eluted from the beads in 100 μl of SDS loading buffer. 25-μl aliquots were subjected to SDS-PAGE and stained with Coomassie Blue (middle gel). To compare ARF6 levels precipitated from extracts, Western blots were carried out with anti-HA antibodies. The density of the immunoreactive ARF6-GTP bands from two independent experiments was quantified. B, PC12 cells expressing GIT1-FLAG and ARF6 were incubated for 10 min in Locke’s solution (R) or in high K⁺ solution (S) and stained with anti-FLAG antibodies (GIT1) and anti-ARF6 antibodies (ARF6). Masks represent the region of colocalization obtained by selecting the double labeled pixels. Scale bar = 5 μm. C, PC12 cells co-expressing GH and GIT1 and/or ARF6(Q67L) were incubated for 10 min in calcium-free Locke’s solution (open bars) or stimulated for 10 min with a depolarizing concentration of K⁺ (filled bars). GH present in the extracellular fluids and in cells was estimated. Data are given as the mean values ± S.E. Similar results were obtained in three independent experiments performed with different cell cultures.

with our results on PC12 cell GH secretion, (Δ45)GIT1 and ARF-GAP1 had no effect on catecholamine release from chromaffin cells (Fig. 5C). Microinjection of GIT1 resulted also in a marked reduction in the number of individual exocytotic events (amperometric spikes) evoked by nicotine over a 50-s period of stimulation. From a series of recordings on separate cells, we found a significant reduction in spike number from 33 ± 4 spikes/cell in control buffer-injected cells to 16 ± 3 spikes/cell in cells injected with 1 μM GIT1 (Fig. 5D). This reduction in spike number was not simply a consequence of the microinjection of an exogenous protein, because it was not observed in cells microinjected with either 1 μM (Δ45)GIT1 or ARF-GAP1 (data not shown). These observations indicate that GIT1 regulates secretion by directly affecting granule exocytosis at a late stage through a mechanism involving its GAP activity.

GIT1 Regulates the Activation Status of ARF6 in PC12 Cells—ARF6 has been described previously as an important modulator of regulated exocytosis in neuroendocrine cells (6, 26). Because in vitro GIT1 stimulates hydrolysis of GTP bound to ARF6 (10) and because the ARF-GAP activity of GIT1 is required for its inhibitory effect on secretion in PC12 and chromaffin cells, we thought to determine whether GIT1 might regulate the activation status of ARF6. Therefore, we adapted a recently described pull-down assay based on the use of MT-2, a ubiquitous 6–7-kDa metal-containing protein that has been shown to interact specifically with GTP-loaded ARF6 in vitro and in a yeast two-hybrid assay (23). MT-2 was cloned from PC12 cells and expressed as a GST-MT-2 fusion protein for the pull-down assay. Cells were co-transfected with HA-tagged ARF6 and either GIT1 or ARNO. As illustrated in Fig. 6A, GTP-loaded ARF6 could be detected only when cells were stimulated with high K⁺, in agreement with our previous finding that activation of ARF6 occurs specifically at the plasma membrane in response to secretagogue-evoked stimulation (6). As expected, over-expression of ARNO, the ARF6 exchange factor present at the plasma membrane in PC12 cells (20), strongly increased the level of GTP-bound ARF6 detected in stimulated cells, whereas expression of the catalytically inactive ARNO(E156K) mutant dramatically reduced it. Over-expression of GIT1 reduced by 65% the level of GTP-bound ARF6 precipitated from stimulated PC12 cells (Fig. 6A), supporting the idea that GIT1 is able to control the activation status of ARF6 in secretagogue-activated cells. Accordingly, expression of the GIT1(R39A) mutant, which in vitro displays no GAP activity on ARF6, does not modify the level of activated ARF6 found in stimulated cells (Fig. 6A). These data suggest that GIT1 could represent the molecular partner that switches ARF6 off during regulated exocytosis.

To further establish that GIT1 is able to act as an ARF6 silencer at the cell periphery in stimulated PC12 cells, we compared the intracellular distribution of ARF6 and GIT1. In resting PC12 cells, ARF6 associates...
with secretory granules as described previously (6), whereas GIT1 is found in the cytosol (Fig. 6B). In contrast, in stimulated cells, ARF6 accompanies the secretory granules to the docking sites at the plasma membrane. Because cytosolic GIT1 is also recruited to the plasma membrane upon cell stimulation, ARF6 and GIT1 co-localize at the cell periphery and are potentially able to interact at the exocytic sites in secreting cells (Fig. 6B). Finally, to demonstrate that the inhibitory effect of GIT1 on secretion lies in its ability to hydrolyze GTP and inactivate ARF6, we examined the effect of the constitutively GTP-loaded ARF6(Q67L) mutant on GIT1-induced reduction of GH release (Fig. 6C). Co-expression of ARF6(Q67L) with GIT1 completely abolished the inhibitory effect of GIT1, confirming that GIT1 plays a negative role in the exocytotic pathway by inactivating ARF6 at the plasma membrane.

**DISCUSSION**

GIT1 has recently been described as a key regulator in membrane trafficking events during neurite outgrowth (17) and synapse formation (18). At the presynaptic plasma membrane, GIT1 has also been proposed as a partner for Piccolo and βPIX in the organization of the cytoskeletal matrix, which defines neurotransmitter release sites (19). From these observations, it is tempting to speculate that GIT1 serves additional synaptic functions, including calcium-evoked neurotransmitter release. Using neuroendocrine chromaffin and PC12 cells, we show here for the first time that GIT1 is an active regulator of calcium-dependent exocytosis. Our results indicate that GIT1 inhibits secretion in an ARF6-GAP-dependent fashion. Moreover, through a series of experiments, we demonstrate that GIT1 fulfills a negative function in the exocytotic pathway by inactivating ARF6. ARF6 plays a central role in regulated exocytosis as it is the direct activator of two lipid-modifying enzymes that provide essential lipids for the exocytotic machinery, i.e. PLD1 and PIP5K producing PA and PIP2, respectively (5, 6, 26). These enzymes that provide essential lipids for the exocytotic machinery, in a calcium-dependent manner (6), whereas interactions between ARF6 and PIP5K seem to be governed by calcium-dependent mechanisms (26). Thus, the calcium-dependent recruitment of GIT1 to exocytotic sites may reduce the production of PA but favor the formation of PIP2. In this respect, GIT1 may be recruited in order to create a lipid environment that is optimal for initiating the fusion reaction at the granule docking site.

Thus the recruitment of βPIX-GIT1 complexes to the plasma membrane may not only serve for the formation of adequate lipids induced by the ARF6-dependent enzymes PLD1 and PIP5K but also for the synthesis of Rho-dependent actin structures necessary for exocytosis. In other words, the Scribbles/βPIX-GIT1 complex represents a serious candidate to act as a molecular device integrating lipid-mediated fusion reactions with accompanying actin dynamics to form the finely tuned machinery for secretion.

The fact that ARF6 is inactivated by GIT1 in response to variations in cytosolic calcium is an appealing feature, as this means that the ARF6-dependent reactions can be stopped in response to extracellular signals. What might be the role of this pathway in the exocytotic machinery? The most obvious interpretation is that GIT1 represents an off signal for the exocytic reaction. For instance, by inactivating the ARF6-dependent PLD1 and thereby reducing the production of PA, a lipid described as a promoter of membrane fusion (5), GIT1 may initiate the closure of the fusion pore. Indeed, recent work has established that the fusion pore opening/closing time for dense-core granules can be modulated by various signaling pathways (29, 30). GIT1 may represent part of the molecular machinery enabling cells to control the amount of hormone released from a single granule. Alternatively, fusion between granule and plasma membranes may require an appropriate lipid composition with a specific equilibrium between PA and PIP2. In this context, it is interesting to recall that ARF6 stimulates PLD1 in a GTP-dependent manner (6), whereas interactions between ARF6 and PIP5K seem to be governed by calcium-dependent mechanisms (26). Thus, the calcium-dependent recruitment of GIT1 to exocytotic sites may reduce the production of PA but favor the formation of PIP2. In this respect, GIT1 may be recruited in order to create a lipid environment that is optimal for initiating the fusion reaction at the granule docking site.

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