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Importance of the Rab3a–GTP Binding Domain for the Intracellular Stability and Function of Rabphilin3a in Secretion

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Abstract: We had previously demonstrated that Rab3a–GTP inhibits and the Rab3a-binding protein Rabphilin3a enhances secretion in bovine chromaffin cells. In this study, we investigated the role of Rab3a–GTP binding in the intracellular expression and the function of Rabphilin3a in regulated exocytosis in bovine chromaffin cells. Using transient transfections, we found that a minimal domain, Rp(51–190), that inhibits secretion coincides with a minimal domain that effectively binds Rab3a–GTP and allows intracellular stability of the construct. This domain includes a cysteine-rich, Zn^{2+}-binding domain whose integrity is also required for Rab3a–GTP binding and the ability to inhibit secretion. A Rabphilin3a mutant, containing both C2 domains but defective in Rab3a–GTP, and wild-type Rabphilin3a both localized to chromaffin granules and stimulated secretion similarly despite lessened intracellular expression of the mutant protein. The data are consistent with a sequence of events in which a Rab3a–GTP · Rabphilin3a complex forms on the secretory granule as a precursor in a pathway that enhances secretion. The complex dissociates (perhaps because of GTP hydrolysis) to permit the enhancement of secretion by Rabphilin3a. **Key Words:** Exocytosis—Rabphilin3a—Rab3a—Adrenal chromaffin cells.
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The Rab class of low-molecular-weight GTPases has been implicated by genetic and biochemical studies to be involved in vesicular trafficking in eukaryotic cells (Novick et al., 1980; Gallwitz et al., 1983; Pfeffer and Rothman, 1987; Goud et al., 1988; Segev et al., 1988; Plutner et al., 1991; Tisdale et al., 1992; Lombardi et al., 1993; Pfeffer, 1994). Distinct Rab proteins are necessary for trafficking in the constitutive secretory (Tisdale et al., 1992; Elazar et al., 1994; Nuoffer et al., 1994) and endocytosis pathways (van der Sluijs et al., 1991, 1992; Bucci et al., 1992; Lombardi et al., 1993; Barbieri et al., 1994) and may regulate docking and/or fusion through as yet unknown mechanisms. There is evidence that Rab proteins may indirectly modulate the function of another set of proteins, the SNARE proteins, that also play a role in vesicular trafficking pathways (Lian et al., 1994; Sogaard et al., 1994).

Rab3a is found mainly in cells, including neurons and chromaffin cells, with a highly differentiated Ca^{2+}-dependent secretory pathway. We (Holz et al., 1994) and others (Johannes et al., 1994) had previously demonstrated that Rab3a is a negative regulator of exocytosis, in experiments with bovine chromaffin cells and PC12 cells. Investigation of the effects of various mutants indicates that GTP-bound rather than GDP-bound Rab3a is inhibitory. Experiments with permeabilized chromaffin cells suggest that Rab3a acts upstream of the final fusion events triggered by Ca^{2+} and may be involved in preparing the granules to undergo exocytosis (Holz et al., 1994).

A possible effector for Rab3a is Rabphilin3a, a Rab3a-binding protein. Rabphilin3a was initially identified as an 85-kDa protein that bound Rab3a with a much greater affinity for the GTP- than the GDP-bound form. Cloning of the Rabphilin3a revealed a 704-amino-acid protein in bovine brain (Shirataki et al., 1993) and a 710 splice variant in bovine chromaffin cells (Chung et al., 1995). Both Rab3a and Rabphilin3a are found on synaptic vesicles and chromaffin granules (Darchen et al., 1990, 1995; Mollard et al., 1990; Li et al., 1994; Mizoguchi et al., 1994; Chung et al., 1995), which suggests that the proteins interact on the secretory vesicle or chromaffin granule membrane. Another Rabphilin3a-binding protein is also associated with synaptic vesicles (Shirataki et al., 1994).

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Abbreviations used: CMV, human cytomegalovirus; DMPP, 1,1-dimethyl-4-phenylpiperazinium; GST, glutathione-*S*-transferase; HA, hemagglutinin; hGH, human growth hormone; Rp, Rabphilin3a; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

A transient transfection technique based on the expression of human growth hormone (hGH) as a reporter for the regulated secretory pathway was used to study the effects on regulated secretion of cotransfected Rabphilin3a in bovine chromaffin cells (Chung et al., 1995). Overexpression of Rabphilin3a enhanced 1,1-dimethyl-4-phenylpiperazinium (DMPP)-induced hGH secretion ~30%. Secretion by chromaffin cells transfected with a plasmid with the entire coding sequence of Rabphilin3a inserted in the antisense orientation was inhibited by ~30%, indicating that endogenous Rabphilin3a regulates Ca^{2+}-dependent secretion (Chung et al., 1995). The N-terminal half of Rabphilin3a binds Rab3a–GTP and the C-terminal half has tandem C2 domains (Shirataki et al., 1993) that are responsible for Ca^{2+} and phospholipid binding of the protein. Deletion of one or both of the C2 domains converts the protein from an enhancer to a strong inhibitor of Ca^{2+}-dependent exocytosis.

The opposite effects on secretion of Rab3a and Rabphilin3a suggested a sequence of events in which a Rab3a–GTP·Rabphilin3a complex forms on the secretory granule as a precursor in a pathway that enhances secretion. The complex dissociates (perhaps because of GTP hydrolysis) to permit the enhancement of secretion by Rabphilin3a. Overexpressed Rab3a bound to GTP may inhibit secretion by binding and reducing the amount of activated Rabphilin3a on the chromaffin granule.

The sequential model is compatible with the inhibition of secretion caused by expression of the Rabphilin3a deletion mutants lacking one or both of the C2 domains. Because these mutants bind Rab3a–GTP, they may inhibit secretion by competing with endogenous Rabphilin3a for interaction with Rab3a–GTP without being able to mimic the functional effects of full-length Rabphilin3a. Reduced interaction of endogenous Rabphilin3a with Rab3a may also result in increased degradation of endogenous Rabphilin3a and/or Rab3a (Geppert et al., 1994; Li et al., 1994).

In this study, we tested the model by analysis of the Rabphilin3a mutants that are unable to bind Rab3a–GTP. We defined Rabphilin3a mutations that interfere with Rab3a–GTP binding. We determined the intracellular stability and function in secretion of constructs with and without the C2 domains that were deficient in Rab3a–GTP binding.

MATERIALS AND METHODS

Chromaffin cell preparation, transfection, and secretion experiments

Chromaffin cell preparation, transient transfection, and secretion experiments were performed as described previously (Wick et al., 1993; Holz et al., 1994, 1995; Chung et al., 1995). Ca^{2+} phosphate precipitation was used for transfections according to Wilson et al. (1996) in 12-well plates (22.6-mm well diameter). Secretion experiments were generally performed 5–6 days after transfection at 27°C. hGH was measured with a high-sensitivity chemiluminescence assay from Nichols Institute (San Juan Capistrano, CA, U.S.A.). Endogenous catecholamine secretion was measured with a fluorescence assay (Holz et al., 1982). Because only 1–4% of the cells are transfected, catecholamine secretion mainly reflects secretion from nontransfected cells. It is not altered by transfection and served as a control in the hGH-secretion experiments. Secretion was expressed as the percentage of the total cellular hGH (or catecholamine) that was released into the medium. There was usually 0.5–2.0 ng hGH and 20–40 nmol catecholamine/22.6-mm-diameter well.

Construction of expression vectors

The deletion mutants of Rabphilin3a were made by PCRs from plasmid containing full-length bovine chromaffin cell Rabphilin3a (Chung et al., 1995). Primers were designed to generate 5′-*Bam*HI and 3′-*Eco*RI restriction sites. PCR products were digested with *Bam*HI and *Eco*RI and subcloned into pCMV-hemagglutinin (pCMV-HA), a mammalian expression vector that is under the control of human cytomegalovirus (CMV) promoter and contains the HA_1 epitope (YPYDVPDYA), and pGEX-2T (Pharmacia). The construction of internal cysteine deletions of Rabphilin3a(1–206) [Rp(1–206)] has been described previously (McKiernen et al., 1996).

Rab3a-binding assays

Rab3a-binding assays were performed as described previously (McKiernen et al., 1996). The glutathione-*S*-transferase–Rabphilin3a (GST–Rabphilin3a) fusion proteins and GST protein were attached to glutathione–Sepharose beads (Pharmacia) and incubated with Rab3a–[γ-^{32}P]GTP in 50 m*M* HEPES, pH 7.5, 150 m*M* NaCl, 10 m*M* $MgCl_2$, 0.2 mg/ml bovine serum albumin, and 1 m*M* GTP. After incubation on ice for 10 min, the beads were rinsed with wash buffer (50 m*M* HEPES, pH 7.5, 150 m*M* NaCl, 10 m*M* $MgCl_2$). The amount of Rab3a bound to beads was determined by scintillation counting.

Full-length Rabphilin3a and short N-terminal deletions were not well expressed in bacteria. Instead, HA_3–Rabphilin3a [HA_3–Rp(1–710)] and HA_3–Rp(71–710) were expressed in COS cells in 100-mm-diameter plates. Two days after transfection, the proteins were immunoprecipitated from homogenates by using protein A–Sepharose. The binding of Rab3a–[γ-^{32}P]GTP to the immunoprecipitates was determined.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), western analysis, and immunocytochemistry

The GST–Rabphilin3a fusion proteins bound to glutathione–Sepharose beads were eluted with 10 m*M* glutathione (Sigma) in 50 m*M* Tris-HCl, pH 8, and separated by SDS-PAGE.

Protein expression was examined by transient transfection of bovine chromaffin cells in 35-mm-diameter dishes by calcium phosphate precipitation (Wilson et al., 1996). After 6 days, cells were harvested into sample buffer and subjected to 12 or 15% SDS-PAGE, followed by immunoblotting with anti-HA1 antibody (1:5,000 dilution). The enhanced chemiluminescence detection method (Amersham) was used according to the manufacturer's instructions.

Immunocytochemistry to determine expression of hGH and the HA_1 epitope was performed with conventional fluorescence microscopy as previously described (Holz et al.,

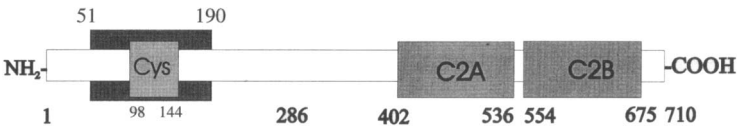

FIG. 1. Chromaffin cell Rabphilin3a. The N-terminal region of Rabphilin3a binds Rab3a-GTP. The C-terminal region contains two C2 domains, which are responsible for Ca^{2+}-dependent phospholipid binding. The two domains function independently in vitro. The N-terminal domain has a cysteine-rich, Zn^{2+}-binding domain (amino acids 98–144). The small fragment Rp(51–190) binds Rab3a-GTP and inhibits secretion. See text for details.

1994). Confocal microscopy was performed as previously described (Schroeder et al., 1994) to examine the colocalization of hGH-containing granules and Rabphilin3a and mutants in transfected chromaffin cells. Overlap in the FITC channel (hGH) and the lissamine-rhodamine channel (HA_1-Rabphilin3a) was determined by multiplication of the images, pixel by pixel, in the two channels by using NIH Image (version 1.59). The resulting pixel intensities were normalized to fit an eight-bit scale.

RESULTS

Specific amino-terminal domains are necessary for the binding of Rabphilin3a to Rab3a-GTP

Figure 1 is a schematic of chromaffin cell Rabphilin3a. Several Rabphilin3a truncation mutants were expressed as GST fusion proteins and tested for their ability to bind Rab3a-[γ-^{32}P]GTP. The GST fusion proteins were attached to glutathione-Sepharose beads and incubated with Rab3a-[γ-^{32}P]GTP. The amount of bound Rab3a was determined after rapid washing of the beads. GST-Rp(1–206) bound 100-fold more Rab3a-[γ-^{32}P]GTP than GST alone (Table 1). Similar binding was obtained with Rp(40–206), Rp(51–206), and Rp(51–190), which indicates that the first 50 amino-terminal amino acids are not important for Rab3a-GTP binding. It is noteworthy that deleting nine additional amino-terminal amino acids [GST-Rp(60–190) and GST-Rp(60–206)] completely eliminated Rab3a-GTP binding.

TABLE 1. *The ability of N- and C-terminal deletion mutants to inhibit secretion requires binding to Rab3a*

	Rab3a-[γ-^{32}P]GTP bound (cpm)	% of inhibition of DMPP-induced hGH secretion
GST	2,765	
GST-Rp(1–206)	333,400	48 ± 6 (3)
GST-Rp(40–206)	326,700	60 ± 4 (6)
GST-Rp(51–206)	279,300	61 ± 5 (2)
GST-Rp(51–190)	267,000	48 ± 8 (2)
GST-Rp(60–206)	3,225	4 ± 11 (4)
GST-Rp(60–190)	3,570	−2 ± 8 (3)

Rab3a-[γ-^{32}P]GTP binding to GST-Rabphilin3a mutants was determined. The mean of duplicate determinations is presented. Inhibition of DMPP-induced hGH secretion was determined in transfection experiments. Data are mean ± SEM values from individual experiments. The numbers in parentheses indicate the numbers of individual experiments.

The binding of Rab3a-GTP to Rp(71–710) containing both C2 domains was also examined. The binding of Rab3a-[γ-^{32}P]GTP to immunoprecipitated Rp(71–710) was 1% of the binding to Rp(1–710) (n = 2). Thus, as with shorter constructs (Table 1), residues that are N-terminal to amino acid 71 in a Rabphilin3a mutant containing both C2 domains are important for the binding of Rab3a-GTP.

While this study was in progress, Stahl et al. (1996) and McKiernen et al. (1996) presented a mutational analysis of components of Rabphilin3a structure necessary for binding to Rab3a-GTP. Although amino acids 51–59 were not investigated, the regions found to encompass the Rab3a-GTP binding domain in these studies, i.e., residues 40–170 (Stahl et al., 1996) and 45–170 (McKiernen et al., 1996), are in excellent agreement with the present study.

Rabphilin3a constructs unable to bind Rab3a-GTP have reduced stability when expressed in chromaffin cells

To investigate the importance of Rab3a-GTP binding for the intracellular stability of Rabphilin3a, plasmids encoding HA-tagged Rabphilin3a mutants were transiently transfected together with pXGH5 (which encodes hGH) in chromaffin cells. Five to 6 days later, cell homogenates were subjected to SDS-PAGE and immunoblotting with anti-HA antibody. Despite transfection of only 1–4% of the chromaffin cells, mutants that bind Rab3a-GTP could be readily detected. Figure 2A demonstrates the expression of Rp(40–206), Rp(51–206), Rp(51–190), and Rp(1–206). However, Rabphilin3a mutants that do not bind Rab3a-GTP, i.e., Rp(60–190) and Rp(60–206), were not well expressed.

The poor expression of constructs that do not bind Rab3a-GTP was not caused by low transfection efficiency, because cotransfected hGH was expressed similarly in experiments with Rabphilin3a constructs with and without the ability to bind Rab3a-GTP. For example, in Fig. 2A1, Rp(40–206), Rp(51–190), Rp(60–190), and CMV.neo expressed 3.0, 4.3, 4.9, and 5.4 ng hGH/well, respectively. The Rabphilin3a constructs without the ability to bind Rab3a-GTP are not intrinsically unstable, because they could be expressed as GST fusion proteins in bacteria similarly to mutants that bind Rab3a-GTP (Fig. 2B).

The actual coexpression of hGH and HA-tagged Rabphilin3a mutants in individual cells was examined

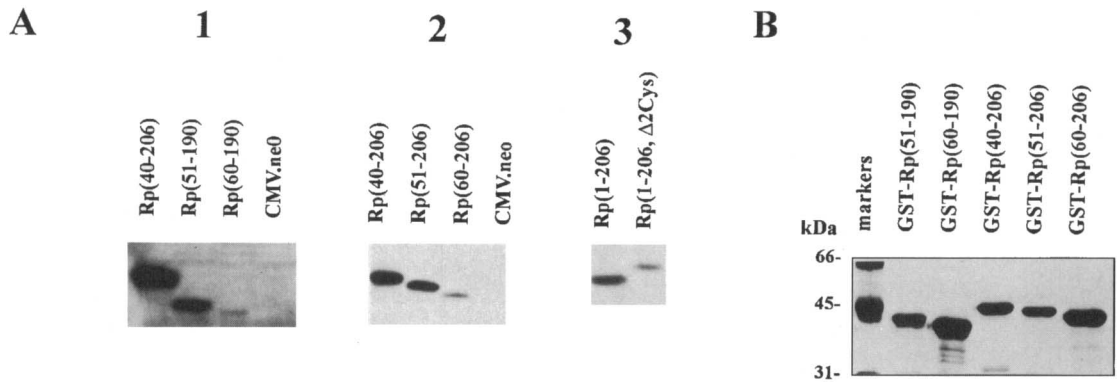

FIG. 2. Expression of Rabphilin3a mutants in chromaffin cells (A) and *E. coli* (B). **A:** Chromaffin cells were cotransfected with plasmids encoding hGH and the indicated Rabphilin3a mutants (HA tagged). The expression of the various proteins was determined by immunoblots using anti-HA_1 on homogenates prepared 6 days after transfection. Three different experiments are shown. Mutants that do not bind Rab3a–GTP readily [Rp(60–190), Rp(60–206), and Rp(1–206Δ2cys)] were usually poorly expressed in the cells despite excellent hGH expression. For example, in panel 1, Rp(40–206), Rp(51–190), Rp(60–190), and CMV.neo expressed 3.0, 4.3, 4.9, and 5.4 ng hGH/well, respectively. **B:** The indicated GST fusion constructs were expressed in *E. coli* and demonstrated on SDS-PAGE with 1.5 μg of protein/lane.

by immunocytochemistry using conventional fluorescent microscopy (Fig. 3). Cultures were transfected with hGH and either HA–Rp(51–206) or HA–Rp(60–206). The Rab3a–GTP-binding mutant HA–Rp(51–206) was almost always expressed in hGH-containing cells. Figure 3A shows two hGH-expressing

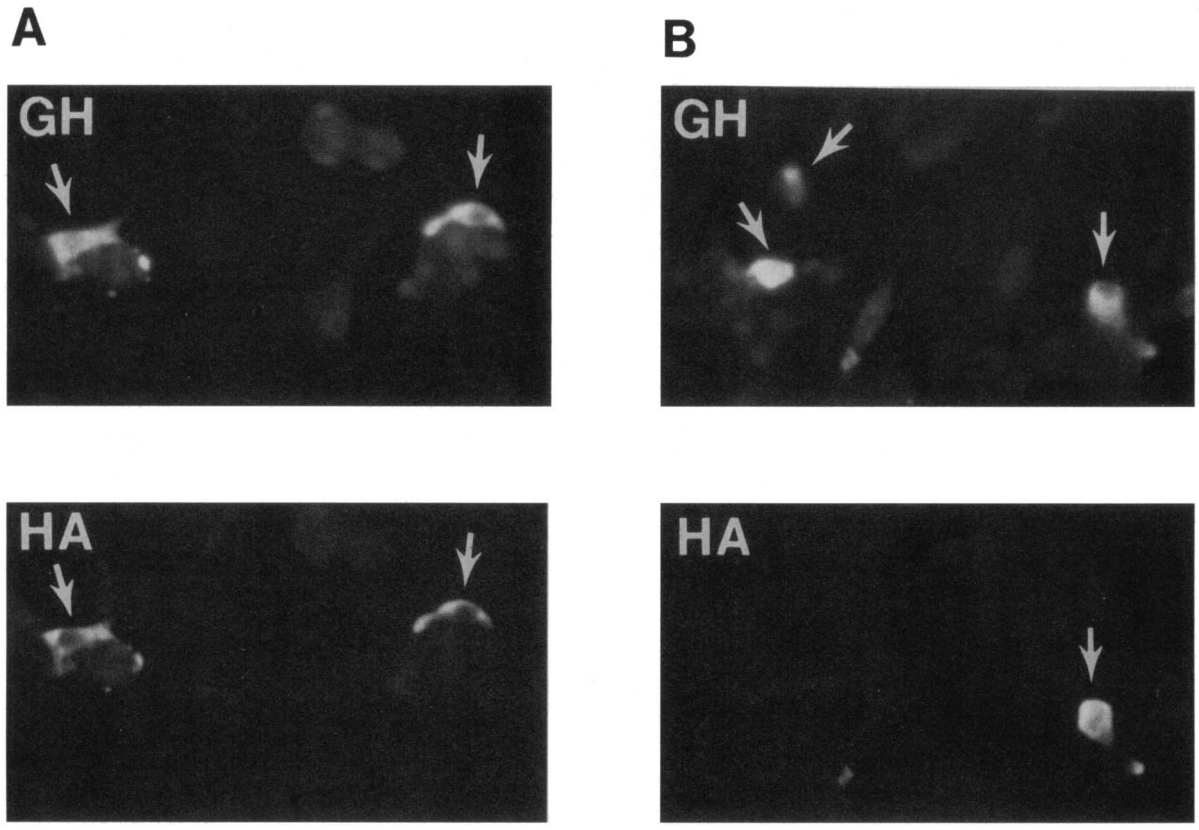

FIG. 3. Deficient expression of a Rabphilin3a mutant that is unable to bind Rab3a–GTP in hGH-expressing chromaffin cells. Chromaffin cells were cotransfected with a plasmid encoding hGH and a plasmid encoding either HA–Rp(51–206) (A) or HA–Rp(60–206) (B). **A:** Two hGH-expressing cells expressed HA–Rp(51–206). **B:** Only one of three hGH-expressing cells expressed detectable amounts of HA–Rp(60–206). hGH- (GH) and HA_1-expressing cells (HA) are indicated by arrows.

TABLE 2. *Analysis of coexpression of Rabphilin3a mutants and hGH in chromaffin cells*

HA-tagged mutant	Binds Rab3a–GTP	% of hGH-expressing cells that coexpress HA–Rabphilin3a mutant
CMV.neo		0
Rp(1–206)	Yes	96
Rp(40–206)	Yes	97
Rp(51–206)	Yes	96
Rp(51–190)	Yes	91
Rp(60–206)	No	39
Rp(60–190)	No	23
Rp(1–206Δ2cys)	Weakly	35
Rp(71–710)	No	48

Chromaffin cells were cotransfected with pXGH5 encoding hGH and plasmids encoding HA-tagged Rabphilin3a mutants. The cultures were prepared for immunocytochemistry 6 days later. The expression of HA–Rabphilin3a mutants was evaluated in 30–40 hGH-expressing cells in each transfected culture. Rp(1–206Δ2cys) is HA–Rp(1–206) with amino acids 97–102 deleted, which contain the first two cysteines in the Zn-binding domain. This deletion reduces the Rab3a–GTP binding by 83% compared with Rp(1–206) (McKiernen et al., 1996). The immunofluorescence due to Rabphilin3a mutants that do not bind Rab3a–GTP well tended to be less bright in expressing cells than immunofluorescence due to Rab3a–GTP-binding mutants.

cells that also expressed HA–Rp(51–206). In contrast, HA–Rp(60–206) that does not bind Rab3a–GTP was not significantly expressed in many hGH-containing chromaffin cells. In Fig. 3B, only one of three hGH-expressing cells expressed detectable amounts of HA–Rp(60–206). Quantitative evaluation of coexpression of hGH and various HA–Rabphilin3a mutants is shown in Table 2. Mutants that bound Rab3a–GTP were expressed in >90% of the hGH-positive chromaffin cells. HA–Rabphilin3a mutants that do not bind Rab3a–GTP were expressed in only 20–48% of the hGH-positive cells. The immunofluorescence of HA in hGH-positive cells with Rp(60–190), Rp(60–206), and Rp(71–710) was often less intense than with Rab3a–GTP-binding mutants. These results confirm that the lower expression of Rabphilin3a mutants that do not bind Rab3a–GTP results from poor expression in individual transfected cells.

In four experiments, the coexpression of HA–Rp(71–710) and hGH was evaluated by immunocytochemistry. The percentage of the hGH-expressing cells that coexpressed the mutant protein 3 or 4 days after transfection was 48 ± 9%. The coexpression is significantly less than the 90% for Rabphilin3a constructs that bind Rab3a–GTP including both C2 truncations (Table 2) and full-length Rabphilin3a (Chung et al., 1995).

Because an endogenous protein gave a signal with the anti-HA antibody that coincided with the expected band from HA–Rp(71–710), immunoblots could not detect the transfected protein.

Serial N- and C-terminal deletions of Rp(1–536) define a minimal region necessary for inhibition of secretion that corresponds to the Rab3a–GTP binding domain

In a previous study, we found that deletion of one or both of the C-terminal C2 domains of Rabphilin3a converted the protein from one that enhanced to one that inhibited secretion when transiently expressed in bovine chromaffin cells (Chung et al., 1995). To determine the minimal region necessary for inhibition, N- and C-terminal deletion mutants were constructed from Rp(1–536). Rp(1–536) lacks the region encoding the most carboxy-terminal C2 domain. The plasmids were tested for their effects on hGH secretion after transient expression (together with hGH) in bovine chromaffin cells. The inhibitory effects on secretion of various constructs from a large number of experiments are summarized in Fig. 4. Stimulation with the nicotinic agonist DMPP for 2 min in cells not expressing exogenous Rabphilin3a constructs caused vigorous hGH secretion (10–15% of the total expressed hGH, which was normalized to 100% for each experiment). Rp(1–536) inhibited hGH secretion 60%, a result that is consistent with a previous study (Chung et al., 1995). Deletion of the N-terminal 50 amino acids had little or no effect on the ability of the constructs to

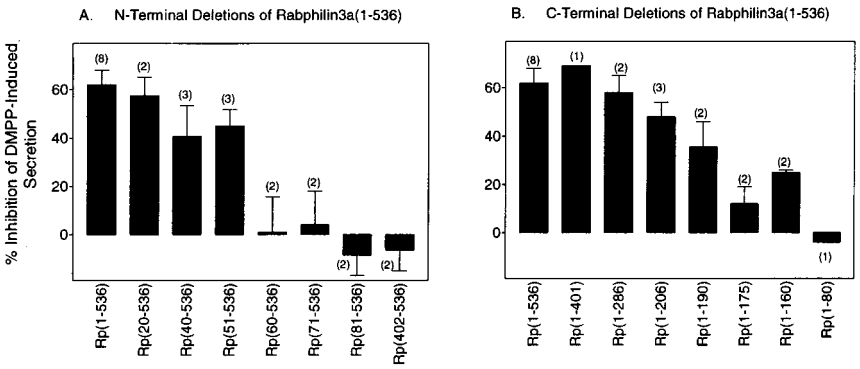

FIG. 4. Effects on secretion of serial deletions from the N-terminus (A) and C-terminus (B) of RpΔC2B [Rp(1–536)]. **A:** Chromaffin cells were cotransfected with a plasmid encoding hGH (pXGH5) and either pCMVneo or serial N-terminal deletions of Rp(1–536) that encodes Rabphilin3a without the one C2 domain. **B:** Chromaffin cells were cotransfected with a plasmid encoding hGH (pXGH5) and either pCMVneo or serial C-terminal deletions of Rp(1–536). All constructs were HA_1 tagged at the amino terminus. Five to 6 days after transfection, cells were incubated ±20 μ*M* DMPP for 2 min. Data are presented as percent inhibition values of DMPP-dependent secretion compared with hGH secretion from cells transfected with control plasmid pCMVneo. The numbers of experiments performed are indicated in parentheses adjacent to error bars.

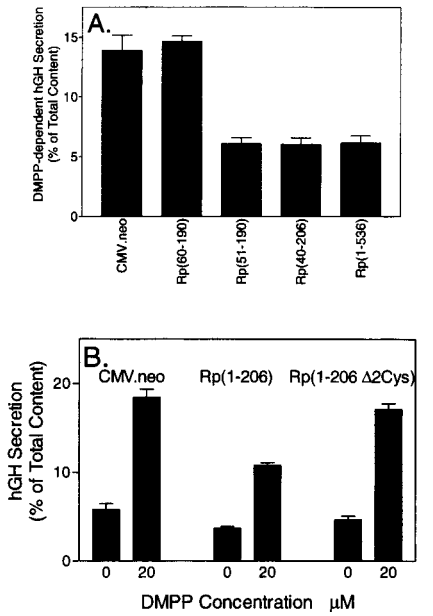

FIG. 5. The minimal domain necessary to inhibit secretion includes a Zn^{2+}-binding domain. **A:** A minimal domain without C2 regions that is necessary for inhibition of secretion. hGH secretion was determined from chromaffin cells that were cotransfected with a plasmid encoding hGH and either N- and C-terminal deletion mutants of Rp(1–536) or the parent plasmid pCMVneo. Six days later, cells were incubated in physiological salt solution for 2 min with and without 20 μM DMPP. There were four wells per group. **B:** Deletions of cysteines in the Zn^{2+}-binding domain prevent inhibition of secretion by transfection with plasmid encoding truncated Rabphilin3a. hGH secretion was determined from chromaffin cells that were cotransfected with a plasmid encoding hGH and either Rp(1–206), Rp(1–206Δ2cys) without the first two cysteines in the Zn^{2+}-binding domain, or the parent plasmid CMV.neo. Cells were incubated in physiological salt solution for 2 min with and without 20 μM DMPP. There were eight wells per group for the groups transfected with pCMVneo, and the plasmid encoding Rp(1–206Δ2cys). There were four wells per group for groups transfected with the plasmid encoding Rp(1–206).

inhibit secretion. However, deletion of the next nine amino acids almost completely prevented the inhibition, with further N-terminal deletions having no effect on secretion (Fig. 4A).

C-terminal deletions of Rp(1–536) also revealed a region necessary for the inhibition of secretion (Fig. 4B). As shown in a previous study (Chung et al., 1995), removal of both C2 domains [Rp(1–286)] results in a protein that inhibits secretion similarly to Rp(1–536). Rp(1–206) and Rp(1–190) also significantly inhibited secretion. The inhibition decreases with larger C-terminal deletions.

The data suggest that the small fragment Rp(51–190) should inhibit secretion. Figure 5A demonstrates that this is, indeed, the case. Whereas Rp(40–206) and Rp(51–190) are equally effective as Rp(1–536) in inhibiting secretion, Rp(60–190) is without effect.

As summarized in Tables 1 and 2, the minimum domain of Rabphilin3a that inhibits secretion binds Rab3a–GTP and is well expressed in transfected chromaffin cells. Constructs that do not bind Rab3a–GTP have low intracellular stability and do not inhibit secretion.

Deletion in the Zn^{2+}-binding domain of Rabphilin3a prevents inhibition of secretion by truncated Rabphilin3a [Rp(1–206)]

Rabphilin3a binds Zn^{2+} in a region from C98 to C144 that contains nine cysteines in CXXC motifs (McKiernen et al., 1996; Stahl et al., 1996). Deletion of amino acids R97–G102, which removes the first CXXC motif [Rp(1–206Δ2cys)] reduces by 70% the ability of the protein to bind Zn^{2+} and reduces the Rab3A–GTP binding by 83% (McKiernen et al., 1996). This construct is not well expressed when transfected into chromaffin cells (Table 2) and was not able to inhibit secretion (Fig. 5B). In five experiments, secretion was inhibited by 5 ± 10%.

Carboxyl terminal–containing constructs deficient in Rab3a binding are located on secretory granules and are able to enhance secretion

Full-length Rabphilin3a has been previously shown to be associated with synaptic vesicles and chromaffin granules. The localization of an N-terminal deletion, HA–Rp(71–710), which is unable to bind Rab3a–GTP, was investigated by confocal microscopy. When present in an hGH-expressing cell, HA–Rp(71–710) (Fig. 6B) as well as HA–Rp(1–710) (Fig. 6E) had distinctly punctate appearances in chromaffin cells. Both colocalized with hGH-containing secretory granules (Fig. 6C and F).

The role of Rab3a–GTP binding in the enhancement of secretion by Rabphilin3a was investigated. Because the enhancement of secretion by wild-type Rabphilin3a is relatively small (~30%) (Chung et al., 1995), a large number of experiments were required to determine the effects of various constructs on secretion. Two mutants were investigated with deletions of N-terminal segments that are required for Rab3a–GTP binding, i.e., HA–Rp(71–710) (six experiments) and HA–Rp(81–710) (three experiments) (Table 3). Secretion stimulated by either 20 μM DMPP or elevated K^+ for 8, 10, or 20 min was increased in all experiments with an average enhancement of 29.4 ± 2.7% ($p < 0.0001$, vs. transfection with pCMVneo, paired t test). This is similar to the ~30% enhancement of full-length Rabphilin3a reported previously (Chung et al., 1995). Thus, although the proteins were relatively unstable within the cell, they were, nevertheless, able to enhance secretion. Because only 48% of the hGH-containing cells coexpressed HA–Rp(71–710) (see above), the protein may have enhanced hGH secretion 60% in those cells expressing the mutant.

DISCUSSION

In this study, we investigated the role of Rab3a–GTP binding in the function of Rabphilin3a in regu-

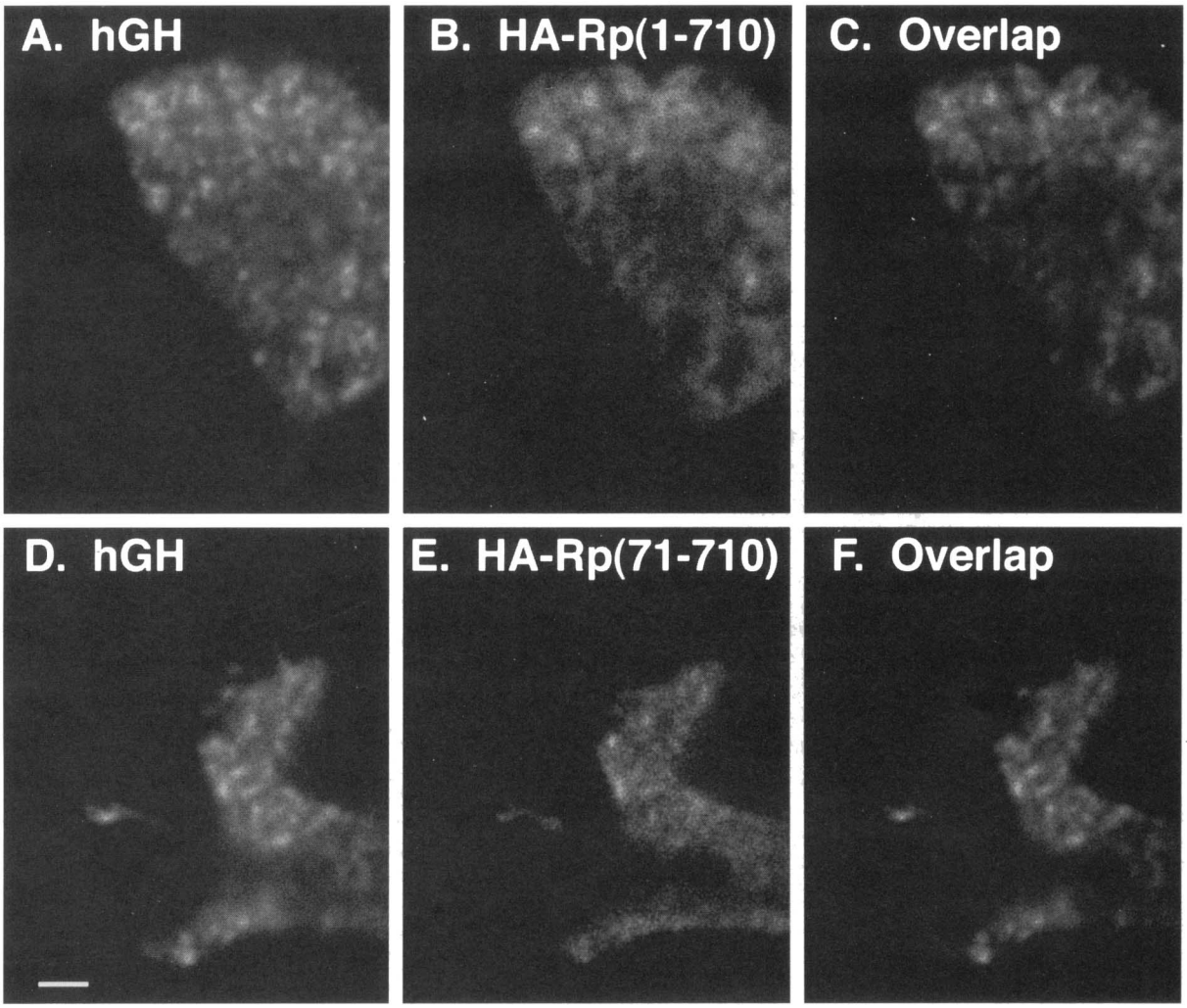

FIG. 6. Full-length HA–Rabphilin3a (**A–C**) and HA–Rp(71–710) (**D–F**) are associated with hGH-containing secretory granules. hGH and Rabphilin3a channels are indicated. Overlapping structures in A and B, and in D and E, are shown in C and F, respectively. Overlapping structures were determined by pixel-by-pixel multiplication in A and B, or in C and D, as described in Materials and Methods. The calibration bar corresponds to 2 μm.

lated exocytosis. We found that constructs with decreased ability to bind Rab3a–GTP have decreased stability when expressed in chromaffin cells. The inability to bind Rab3a–GTP was associated with the loss of inhibitory action of C2 deletion mutations. Rabphilin3a mutants containing both C2 domains but defective in Rab3a–GTP binding enhanced secretion similarly to the wild-type protein despite decreased intracellular stability. These results are consistent with a sequential model for the action of Rab3a and Rabphilin3a and suggest that Rabphilin3a not bound by Rab3a–GTP enhances secretion (see below).

The minimal inhibitory region of Rabphilin3a coincides with the minimal Rab3a binding domain

In a previous study, we demonstrated inhibitory effects of Rabphilin3a mutants lacking one or both carboxyl-terminal C2 domains in Ca^{2+}-dependent exocytosis in bovine chromaffin cells (Chung et al., 1995). In the present study, the minimal inhibitory domain was mapped to residues 51–190 of Rabphilin3a. It coincided with the minimal Rab3a–GTP binding domain. Deletion of just nine additional amino-terminal amino acids resulted in a construct [Rp(60–190)] unable to bind to Rab3a–GTP and unable to inhibit secretion.

Rabphilin3a mutants defective in Rab3a–GTP binding [including Rp(60–190)] were poorly expressed compared with Rab3a–GTP binding constructs. The low expression was not caused by poor transfection, because the plasmid encoding hGH that was cotransfected with these constructs was well expressed. The low expression probably reflects poor intracellular stability of the protein. At this time, we cannot determine whether the loss of inhibitory effect on secretion results

TABLE 3. *Enhancement of secretion by Rabphilin3a with N-terminal deletions that prevent Rab3a–GTP binding*

Construct	Stimulation, time	Stimulated secretion with pCMVneo	Stimulated secretion with construct	Relative enhancement (%)
Rp(71–710)	DMPP, 10 min	17.7	20.9	18.1
Rp(71–710)	KCl, 8 min	13.7	16.9	23.4
Rp(71–710)	KCl, 8 min	12.2	17.3	41.8
Rp(71–710)	KCl, 8 min	8.8	12.0	36.4
Rp(71–710)	KCl, 8 min	10.4	13.3	27.9
Rp(71–710)	KCl, 8 min	15.8	20.6	30.4
Rp(81–710)	DMPP, 20 min	17.7	21.3	20.3
Rp(81–710)	DMPP, 20 min	25.3	34.8	37.5
Rp(81–710)	DMPP, 10 min	17.7	22.8	28.8
Average ± SEM				29.4 ± 2.7

Chromaffin cells were transfected with a plasmid encoding hGH and either pCMVneo or plasmids encoding HA_1–Rabphilin3a(71–710) or HA_1–Rabphilin3a(81–710). These Rabphilin3a constructs contain both C2 domains but not the amino-terminal domain necessary for Rab3a–GTP binding. Three to 6 days after transfection secretion was stimulated with either elevated K^+ (56 m*M*) or DMPP (20 μM) for 8, 10, or 20 min as indicated. Data from nine experiments are shown. Stimulated secretion is indicated and is expressed as the percentage of the total cellular hGH that is secreted into the medium. The relative enhancement is calculated in the far right column. The average enhancement was 29.4 ± 2.7%. $p < 0.0001$, vs. transfection with pCMVneo with a paired *t* test.

solely from poor stability of the mutant proteins in the cell or, in addition, from the inability of the expressed protein to bind Rab3a–GTP. The effect of Rab3a binding to confer intracellular stability to Rabphilin3a is consistent with the decrease of Rabphilin3a expression in the CNS of Rab3a knockout mice (Geppert et al., 1994).

In a previous study, Rabphilin3a mutants deficient in Rab3a–GTP binding were relatively well expressed in transiently transfected PC12 cells (McKiernen et al., 1996). The seemingly greater stability in PC12 cells than in chromaffin cells may be the result of a larger N-terminal epitope (three consecutive HA peptides) used in the former study compared with the smaller N-terminal epitope (a single HA peptide) in the present experiments. The large N-terminal addition may have protected against proteolysis. An alternative possibility is that the different stabilities could reflect differences in the cell types.

Rab3a–GTP binding is not necessary for full-length Rabphilin3a to enhance secretion

To investigate the role of Rab3a–GTP binding in the enhancement of secretion by full-length Rabphilin3a, two mutants were investigated with deletion of N-terminal segments that are necessary for Rab3a–GTP binding, i.e., Rp(71–710) and Rp(81–710). The mutants were able to enhance hGH secretion despite their deficient expression in hGH-containing cells. These results are consistent with the finding that Doc2 also enhances secretion when overexpressed in PC12 cells (Orita et al., 1996). Doc2 is a synaptic vesicle protein that has two C2 domains with sequence homology to that of Rabphilin3a (62% identity over 323 amino acids) but does not bind to Rab3a–GTP (Orita et al., 1995). The data suggest that endogenous Rabphilin3a can enhance secretion after binding to Rab3a is terminated by GTP hydrolysis.

The inability of Rp(71–710) to bind Rab3a–GTP did not prevent the protein from associating with secretory granules (Fig. 6). A previous study also demonstrated that Rabphilin3a mutants deficient in Rab3a–GTP binding could associate with Rab3a-containing membrane organelles in PC12 cells (secretory granules), although to a somewhat lesser degree than wild-type protein (McKiernen et al., 1996). Indeed, a protein binding site, in addition to Rab3a–GTP, is probably important for the association of Rabphilin3a to secretory vesicles (Shirataki et al., 1994).

We had found that the Rp(287–710) with a large N-terminal deletion did not enhance secretion (Chung et al., 1995). The ability of Rp(81–710) to enhance secretion suggests that the region between amino acids 81 and 287 is necessary for the enhancement of secretion. The overlap of this region with the Rab3a–GTP binding domain (amino acids 51–190) raises the possibility that Rab3a–GTP controls the accessibility of the region to other components in the secretory pathway.

A model for the role of Rabphilin3a in regulated secretion

Recent cross-linking experiments with synaptosomes (Stahl et al., 1996) and confocal immunocytochemical experiments with PC12 cells (McKiernen et al., 1996) provide strong evidence that Rab3a and Rabphilin3a are colocalized and interact on secretory granules and vesicles. The opposite effects of Rab3a–GTP to inhibit secretion and full-length Rabphilin3a to enhance secretion (Holz et al., 1994; Johannes et al.,

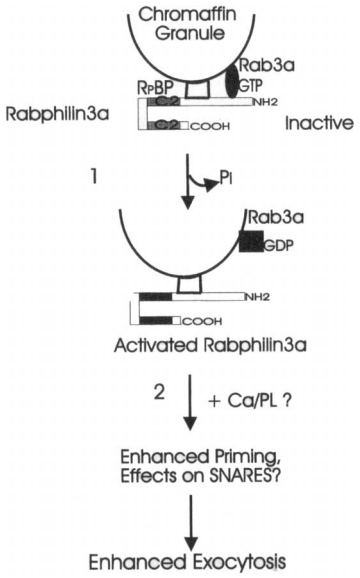

FIG. 7. Sequential events in Rab3a/Rabphilin3a control Ca^{2+}-dependent exocytosis. Rab3a–GTP and Rabphilin3a interact on the secretory granule membrane. Rabphilin3a mutants without one or both C2 domains compete with endogenous Rabphilin3a for binding to Rab3a–GTP. RpBP is a putative Rabphilin3a-binding protein distinct from Rab3a that is suggested to participate, together with Rab3a–GTP, in the binding of Rabphilin3a to secretory granules (Shirataki et al., 1994). GTP hydrolysis (step 1) results in dissociation of Rabphilin3a and Rab3a and the activation of Rabphilin3a function. This is consistent with Rabphilin3a mutants containing both C2 domains but defective in Rab3a–GTP binding enhancing secretion similarly to the wild-type protein. Step 2 represents unknown effector pathways for activated Rabphilin3a. Overexpression of Rab3a in transfection experiments results in inhibition of secretion because of rapid rebinding of activated Rabphilin3a by abundant Rab3a–GTP to functionally reverse step 1. Because overexpression of Rab3a and mutants preferentially alters the initial secretory response, it is suggested that the Rab3a/Rabphilin3a pathway regulates the number of primed granules.

1994; Chung et al., 1995) suggest a sequence of events in which Rab3a–GTP and Rabphilin3a form a complex on the secretory granule that is a precursor in a pathway that enhances secretion (Fig. 7). Rabphilin3a then dissociates from Rab3a, possibly because of GTP hydrolysis, in order for enhanced secretion to occur. The findings of this study support the model. Rabphilin3a mutants containing both C2 domains but defective in Rab3a–GTP binding [Rp(71–710) and Rp(81–710)] were able to enhance secretion. This effect was observed despite limited stability of the protein in transfected cells.

The ability of the mutants to enhance secretion eliminates an alternative model in which Rabphilin3a would enhance secretion by preventing direct, inhibitory effects of Rab3a–GTP on secretion.

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