High affinity Rab3 binding is dispensable for Rabphilin-dependent potentiation of stimulated secretion

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Accepted 5 August; published on WWW 30 September 1999

SUMMARY

Rabphilin is a protein that associates with the GTP-bound form of Rab3, a small GTPase that controls a late step in Ca2+-triggered exocytosis. Rabphilin is found only in neuroendocrine cells where it co-localises with Rab3A on the secretory vesicle membrane. The Rab3 binding domain (residues 45 to 170), located in the N-terminal part of Rabphilin, includes a cysteine-rich region with two zinc finger motifs that are required for efficient interaction with the small GTPase. To determine whether binding to Rab3A is necessary for the subcellular localisation of Rabphilin, we synthesised point mutants within the Rab3-binding domain. We found that two unique mutations (V61A and L83A) within an amphipathic α-helix of this region abolish detectable binding to endogenous Rab3, but only partially impair the targeting of the protein to secretory vesicles in PC12 and pancreatic HIT-T15 cells. Furthermore, both mutants transfected in the HIT-T15 beta cell line stimulate Ca2+-regulated exocytosis to the same extent as wild-type Rabphilin. Surprisingly, another Rabphilin mutant, R60A, which possesses a wild-type affinity for Rab3, and targets efficiently to membranes, does not potentiate regulated secretion.

High affinity binding to Rab3 is therefore dispensable for the targeting of Rabphilin to secretory vesicles and for the potentiation of Ca2+-regulated secretion. The effects of Rabphilin on secretion may be mediated through interaction with another, unknown, factor that recognizes the Rab3 binding domain.

Key words: Rab3 GTPase, Rabphilin3A, Ca2+-regulated secretion

INTRODUCTION

Rabphilin is a 78 kDa protein identified by its ability to interact with the GTP-bound form of the small GTPase Rab3A (Shiritaki et al., 1993; McKiernan et al., 1993). The amino acid sequence of the protein defines three regions (Kishida et al., 1993). The N-terminal region contains the Rab3 binding domain (amino acids 45 to 170) that includes a cysteine-rich sequence with two zinc finger motifs that are required for efficient interaction with the small GTPase, and Rabphilin is an in vitro substrate for protein kinase A (McKiernan et al., 1996; Stahl et al., 1996; Ostermeier and Brunger, 1999). In a previous study, we showed that the binding of two Zn2+ ions was necessary but not sufficient for efficient binding of Rabphilin to Rab3A (McKiernan et al., 1996). The central region contains multiple consensus sequences for phosphorylation by protein kinases, and Rabphilin is an in vitro substrate for protein kinase A (Fykse et al., 1995; Lonart and Südhof, 1998). The C-terminal region contains two C2 domains, related to those of protein kinases C and synaptotagmin, that can bind phospholipids in a Ca2+-dependent manner (Chung et al., 1998). Despite the fact that Rabphilin lacks a transmembrane domain or consensus sequences for lipidic modifications, the protein is mainly bound to membranes. The C2 domains are required for efficient membrane attachment of Rabphilin in PC12 cells (Yamaguchi et al., 1993). Rabphilin is only found in neuroendocrine cells (Shiritaki et al., 1993), and one splice variant has been described (Chung et al., 1995). Rabphilin co-localises with the membrane-bound fraction of Rab3A on dense-core secretory granules in both PC12 and chromaffin cells (Chung et al., 1995; Shiritaki et al., 1994).

Rab3A belongs to the Rab/YPT group of the Ras superfamily (for reviews, see Valencia et al., 1991; Macara et al., 1996). Rab proteins are involved in vesicular transport and may regulate the vesicle docking step prior to membrane fusion (Mayer and Wickner, 1997; Rybin et al., 1996). Rab proteins are located in specific intracellular compartments (for review see Simons and Zerial, 1993). Rab3A is expressed only in cells that are capable of regulated secretion (Olofsson et al., 1988; Fischer von Mollard et al., 1990; Darchen et al., 1995; Regazzi et al., 1996). Three additional isoforms of Rab3 have been described (Matsui et al., 1988; Baldini et al., 1992). In brain, Rab3C presents a localisation similar to that of Rab3A with, however, a more heterogeneous distribution among synapses (Fischer von Mollard et al., 1994; Li et al., 1994; Castillo et al., 1997). Rab3B and Rab3D are expressed in many different tissues (Baldini et al., 1992; Weber et al., 1994; Regazzi et al., 1996).
The nucleotide-bound state of Rab3A is regulated by a GTPase activating protein (Rab3A-GAP), exchange factors (MSS4, Rab3-GRF, Rab3-GEP) and by binding proteins such as Rab-GDI, Rim and Rabphilin (Burstein et al., 1991; Fukui et al., 1997; Burton et al., 1993; Burstein and Macara, 1992; Wada et al., 1997; Matsui et al., 1990; Oishi et al., 1998; Wang et al., 1998). Rabphilin itself inhibits the GTPase activity of Rab3A promoted by Rab3-GAP and possesses a weak nucleotide exchange activity toward Rab3A. The physiological significance of these activities is not known (Kishida et al., 1993). Another protein, Rabin3, interacts with Rab3 proteins but its function is still unknown (Broydy et al., 1995).

When overexpressed in chromaffins cells, Rab3A inhibits DMPP-induced, Ca$^{2+}$-regulated secretion (Holz et al., 1994; Chung et al., 1997) while Rabphilin potentiates secretion (Holz et al., 1994; Chung et al., 1995). Fragments of Rabphilin lacking one or both C2 domains, however, act as potent dominant inhibitors of secretion (Chung et al., 1995). Microinjection of the isolated N- or C-terminal parts of Rabphilin also inhibit Ca$^{2+}$-triggered cortical granule exocytosis in metaphase II mouse eggs at fertilization (Masumoto et al., 1996). These results, with others (Johannes et al., 1994, 1996), suggest that Rab3A may form an inhibitory complex with Rabphilin. Dissociation would release Rabphilin and allow it to interact with other factors leading to fusion and secretion of the vesicular cargo. Recent studies suggest a particular involvement of Rab3A in neuronal responses to repetitive stimulation. Long-term potentiation is a synaptic plasticity phenomenon resulting in a stable increase in synaptic transmission following repetitive activation of excitatory synapses. This process is abolished at hippocampal mossy fibre synapses in transgenic mice lacking Rab3A (Castillo et al., 1997). These mice also show impaired responses to repetitive stimuli (Geppert et al., 1994). In a similar way, the microinjection of antisense oligonucleotides directed against rab3A messengers in chromaffin cells increases the response to repetitive stimulations, when a desensitisation phenomenon is observed in the control population (Johannes et al., 1994). In the Rab3A deficient mice, the size of the readily releasable pool of vesicles is normal, but Ca$^{2+}$-triggered fusion is altered with a greater number of exocytotic events occurring immediately following the nerve impulse (Geppert et al., 1997). These results suggest that Rab3A may regulate a late step in synaptic fusion.

One function of Rab3A may be to recruit Rabphilin to the secretory vesicle membrane, where it can interact with other factors after its release from Rab3A. This hypothesis has received experimental support (McKiernan et al., 1996; Stahl et al., 1996), although our study suggested that factors other than Rab3A may contribute to Rabphilin targeting (McKiernan et al., 1996).

To determine the importance of the Rab3 binding domain of Rabphilin for targeting, we synthesised point mutants within the N-terminal region of that domain. We show that two point mutants within an amphipathic α-helix abolish detectable interaction with endogenous Rab3 but the proteins still partly co-localise with Rab3A in PC12 cells and enhance Ca$^{2+}$-regulated secretion in pancreatic beta cells. Interestingly, a third point mutant, which did not interfere with the binding of Rab3A, nonetheless abrogated the ability of Rabphilin to potentiate secretion.

**MATERIALS AND METHODS**

**Synthesis of Rabphilin point mutant cDNAs**

Point mutants in Rabphilin were created by the PCR-based megaprimer method (White, 1993) using the bovine Rabphilin sequence. PCR products were digested and subcloned into pGEX-2T (Pharmacia) to express glutathione-S-transferase(GST)-fusion proteins in bacteria; and into eukaryotic expression vectors pKH1 and pK-GFP (McKiernan et al., 1996), to express proteins preceded by a triple epitope hemaglutinin1 (HA1)-tag or fused with the green fluorescent protein, respectively. Mutations were confirmed by DNA sequencing. Cloning and expression of Rab3A as well as the purification and cleavage of GST fusion proteins were performed as previously described (McKiernan et al., 1996).

**Rabphilin-Rab3A binding assay**

Recombinant Rab3A cleaved from GST was loaded with [$\gamma$-32P]GTP (10 μCi/ml at 5 μCi/mol) in 20 mM Hepes, pH 7.4, 150 mM NaCl, 1 mM DTT, 2 mM EDTA, pH 8.0, 0.2 mg BSA/ml) and the nucleotide was trapped on the protein by addition of MgCl$_2$ to 10 mM. Counts associated with Rab3A were determined by nitrocellulose filter binding after extensive washing (McKiernan et al., 1996). Fifty pmoles of GST-Rabphilin (1-206) fusion protein were bound to 5 μl of glutathione-Sepharose beads in TBS (20 mM Tris-HCl, pH 7.4, 150 mM NaCl). The beads were washed 3 times in TBS and resuspended in binding buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 10 mM MgCl$_2$, 0.2 mg/ml BSA and 1 mM GTP) such that the final reaction volume was 100 μl. An equal amount of radiolabelled Rab3A-GTP was then added to each tube and the mixture was incubated on ice for 10 minutes. In preliminary experiments, maximal binding was observed within 5 minutes; negligible [$\gamma$-32P]GTP release from Rab3A was detected over a period of 15 minutes after initial mixing, either in the absence or presence of Rabphilin. The bead suspension was transferred to an Octavac 8-well strip attached to a vacuum apparatus. Each well, fitted with a cellulose acetate filter (pore size 0.45 μm), had been pre-rinsed with binding buffer to reduce non-specific binding. Filters were washed and the relative amount of 32P-labeled Rab3A bound to the beads in each well was determined by scintillation counting.

**Transfection of PC12 cells and immunofluorescence**

PC12 cells were grown in DMEM (Gibco-BRL) supplemented with 5% fetal calf serum, 5% bovine serum, penicillin (100 U/ml) and streptomycin (100 µg/ml). Transfection of PC12 cells and immunofluorescence were carried out as previously described (McKiernan et al., 1996). Briefly, cells were electroporated with a total of 10-20 μg plasmid DNA per transfection at 280 μF/960 μF in a Bio-Rad Gene Pulser. Cells were plated onto dishes or glass Labtek chamber slides (Nunc) coated with poly-L-lysine (Sigma). Medium was changed after several hours and the cells were grown for two days. Cells used for immunofluorescence were grown in the presence of 40 ng recombinant human nerve growth factor (NGF, a gift from Genentech) per ml to induce neurite outgrowths. For immunofluorescence studies, cells were fixed in paraformaldehyde (4%, w/v, in phosphate-buffered saline, PBS) and permeabilized with cold methanol. GFP-Rabphilin fusion proteins were visualised directly by the intrinsic fluorescence of the GFP(S65T). HA1-tagged Rab3A was visualised using the 12CA5 anti-HA1 antibody and a Cy3-linked goat anti-mouse antibody (Jackson Immunoresearch Laboratories). Cells were imaged on a Bio-Rad MRC1000 confocal microscope. To determine the relative degrees of overlap of the GFP and Cy3 fluorosences within the transfected cells, an overlap quotient
Partitioning of full-length Rabphilin mutants in cell extracts

PC12 cell fractions were prepared as described (McKienan et al., 1996). HIT-T15 cell fractions were prepared in a similar manner, after lysing the cells by sonication for 1 second. After SDS-PAGE and transfer on a nitrocellulose membrane, proteins were submitted to immunoblotting using a rabbit anti-GFP antibody (Clontech) or the mouse monoclonal 12CA5 antibody and detected by chemiluminescence (KPL) using an horseradish peroxidase (HRP)-conjugated secondary antibody (Jackson Immunoresearch Laboratories).

Binding of endogenous Rab3C from PC12 cell extracts to GST-Rabphilin (1-206)

PC12 extracts were prepared as follows. All steps were performed at 4°C. Cells were washed in PBS and lysed in lysis buffer (50 mM Hepes, pH 7.4, 150 mM NaCl, 5 mM MgCl2, 2 mM DTT, 1 mM PMSF, 25 μg/ml aprotinin and 25 μg/ml leupeptin). Lysates were centrifuged at 500 g for 2 minutes to eliminate nuclei and unlysed cells and then at 100,000 g for 1 hour. Extracts were incubated 30 minutes with GMPPNP (1 mM) in the presence of EDTA (5 mM). Supernatants were then supplemented with MgCl2 to a final concentration of 12 mM. The cell content of a 150 mm diameter plate was the input for each experiment. The pellets were washed three times in buffer containing 50 mM Hepes, pH 7.4, 150 mM NaCl, pH 7.4, 5 mM KCl, 0.5% v/v Triton X-100, 2 mM DTT, 1 mM DTT, 1 mM PMSF, 25 μg/ml aprotinin and 25 μg/ml leupeptin. Lysates were centrifuged at 500 g for 2 minutes to eliminate nuclei and unlysed cells and then at 100,000 g for 1 hour. Extracts were incubated 30 minutes with GMPPNP (1 mM) in the presence of EDTA (5 mM). Supernatants were then supplemented with MgCl2 to a final concentration of 12 mM. The cell content of a 150 mm diameter plate was the input for each binding assay. GST-GFP or GST-Rabphilin (1-206) (10 μg) was bound to glutathione-Sepharose beads and then added to the supernatants for an incubation time of 1 hour with rocking. Beads were washed three times in buffer containing 50 mM Hepes, pH 7.4, 150 mM NaCl, 5 mM MgCl2, 0.5% Triton X-100, 2 mM DTT, 1 mM PMSF, and twice in the same buffer without Triton. Bound proteins were eluted with 15 to 20 mM glutathione (pH 8.0-8.5). Eluted proteins were subjected to SDS-PAGE and then transferred to nitrocellulose. Rab3C was detected using a specific, anti-Rab3C antibody. This rabbit anti-peptide antibody, made against unique residues of Rab3C (amino acids 195 to 209), does not recognise Rab3B or Rab3D, and shows only very weak cross-reactivity with Rab3A (G. Joberty and A. Zahraoui, unpublished data). GST fusion proteins were detected with a monoclonal antibody against GST (Santa Cruz). HRP-linked secondary antibodies (Jackson Immunoresearch Laboratories) were used to visualise proteins by chemiluminescence.

Secretion from transfected HIT-T15 cells

HIT-T15 cells were cultured as described (Regazzi et al., 1990). On the day of the experiment, 3×10⁶ cells, were resuspended in 300 μl of serum-free RPMI 1640 and were electroporated in the presence of 30 μg of a plasmid encoding human growth hormone (hGH) and 30 μg of the plasmids encoding the full-length Rabphilin constructs. Immediately after transfection the cells were diluted in culture medium and seeded in 24 multiwell plates. Three days after transfection the cells were pre-incubated during 30 minutes in 20 mM Hepes, pH 7.4, 128 mM NaCl, 5 mM KCl, 2.7 mM CaCl2, 10 mM glucose and 1 mM MgCl2. The medium was then aspirated and the cells stimulated for 10 minutes with the same buffer but containing 53 mM NaCl and 80 mM KCl. Exocytosis from transfected cells was determined by measuring by measuring by ELISA (Boeringer Mannheim) the amount of hGH secreted into the medium during the incubation period. To verify the expression of the HA-Rabphilin proteins the cells used in the secretion experiments were collected in SDS buffer and analyzed by western blotting using the anti-HA antibody. Targeting of the HA-Rabphilin proteins in HIT-T15 cells was assessed byconfocal microscopy using the anti-HA antibody to label the transfected proteins and a polyclonal anti-insulin antibody to label the secretory vesicles (Iezzi et al., 1999).

RESULTS

Residues within the N-terminal domain of Rabphilin are critical for association with Rab3 proteins

Analysis of the N-terminal, conserved Rab3 binding domain of Rabphilin using the coiled-coil algorithm of Lupas et al. (1991) showed that this region contains a putative α-helix (amino acids 44 to 90, Fig. 1, top). A projection along the axis of this helix reveals a distinct amphipathic bias (Fig. 1, bottom). The structural prediction has been recently confirmed by the publication of the crystal structure of a Rab3A-Rabphilin complex (Ostermeier and Brunger, 1999). To identify residues important for Rab3 binding we mutated to alanines a number of conserved amino acid residues within the putative α-helix. Polar (basic residues R60 and R78, acidic residues D81 and D84) as well as aliphatic amino acids (L83 and V61) were chosen.

Mutated GST fusion proteins of Rabphilin (1-206) were attached to GSH-Sepharose and assayed for binding to recombinant Rab3A.[γ-32P]GTP. The Rabphilin (1-206) fragment was used because it is resistant to proteolysis and has been shown to bind Rab3A with the same affinity as the full-length protein (McKienan et al., 1996). Mutations of the polar amino acids did not noticeably affect the association with Rab3A, but mutations of both aliphatic residues, L83A and V61A, showed, respectively, a 30% reduction and an almost complete abolition of the binding to Rab3A (Fig. 2).

To determine whether endogenous, prenylated Rab3 proteins would show the same differential interaction with the mutants of Rabphilin, we incubated the GST-Rabphilin (1-206) constructs attached on glutathione-Sepharose beads with extracts of PC12 cells. GST-GFP (a fusion protein between GST and the Green Fluorescent Protein) was used as a negative control. Because Rabphilin interacts preferentially with the GTP-bound form of Rab3A, the PC12 extracts were incubated with GMPPNP, a slowly hydrolysable analogue of GTP, prior to addition of the GST fusion proteins, to allow conversion of endogenous Rab3 proteins to the active form. The proteins retained by the beads were immunoblotted with a specific anti-Rab3C antibody. We have confirmed that all four Rab3 isoforms (A-D) bind with similar affinities to Rabphilin (Chung et al., 1999). We checked that equal amounts of fusion protein were bound to the beads using an anti-GST antibody. As shown in Fig. 3, the wild-type Rabphilin (1-206) and mutant R60A fusion proteins formed stable complexes with Rab3C produced by PC12 cells, while the mutant V61A and GST-GFP control did not. These data confirm the results obtained with recombinant Rab3A. However, Rab3C did not detectably bind to the L83A mutant of Rabphilin (1-206). This difference is likely to be related to the much lower concentration of Rab3C present in PC12 extracts, as compared to the assay in which recombinant Rab3A was used.

The results demonstrate that certain hydrophobic residues within a putative amphipathic α-helical region of the Rab3...
Fig. 1. Putative amphipathic α-helix of Rabphilin Rab3-binding domain. Top: schematic structure of Rabphilin 3A protein showing the N-terminal Rab3 binding domain with the two Zn$^{2+}$ binding motifs. An amphipathic α-helical region, identified using the coiled-coil algorithm of Lupas et al. (1991), is located just upstream of these motifs. Bovine and C. elegans sequences of that region are aligned. Identical amino acids among both sequences are indicated via straight lines and conserved ones by double-dots. Numbering corresponds to the bovine sequence of Rabphilin. Residues with numbers from position 60 to 84 indicate amino acid residues that were mutated to alanines. Bottom: helical wheel projection of residues 45-90 generated by the Protein Analysis program in the Lasergene Software package (DNA-Star). The amphipathic bias is revealed by the presence of most aliphatic residues (in black) on the right part of the helix, whereas the charged amino acids (in grey) are located primarily on the other side. Arrows and arrowheads show respectively aliphatic and charged residues that were mutated to alanines. Asterisks show residues that interact directly with Rab3A, as shown by the crystal structure of the Rabphilin:Rab3A complex (Ostermeier and Brunger, 1999).

binding domain of Rabphilin are essential for high affinity association with the small GTPase.

Rabphilin targeting is reduced but not abolished by loss of Rab3 binding domain

To determine the role of Rab3 binding in targeting Rabphilin to vesicle membranes, we compared by confocal microscopy the cellular localisation with Rab3A of the R60A, V61A and L83A mutants of full-length Rabphilin fused with GFP. Vectors encoding the GFP-Rabphilin constructs were co-transfected into PC12 cells together with a vector encoding the triple HA1-tagged Rab3A protein. As described previously, HA$_3$-Rab3 localises to vesicles concentrated at the cell periphery and in the neurite extensions induced by treatment of PC12 cells with NGF. Wild-type GFP-Rabphilin, co-localises with the HA$_3$-Rab3 (Fig. 4A). GFP-Rabphilin (R60A), which binds Rab3 with wild-type affinity, was detected mainly in the neurite outgrowths. In contrast, the labelling of the V61A mutant, which can not bind Rab3A, was more diffused. Nonetheless the protein still displayed significant co-localisation with Rab3A, especially in the neurite extensions. The L83A mutant, which has a reduced affinity for Rab3 in vitro, gave an intermediate phenotype. Immunofluorescence images were quantitated as previously described (Mc Kiernan et al., 1996; see also Materials and Methods). As shown in Table 1, the overlap quotient for the mutant R60A (0.136) is essentially the same as for wild-type GFP-Rabphilin (0.127), indicating a high degree of co-localisation with HA$_3$-Rab3. The partial co-localisation of the mutant V61A with HA$_3$-Rab3A is reflected by an overlap quotient (0.077) that is intermediate between those of wild-type GFP-Rabphilin and of free GFP (0.018). The overlap quotient between mutant L83A and Rab3A is close to that of mutant V61A (0.095). Localisation studies were also performed using a pancreatic beta cell line, HIT-T15. These cells accumulate insulin within secretory granules and have been used previously to study the effects of Rabphilin on stimulated exocytosis (Arribas et al., 1997). Confocal microscopy demonstrated a high degree of co-localisation between insulin-containing granules and HA-Rabphilin (full-length) in cells transfected with wild-type or with the R60A or V61A mutant Rabphilin proteins (Fig. 4B).

Fractionation studies were performed on extracts of both PC12 and HIT-T15 cells co-expressing GFP-Rabphilins
Fig. 2. Identification of residues within the Rab3 binding domain of Rabphilin that are important for Rab3A binding. GST-Rabphilin fusion proteins were attached to glutathione-Sepharose beads and incubated with [γ-32P]GTP-Rab3A. The beads were washed and the amount of associated Rab3A was determined by scintillation counting. Binding is expressed as percentage relative to GST-Rabphilin(1-206). Binding data are the means of two experiments performed in duplicate. Non-specific binding obtained with the GST protein alone (less than 2%) was deducted from all other values. The range for each experiment was ±1%.

Fig. 3. Mutations to alanine of aliphatic residues 61 and 83 of Rabphilin abolish the binding to endogenous Rab3 proteins. GST-GFP or GST-Rabphilin(1-206) were bound to glutathione-Sepharose beads and incubated with PC12 extracts in which the GTP-binding proteins were previously loaded with GMPPNP. After washing, GST fusion proteins and Rab3C were analysed by immunoblotting using, respectively, a monoclonal anti-GST antibody and a polyclonal anti-Rab3C antibody. Lane 1, corresponds to an aliquot (10% of input) of the PC12 extract and is used as a negative control for the anti-GST antibody and as a positive control for the Rab3C antiserum.

Table 1. Quantitation of co-localisation of HA1-Rab3A and GFP-Rabphilin proteins in transiently co-transfected PC12 cells

<table>
<thead>
<tr>
<th>Red channel</th>
<th>Green channel</th>
<th>Overlap quotient ± s.e. (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA1-Rab3A</td>
<td>GFP-Rabphilin (wild-type)</td>
<td>0.127±0.009</td>
</tr>
<tr>
<td>HA1-Rab3A</td>
<td>GFP</td>
<td>0.018±0.005*</td>
</tr>
<tr>
<td>HA1-Rab3A</td>
<td>GFP-Rabphilin R60A</td>
<td>0.136±0.016</td>
</tr>
<tr>
<td>HA1-Rab3A</td>
<td>GFP-Rabphilin V61A</td>
<td>0.077±0.010**</td>
</tr>
<tr>
<td>HA1-Rab3A</td>
<td>GFP-Rabphilin L83A</td>
<td>0.095±0.014</td>
</tr>
</tbody>
</table>

*P value ≤0.01 (difference very significant)

**P value ≤0.001 (difference extremely significant)

Overlap immunofluorescence quotients were determined by dividing the number of pixels in the multiplied image by the sum of pixels in the two original images as described in Material and Methods. A minimum of five cells were analysed per transfection condition. P values were calculated by an unpaired t-test as compared to the overlap quotient for pKH3-Rab3A and GFP-Rabphilin (wild-type) to determine if there was a significant difference in the overlap quotient.

cells in comparison with the PC12 cells (Fig. 5B). Replacing the GFP tag by a HA3 tag did not change significantly the results of the partitioning in PC12 cells (data not shown), and the difference may therefore be cell-specific. Similar to the PC12 cells, the distribution of the V60A mutant in HIT-T15 cells was close to those obtained with the wild-type protein, whereas both mutants V61A and L83A, that do not bind Rab3 with a high affinity, showed a slightly higher accumulation in the soluble fraction (Fig. 5B).

We conclude that the effects of the mutations or targetting are independent of cell type. The results indicate that high affinity binding to Rab3A is required for efficient targetting of Rabphilin to secretory vesicle membranes, but that targetting is still possible in the absence of detectable Rab3A binding.

Rabphilin mutants that do not bind Rab3 are capable of enhancing evoked secretion

To assess how the different mutants of Rabphilin affect Ca2+-regulated secretion we chose to test the effect of the Rabphilin mutants on stimulated exocytosis of HIT-T15. HIT-T15 cells were transiently co-transfected with each of the constructs and with the gene encoding hGH. Ectopically expressed hGH is targeted to dense core insulin-containing secretory granules (T. Coppola and R. Regazzi, unpublished observation). Consistent with previous studies, we observed that ectopic expression of full-length Rabphilin enhances K+-stimulated hGH secretion (Fig. 6A). Surprisingly, however, the two mutants of Rabphilin that do not bind efficiently to Rab3, V61A and R83A, stimulated secretion to comparable extents (54% and 65%, respectively). In contrast, the R60A mutant, which binds Rab3A with an efficiency undistinguishable from that of wild-type Rabphilin and is targeted to the secretory vesicle membrane, did not enhance regulated secretion. This lack of effect was not a consequence of reduced expression of the R60A mutant, as determined by immunoblotting (Fig. 6B). To determine whether the loss of function was specific for the mutation in position 60, we tested two others mutants, R78A and D81A, located on the same side of the amphipathic helix.

As was shown in Fig. 2, both mutants are able to interact normally with Rab3A; in HIT-T15 cells, they both show a partitioning similar to those obtained for the wild-type and
We found that the R78A and D81A mutants potentiated K⁺-stimulated hGH secretion as well as wild-type Rabphilin (Fig. 6A).

**DISCUSSION**

We have identified two point mutations in the N-terminal region of Rabphilin that abolish (V61A) or substantially reduce (L83A) binding to recombinant Rab3A and to endogenous Rab3 from PC12 cells extracts. Both mutations affect hydrophobic residues that are conserved between the mammalian Rabphilin and its putative homolog in the nematode *C. elegans* (Wilson et al., 1994). Four other mutations of charged amino-acid residues on the opposite face of the predicted α-helix and that are not conserved throughout evolution, had no effect on Rab3 binding. The recently published crystal structure of the Rabphilin-Rab3A complex confirms that this region forms an extended α-helix that interacts with Rab3A (Osteimeier and Brunger, 1999). The structure clearly shows that amino acid residue V61 contacts directly the C-terminal region of Rab3A whereas the adjacent residue, R60, does not, accounting for the differences in binding that we observed. The reason why the mutation of residue L83 strongly reduces Rab3 binding is less obvious. The structural data suggest that a mutation in that position might modify either the interaction between α-helices 1 and 2 or the
Rabphilin seems to be unstable when not bound to Rab3A. Neurons from genetically-engineered Rab3A<sup>−/−</sup> mice, for example, express substantially reduced levels of Rabphilin. Similarly, the Rabphilin mutants that are defective in Rab3 binding (V61A, L83A) could not be expressed in primary chromaffin cells (R. Holz, unpublished observation). One
interpretation of these data is that undocked Rabphilin is rapidly proteolysed. This problem of instability did not occur in either PC12 or HIT-T15 cells, perhaps because, in these cell types, proteins other than Rab3 can promote docking to the vesicle membrane. Accordingly, Rabphilin can bind to synaptic vesicles in which Rab3A has been biochemically stripped (Shiratani et al., 1994).

Apart from mutant R60A, all Rabphilin mutants stimulated exocytosis regardless of their ability to bind Rab3, arguing that the interaction is not required for this function. It can not be completely ruled out that mutants V61A and R83A are able to bind Rab3 in vivo but this interpretation is unlikely. First, these mutants were unable to pull down endogenous Rab3C from PC12 extracts, although wild-type Rabphilin shows similar affinities for all Rab3 proteins. Second, residue 61 is now known to directly interact with Rab3A (Ostermeier and Brunger, 1999). A previous study in a comparable system also suggested that the Rab3 binding domain was not required for Rabphilin to potentiate regulated secretion (Arribas et al., 1997). This study was made using N-terminal deletions of Rabphilin. Here, we show that unique point mutations within full-length Rabphilin can considerably impair Rab3 binding, and that a significant fraction of these proteins is correctly targeted and still potentiates Ca2+-dependent exocytosis in pancreatic beta-cells. Remarkably, another point mutant, R60A, was unable to potentiate exocytosis. This mutant was expressed and targeted to secretory vesicles to a degree similar to that of the V61A and L83A mutants. This result suggests that multiple factors bind to the N-terminal region of Rabphilin and regulate its function. The conclusion that Rabphilin-mediated effects on exocytosis are independent of Rab3A binding (Arribas et al., 1997; this study) are supported by an independent recent report in which a series of mutants of Rab3A were used (Chung et al., 1999). The authors found that there is no correlation between the binding of Rab3A to Rabphilin and the ability of the Rab3A effector-domain mutants to inhibit Ca2+-dependent exocytosis in primary chromaffin cells. In particular, Rab3A mutants T54A and F59S do not efficiently bind Rabphilin but do inhibit K+-evoked secretion like the wild-type Rab3A. Thus, the inhibitory effects of ectopically-expressed Rab3A may be a consequence of binding to and titrating out key factors other than Rabphilin that are involved in the secretory response.

Overall, these data suggest that targeting of Rabphilin to the secretory membrane and regulation of its function is more complex than previously thought. Both the targeting of Rabphilin to the secretory vesicle and its capability to potentiate regulated secretion in different cell types are not dramatically affected in the absence of high affinity Rab3A binding. We propose that both events may occur in two different ways: one dependent on Rab3 binding and another that is independent of Rab3. Although Rab3 binding is probably the predominant mechanism that localises Rabphilin to the secretory vesicle membrane in neurons, interaction of the protein with other proteins, via its proline rich regions or C2 domains, may attain the same goal. To potentiate secretion evoked by K+-depolarisation, Rabphilin may need to bind other protein(s); an interaction that would be impaired in the case of the R60A mutant. There are several possible candidate proteins. Rabphilin, via its binding to Rabaptin, an effector of Rab5 (Stenmark et al., 1995), is known to inhibit receptor-mediated endocytosis of transferrin in PC12 cells (Ohy et al., 1998). Another protein, α-actinin, has been reported to interact with the N-terminal region of Rabphilin (Kato et al., 1996). The interaction stimulates the ability of α-actinin to cross-link filament bundles, and this effect is inhibited by GTPγS-Rab3A. However, the relationship of α-actinin binding to vesicle docking and fusion is unknown. In any case, it appears that the effects of Rabphilin and Rab3 on secretion are separable and distinct, and that Rabphilin may not be the effector through which ectopically-expressed Rab3 exerts its inhibitory effect on exocytosis.

The authors thank Dr Paula Tracy, University of Vermont, for the gift of purified thrombin and Véronique Perret-Menoud for technical assistance. This work was supported by NIH grant CA56300 from the National Institute of Health (DHHIS) to I.G.M and by grant 3100-050640.97 from the Swiss National Science Foundation to R.K.F.S. was supported by an NIH Cancer Biology training grant (T32CA09286).

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