Rab3a Binding and Secretion-enhancing Domains in Rim1 Are Separate and Unique

STUDIES IN ADRENAL CHROMAFFIN CELLS*

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Rim1 was identified in brain by its ability to bind Rab3a-GTP and has been postulated to be a Rab3a effector protein. Like Rabphilin3, it modulates secretion and contains a zinc finger and two C2 domains. We have investigated the structural basis for the ability of Rim1 to bind Rab3a-GTP and to stimulate exocytosis in chromaffin cells. Both full-length and N-terminal Rim1 enhance secretion 40–50% in both intact and permeabilized cells. The abilities of Rim1 to enhance secretion and to bind Rab3a-GTP reside on distinct and relatively small domains that act independently. A ~30-amino acid sequence immediately N-terminal of the zinc finger constitutes the minimal Rab3a-GTP binding domain. This short sequence is not found in Rabphilin3 and is entirely different from the zinc finger and flanking regions of Rabphilin3 that bind Rab3a-GTP. The zinc finger domain in Rim1 is unnecessary for Rab3a-GTP binding but, alone, enhances secretion. An analysis of the characteristics of the enhancement of secretion in permeabilized chromaffin cells indicates that N-terminal Rim1 does not alter the sensitivity of secretion to Ca^{2+} but, instead, increases the rate of ATP-dependent priming of secretion.

Rab proteins are Ras-like GTPases that play important roles in mediating vesicular membrane trafficking in eucaryotic cells (1–3). More than 40 mammalian and 11 yeast Rab proteins have been characterized. They are localized in different compartments and control distinct transport systems. The Rab3 subfamily is associated with secretory granules and vesicles and plays a role in regulated secretion. Rab3a and Rab3c are associated with synaptic vesicles and chromaffin granules (4–8). Rab3a inhibits secretion when overexpressed in chromaffin cells or PC12 cells (9, 10). Experiments with various protein mutants indicate that the GTP-bound rather than the GDP-bound Rab3a is the inhibitory form. Mice with Rab3a genetically removed show a variety of alterations in synaptic transmission consistent with Rab3a modulating vesicular trafficking in the nerve terminal (11–13).

Like other GTP-binding proteins, Rab3a is thought to regulate vesicular trafficking by interacting with effector proteins. To date, three possible Rab3a effectors have been identified: Rabin (14), Rabphilin3 (15–17), and Rim family members (18, 19). All preferentially bind to GTP-bound Rab3a. Rabin has no apparent effect on secretion. Rabphilin3 and Rim1 both enhance secretion and possess similar domains. Both contain an N-terminal zinc finger domain and two C-terminal C2 domains. The C2 domains of Rabphilin3 bind calcium and acidic phospholipids (20, 21) with especially strong and specific interactions with phosphatidylinositol 4,5-bisphosphate (20). The C2 domains of Rabphilin3 and Rim1 are not highly homologous, suggesting that the lipid and Ca^{2+} binding characteristics are different. Rim, but not Rabphilin3, contains a PDZ domain that may contribute to its reported localization to presynaptic release sites (18).

Rabphilin3 is present on synaptic vesicles and secretory granule membranes and binds Rab3a in situ (22). Overexpression of full-length Rabphilin3 increases secretion by 30% in chromaffin cells (23) and 2-fold in insulin-secreting cells (24, 25). Binding of Rab3a-GTP stabilizes Rabphilin3 in chromaffin cells (23) and probably in brain (17) but is not necessary for the enhancement of secretion (23, 25).

Although Rim1 and Rabphilin3 have structural homologies, the proteins have different functions and characteristics in situ. Rim1 is stable in Rab3a knockout mice, and its localization in nerve terminals is reported to be at active zones and not on synaptic vesicles (18). N-terminal Rim1, which contains the Rab3a binding domain, enhances secretion in PC12 cells (18), in contrast to the homologous domain of Rabphilin3, which inhibits secretion (23, 27). The zinc finger-containing region in Rabphilin3 is also required for binding to Rab3a-GTP (22, 23, 28).

In this study, we determined the minimal domains of Rim1 necessary for Rab3a binding and for the enhancement of secretion. We found that the domains are distinct, are both located in the N terminus of Rim1, and can function independently. Surprisingly, the Rab3a binding domain of Rim1 does not include the zinc finger domain but requires only a short segment of ~30 amino acids N-terminal to the zinc finger domain. Finally, we determined the step in the secretory pathway at which N-terminal Rim1 enhances secretion.

MATERIALS AND METHODS

N-terminal Rim1 Cloning and Sequencing—According to the Rim1 sequence (18), two primers (5’ -cgg gag gat cga tgg ctc gct cgg ccg tg-3’; 5’ -gga att gta ctc tca ctc gct cag ggg ttg gtt gaa ttc-3’) were designed to amplify Rim1 (1–399) from rat brain mRNA (CLONTECH) using reverse transcription-polymerase chain reaction. Polymerase chain reaction products were digested with BamHI and EcoRI and then cloned into pCMV-HA, a mammalian expression vector that contains the hemagglutinin epitope (PDVDY-DYA). Sequencing analysis showed that residues 56–105 were missing in splice site #1 (19).

Construction of Full-length and N-terminal Deletion Mutants—Deletion mutants of Rim1 were made by polymerase chain reaction from Rim1 (1–399). Primers were designed to generate 5’-BamHI and 3’-EcoRI restriction sites. Polymerase chain reaction products were digested with BamHI and EcoRI and subcloned into pGEX-2T (Amersham Phar-
Rim1, Rab3a, and Secretion

RESULTS

N-terminal and Full-length Rim1—We cloned N-terminal Rim1 (Rim1(1–399)) from rat brain mRNA. Its nucleotide sequence was identical to the published sequence (18) except for a larger deletion (residues 56–105) in splice site #1. It encoded ~44-kDa protein (data not shown). Recombinant full-length Rim1, amino acids 1–1553, was constructed as described under “Material and Methods.” It lacked splice sites #1, #4, and #5 and encoded a ~160-kDa protein (data not shown). The HA-tagged proteins were detected both with anti-HA and anti-Rim1 directed against the zinc finger domain.

A schematic of the two constructs is shown in Fig. 1A. Unlike Rabphilin3 (27), no endogenous Rim1 mRNA could be detected in either chromaffin cells or PC12 cells (Northern blot data not shown), consistent with Rim1 being a brain-specific gene.

Full-length and N-terminal Rim1 Enhance Secretion When Transiently Expressed in Chromaffin Cells—Wang (18) reports that the N-terminal region of Rim1 enhanced secretion in PC12 cells. We examined the effects on secretion of transiently expressed full-length and N-terminal Rim1 in primary cultures of bovine chromaffin cells. Secretion stimulated by nictinotic agonist was strongly enhanced (~50%) by both constructs (Fig. 2A).

The enhancement of secretion from intact cells could result from altered Ca\(^{2+}\) signaling upon nictinotic receptor activation or from a direct effect on the intracellular pathway triggered by Ca\(^{2+}\). To investigate the direct effects of the proteins on the secretory pathway, secretion from digitonin-permeabilized cells was examined. Secretion stimulated by 30 μM Ca\(^{2+}\) was strongly enhanced by both the N-terminal construct and full-length Rim1 (Fig. 1B), indicating that the proteins act downstream of the Ca\(^{2+}\) signal.

Effects of Rim1 on the Ca\(^{2+}\) Sensitivity of Secretion and on ATP-dependent and ATP-independent Secretion—During the first few minutes of a constant Ca\(^{2+}\) stimulus in permeabilized cells, several steps in the secretory pathway are evident (31, 34, 35). One step does not require the continuing presence of MgATP and has been termed “primed” secretion. Primed secretion probably reflects the prior effects of ATP in cells before permeabilization. It decays rapidly after permeabilization in the absence of MgATP. Another step requires the continuing presence of MgATP, which maintains the primed state. Once priming has occurred, Ca\(^{2+}\) can trigger secretion. The steps are summarized as follows.

Unprimed → Primed → Exocytosis

\[\text{ATP} \rightarrow \text{Ca}^{2+}\] (Eq. 1)

To determine whether Rim1(1–399) alters the sensitivity of the secretory pathway to Ca\(^{2+}\), the effects of Rim1(1–399) on secretion stimulated by different Ca\(^{2+}\) concentrations were investigated (Fig. 3A). Rim1 enhanced secretion at 1, 3, and 30 μM Ca\(^{2+}\) by 64, 62, and 61%, respectively. The almost identical enhancements of secretion at the different Ca\(^{2+}\) concentrations indicate that Rim1(1–399) does not alter the sensitivity of the secretory pathway to Ca\(^{2+}\).

The effects of Rim1(1–399) on ATP-dependent and -independent secretion from permeabilized cells were determined. Cells transfected with or without Rim1(1–399) were permeabilized for 4 min in the presence or absence of 2 mM MgATP and then were stimulated with 30 μM Ca\(^{2+}\) for 2 min in the continuing presence or absence of MgATP. The expression of Rim1(1–399) more strongly enhanced secretion in the presence of MgATP (Fig. 3B). In three experiments, Rim1(1–399) enhanced ATP-dependent secretion (the difference in secretion in the presence and absence of MgATP) and ATP-independent secretion by 71 ± 3 and 38 ± 5%, respectively (p < 0.005). The protocol used in the

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1 The abbreviations used are: PIPEP, 1,4-piperazinedithanesulfonic acid; KEGP, solution containing potassium glutamate, EGTA, and PIPEP, DMPP, dimethylphosphorimidazol; hGH, human growth hormone; ANP-GFP, atrial natriuretic peptide-green fluorescent protein; HA, hemagglutinin; GTPyS, guanosine 5′-O-(thiodiphosphate).
above experiments gives excellent estimates of ATP-dependent secretion, but because of the run-down of already primed secretion in permeabilized cells (31, 34), the amount of ATP-independent secretion was small. An alternative protocol in which Ca\textsuperscript{2+} is added together with digitonin (in the absence of ATP) greatly increases ATP-independent secretion. Using this protocol, Rim(1–399) enhanced secretion by 28%. Taken together, these results indicate that Rim1 preferentially enhances ATP-dependent secretion.

Minimal Region Required to Enhance Secretion in Chromaffin Cells—To determine the minimal region necessary for the enhancement of secretion, N- and C-terminal deletion mutants were constructed from Rim(1–399). Stimulated secretion from control cells (15–20% of the total expressed hGH) was normalized to 100%. The results of a series of experiments are summarized in Fig. 4. The enhancement of secretion was maintained upon deletion of the N-terminal 50 amino acids (Fig. 4A). Further deletions from the N terminus caused loss of activity, with Rim(1–399) having no effect on secretion.

Constructs Rim(11–399)\textsuperscript{a} and Rim(28–399)\textsuperscript{a} contained the region in splice site #1. Rim(1–214)\#, a different splice variant, had a 23-amino acid deletion in splice site #1. Experiments with these plasmids showed that the spliced regions do not contribute to the enhancement of secretion.

C-terminal deletions revealed that activity was maintained in constructs as short as Rim(1–190) (Fig. 4B). This construct does not contain the SGAWFF motif (residues 198–203) that in Rabphilin3 plays a critical role in binding to Rab3a-GTP. In addition, the enhancement of secretion does not require the high charged region (Rim(226–399)). Rim(1–190) contains the 4 pairs of cysteines that constitute a zinc finger domain. This domain is homologous to those in Rabphilin3, EEA1, and HRS-2. Deletion of even one pair of cysteines (Rim(1–180)) resulted in complete loss of activity. The results indicate that the zinc finger domain is important for the enhancement of secretion by Rim1.

The above results lead to the prediction that a peptide containing residues 51–190 should still enhance secretion. This was indeed the case. Construct Rim(51–190) enhanced hGH secretion in chromaffin cells as effectively as Rim(1–399), whereas Rim(71–190) was without effect (Fig. 5). Because there was a 50-amino acid deletion in splice site #1, Rim(51–190) actually encodes 90 amino acids. Thus, the minimal enhancing domain of Rim1 lacks the highly charged region, the SGAWFF motif and the N-terminal 50 amino acids. The integrity of the entire zinc finger is important for the enhancement of secretion.

Identification of Rab3a Binding Domain in Rim1—N-terminal deletion mutants of Rim1 were put into pGEX.2T vectors and expressed as GST-Rim1 fusion proteins. The expressed proteins were bound to glutathione-Sepharose beads, which were incubated in the presence of either 1 mM GTP\textsubscript{GDP} with lysates from human embryonic kidney 293 cells transiently expressing HA-Rab3a. The proteins on the beads were subjected to 10% SDS-polyacrylamide gel electrophoresis, and the bound Rab3a was detected with anti-HA antibody.

We confirmed that N-terminal Rim1 (Rim(1–399)) binds Rab3a in a GTP-dependent manner (Fig. 6A). Rim1 constructs lacking the N-terminal 19 amino acids still bound Rab3a-GTP,
FIG. 3. Effects of Rim(1–399) on the Ca$^{2+}$ sensitivity of secretion (A) and on ATP-dependent and -independent secretion in permeabilized cells (B). Chromaffin cells were cotransfected with plasmids encoding hGH and Rim(1–399) or pCMV.neo as in Fig. 2. A, cells were permeabilized with 20 mM digitonin in Ca$^{2+}$-free KGEP containing 2 mM MgATP for 4 min followed by incubation with the indicated Ca$^{2+}$ concentrations for 4 min in the continuing presence of MgATP. B, cells were permeabilized with 20 mM digitonin in Ca$^{2+}$-free KGEP with or without 2 mM MgATP for 4 min followed by incubation with or without 30 mM Ca$^{2+}$ in the continuing presence or absence of 2 mM MgATP for 2 min. ATP-dependent secretion was calculated as the difference of secretion in the presence and absence of ATP. *, p < 0.001 versus pCMV.neo.

FIG. 4. Effects on secretion of various N-terminal and C-terminal deletions of Rim1. Chromaffin cells were cotransfected with plasmids encoding hGH and either pCMV.neo or Rim constructs. Four to 5 days later, cells were incubated in physiological salt solution for 2 min = 20 mM DMPP. Data are presented as percent enhancement of DMPP-induced hGH secretion relative to hGH secretion from cells transfected with control plasmid pCMV.neo. The numbers of experiments performed are indicated in parentheses above the error bars. Constructs labeled with an asterisk are without the deletion in splice site #1. Constructs labeled with a number sign (#) have residues 83–105 deleted in splice site #1. All other Rim constructs have residues 56–105 deleted in splice site #1.

whereas further deletion (28 amino acids) totally prevented Rab3a-binding (Fig. 6A). Rim(28–399) does not contain the first splice region, whereas Rim(28–399)* retains all the amino acids in splice site #1. The latter construct also lacked the ability to bind Rab3a-GTP, indicating that the splice insert does not restore specific Rab3a-GTP binding.

The SGAWFF motif (residues 198–203) in Rim1 is immediately C-terminal to the zinc finger. The SGAWFF motif in Rabphilin3 has a similar location and is required for the GDP-dependent binding to Rab3a. Surprisingly, two C-terminal deletions, Rim(1–200) and Rim(1–190) bound Rab3a-GTP as strongly as Rim(1–399) (Fig. 6B), indicating that the SGAWFF motif is not required for binding. Rim(1–190) contains the 4 pairs of conserved cysteines in the zinc finger. Removal of one pair (Rim(1–180)), two pairs (Rim(1–160)), three pairs (Rim(1–150)), and four pairs (Rim(1–150)) of cysteines did not prevent binding to Rab3a. No binding was detected for two shorter constructs, Rim(1–139) and Rim(1–28). We conclude that the minimal domain in Rim1 responsible for GDP-dependent binding to Rab3a lies within residues 19–50. The zinc finger motif is not needed for binding to Rab3a-GTP.

It was demonstrated with the yeast two-hybrid system that Rim(1–345) and Rim(11–399) bind Rab3a and Rab3c but not other Rab family members (18). The present data indicate that a small region, less than 50 amino acids, is responsible for GDP-dependent binding of Rim1 to Rab3a. Although this domain is much smaller than those previously examined, it nevertheless shows specificity for Rab3a. Rim(1–50), although able to bind Rab3a-GTP, is unable to bind Rab5-GTP (Fig. 6C), a GTPase necessary for early endosome trafficking. The abilities of different Rim1 N-terminal constructs to bind to Rab3a-GTP and enhance secretion are compared in Table I.

Immunocytochemical Localization of Rim1 Constructs—Chromaffin cells were cotransfected with plasmids encoding various HA-tagged Rim1 constructs and ANP-GFP to label chromaffin granules (Fig. 7). Cells were fixed and permeabilized, and double immunofluorescence was used to visualize the HA-Rim1 constructs. Full-length Rim1 (Rim(1–1553)) (Fig. 7, A and B) was diffusely distributed in the cytoplasm, without apparent plasma membrane or chromaffin granule localization. There was also full-length Rim1 in the nucleus. Rim(1–190) had a patchy cytoplasmic distribution that partially overlapped (data not shown) with the highly punctate ANP-GFP-containing chromaffin granules (Fig. 7, C and D). Surprisingly, Rim(1–399) was concentrated in the nucleus, with much smaller amounts in the cytosol (Fig. 7, E and F). This nuclear localization was probably caused by the highly charged region in the N terminus that was exposed upon deletion of the C terminus of full-length Rim. Rim(1–190) (without the highly charged region) did not localize to the nucleus (Fig. 7, C and D), whereas the highly charged region (Rim(226–399)), when expressed alone, was concentrated in the nucleus (Fig. 7, G and H). Rim(1–180), which did not alter secretion, had a patchy cytoplasmic distribution (Fig. 7, I and J).

Full-length Rim1 was found both in the cytosol and nucleus. It may shuttle between the nucleus and cytosol using nuclear import and export pathways. Treatment with leptomycin B (200 nM), an inhibitor of the CRM1-dependent nuclear export pathway (36–38), did not cause increased further accumulation of Rim1(1–1553) in the nucleus (data not shown). If Rim1 shuttles between the cytoplasm and nucleus, a CRM1-mediated nuclear export pathway is not involved.

DISCUSSION

Rim1 is a large Rab3a-binding protein with multiple domains including a N-terminal zinc finger domain, a central PDZ domain, potential phosphorylation sites, and two C2 domains at the C terminus (18). In this study, we investigated the structural basis for the ability of Rim1 to bind Rab3a-GTP and to stimulate exocytosis from chromaffin cells. We found that the abilities of Rim1 to enhance secretion and to bind Rab3a-GTP reside on distinct and relatively small domains that act...
Fig. 5. The minimal domain necessary to enhance secretion in chromaffin cells includes the zinc finger. Chromaffin cells were cotransfected with plasmids encoding hGH and either Rim(51–190), Rim(71–190), Rim(1–399), or the parent plasmid pCMV.neo. Secretion experiments in intact cells (A) and in permeabilized cells (B) were performed as described in Fig. 2.

Fig. 6. Binding of various deletion mutants of Rim1 to GTPγS- or GDP-bound Rab3a and Rab5. GST-Rim1 constructs were expressed in E. coli and immobilized on glutathione-Sepharose beads. HA-Rab3a or HA-Rab5 were expressed in human embryonic kidney 293 cells and solubilized with Triton X-100. Cell lysates were incubated with beads in the presence of 1 mM GTPγS or GDP as described under "Materials and Methods." Bound Rab was detected by 10% SDS-polyacrylamide gel electrophoresis and immunoblotting for HA.

TABLE I

Comparison of the ability of Rim1 deletion mutants to bind Rab3a-GTP and to enhance secretion

| HA-tagged mutant | Rab3a-GTP binding | Enhancement of DMPP stimulated secretion |
|------------------|---------------------|-----------------------------------------|
| Rim(1–399)       | Yes                 | ++                                      |
| Rim(10–399)      | Yes                 | +                                       |
| Rim(11–399)      | Yes                 | +                                       |
| Rim(19–399)      | Yes                 | +                                       |
| Rim(28–399)      | No                  | +                                       |
| Rim(28–399)      | No                  | +                                       |
| Rim(51–399)      | No                  | +                                       |
| Rim(71–399)      | No                  | +                                       |
| Rim(1–214)       | Yes                 | +                                       |
| Rim(1–200)       | Yes                 | +                                       |
| Rim(1–190)       | Yes                 | +                                       |
| Rim(1–180)       | Yes                 | –                                       |
| Rim(1–160)       | Yes                 | –                                       |
| Rim(1–150)       | Yes                 | –                                       |
| Rim(1–135)       | Yes                 | –                                       |
| Rim(1–120)       | Yes                 | –                                       |
| Rim(1–50)        | Yes                 | –                                       |
| Rim(1–39)        | No                  | –                                       |
| Rim(1–28)        | No                  | –                                       |
| Rim(51–190)      | No                  | +                                       |
| Rim(71–190)      | No                  | –                                       |
| Rim(71–399)      | No                  | –                                       |
| Rim(51–399)      | No                  | –                                       |
| Rim(28–399)      | No                  | –                                       |

Constructs labeled with asterisk (*) have no deletion in splice site 1. Constructs labeled with a number sign (#) have residues 83–105 deleted in splice site 1. Rab3a binding and effects on secretion require different domains in N-terminal Rim1. The table summarizes the data that indicate that the abilities of Rim1 to bind Rab3a-GTP and to enhance secretion from chromaffin cells reflect the function of distinct domains. The Rab3a-GTP binding domain corresponds to amino acids 19–50. The minimal domain that enhances secretion corresponds to amino acids 51–190.

Fig. 5. The minimal domain necessary to enhance secretion in chromaffin cells includes the zinc finger. Chromaffin cells were cotransfected with plasmids encoding hGH and either Rim(51–190), Rim(71–190), Rim(1–399), or the parent plasmid pCMV.neo. Secretion experiments in intact cells (A) and in permeabilized cells (B) were performed as described in Fig. 2.

Fig. 6. Binding of various deletion mutants of Rim1 to GTPγS- or GDP-bound Rab3a and Rab5. GST-Rim1 constructs were expressed in E. coli and immobilized on glutathione-Sepharose beads. HA-Rab3a or HA-Rab5 were expressed in human embryonic kidney 293 cells and solubilized with Triton X-100. Cell lysates were incubated with beads in the presence of 1 mM GTPγS or GDP as described under "Materials and Methods." Bound Rab was detected by 10% SDS-polyacrylamide gel electrophoresis and immunoblotting for HA.

An analysis of the characteristics of the enhancement of secretion indicates that Rim1 acts at a step after Ca2+ entry in the cells to enhance the ATP-dependent priming of secretion.

Rim1 and Rabphilin3 Possess Different Rab3a Binding Domains—The N termini of Rim1 and Rabphilin3 have similar structures with homologous zinc finger regions and a distinctive SGAWFF motif (Fig. 1). They both preferentially bind the GTP-bound form of Rab3a. Surprisingly, the region of Rim1 that binds Rab3a-GTP is distinct from that in Rabphilin3. In Rabphilin3, an ~140-amino acid residue is responsible for the binding of Rab3a-GTP (Fig. 1B) (22, 23, 28). The domain includes the entire zinc finger and flanking sequences. Ostermeier and Brunger explored the crystal structure of Rab3a-GTP/Rabphilin3 and report two contact areas in the complex (39). Although the zinc finger does not directly contact Rab3a, it serves as a scaffold to hold the Rab3a-interacting domains in a binding conformation. The SGAWFF motif that is C-terminal to the zinc finger directly interacts with Rab3a.

Neither the zinc finger nor the SGAWFF motif of Rim1 is required for binding to Rab3a-GTP. Either or both can be totally removed from N-terminal constructs without altering the specific binding to Rab3a-GTP. The minimal Rab3a binding domain in Rim1 was mapped to residues 19–50, immediately N-terminal to the zinc finger. This domain is predicted to be a coiled-coil domain (40). It has little homology to the corresponding region in Rabphilin3. Thus, Rim1 and Rabphilin3 possess different Rab3a binding domains, suggesting that their interaction sites on Rab3a are distinct.

Full Length Rim1 and Its Zinc Finger Domain Enhance Secretion in Chromaffin Cells—A previous study had demonstrated that the N-terminal domain of Rim1 enhances secretion when transiently expressed in PC12 cells (18). In the present study we demonstrated that full-length Rim1 and as well as truncation mutants without the two C2 domains and the PDZ domain stimulate secretion similarly in chromaffin cells. The ability of full-length Rim1 to stimulate secretion suggests that the endogenous protein normally acts as a positive regulator of exocytosis.

The minimal N-terminal domain required for the enhance-
A. Rim(1-1553)  B. ANP-GFP  C. Rim(1-190)  D. ANP-GFP  E. Rim(1-399)  F. ANP-GFP  G. Rim(226-399)  H. ANP-GFP  I. Rim(1-180)  J. ANP-GFP

Fig. 7. Localization by confocal microscopy of HA-tagged Rim constructs in chromaffin cells. Chromaffin cells were cotransfected with plasmids encoding ANP-GFP, a chromaffin granule marker, and HA-tagged Rim constructs. After 5 days, cells were fixed, permeabilized, and immunocytochemistry performed to detect the HA-tagged Rim. ANP-GFP was visualized in the fluorescein isothiocyanate channel (B, D, F, H, J); HA-tagged Rim was visualized in the lissamine-rhodamine channel (A, C, E, G, I).

ment of secretion was located between residues 51 and 190 and did not require a splice insert. It contains the 90 amino acids comprising the cysteine-rich, zinc finger region. Importantly, the ability of transiently expressed full-length and N-terminal constructs of Rim1 to stimulate secretion in permeabilized cells indicates that the enhancing effects are a direct effect of the proteins on the Ca\(^{2+}\)-dependent secretory pathway and not on Ca\(^{2+}\) entry.

The N-terminal Domain of Rim1 Stimulates Secretion by Enhancing ATP-dependent Priming—We had previously demonstrated that ATP acts before the final Ca\(^{2+}\)-triggering step to prime the cells for exocytosis (31, 34). In the present study we found that Rim1 increased the ability of ATP to prime secretion in permeabilized cells without altering the sensitivity of the secretory response to Ca\(^{2+}\) (Figs. 3 and 4). Rim1 also increased, but to a lesser degree, secretion that did not require the continuing presence of ATP (Fig. 4). This seemingly ATP-independent secretion probably reflects the prior effects of ATP in the intact cell before permeabilization (34). It is likely that the enhancement of ATP-independent secretion reflects an increase in the degree of priming in the intact cells.

ATP-dependent priming is a dynamic process (31, 34). Rim1(1–399) could be either increasing the rate of priming or decreasing the rate of unpriming. If Rim1(1–399) decreased the rate of unpriming, then the primed state would be stabilized in the presence of Rim1(1–399), and the decline in the secretory response in the absence of ATP would be slowed. We investigated the effects of Rim1(1–399) on the stability of the primed state after permeabilization in the absence of ATP. When Ca\(^{2+}\) was added after 4 min of permeabilization, primed secretion decreased similarly in the presence and absence of Rim1(1–399) (75 and 73% declines, respectively, data not shown). The data support the notion that Rim1(1–399) increases the rate of ATP-dependent priming rather than decreasing the rate of unpriming.

Localization of Rim1 Constructs in Chromaffin Cells—Endogenous Rim1 is concentrated to presynaptic active zones in neurons and presynaptic ribbons in ribbon synapses (18). Because of the variety of possible splice variants, the actual protein that was visualized in these experiments is unknown. The full-length Rim1 protein that we constructed had a diffuse, cytoplasmic distribution with no evident plasma membrane association when transiently expressed in chromaffin cells. The localization of endogenous Rim1 to the active zone in neurons may require interaction with active zone-specific proteins that are not present in the chromaffin cell plasma membrane. The different subcellular distributions (diffuse, patchy, or nuclear) of the various deletion mutations investigated in this study suggest that multiple domains could determine localization. Despite differences in subcellular distribution in chromaffin cells, all of the constructs with an intact zinc finger domain similarly enhanced secretion.

Although Rim1(1–399) was detected in the cytoplasm, its predominant localization to the nucleus was unexpected and raised the possibility that Rim1 enhances secretion because of an effect on gene expression. However, an analysis of other Rim1 mutants revealed that nuclear localization was unnecessary for the enhancement of secretion. Highly charged Rim1(226–399) localized entirely to the nucleus, indicating that this sequence contains a nuclear localization domain. It had no effect on secretion. Rim1(1–190), a construct without the nuclear localization domain, was entirely cytoplasmic and enhanced secretion. Thus, it is likely that cytoplasmic rather than nuclear Rim1 enhances secretion.

Full-length Rim1 (Rim1(1–1553)) was not concentrated in the nucleus. Its cytoplasmic localization was not altered significantly by leptomycin B, an inhibitor of CRM1-dependent nuclear export. It is possible that deletion of the C terminus in Rim1(1–399) exposed a nuclear localization signal whose function is suppressed in the full-length protein.

Comparison of the Effects on Secretion of Rim1 and Rabphilin3—The domains required for the enhancement of secretion by Rim1 and Rabphilin3 are strikingly different. In previous studies, we found that both C2 domains as well as the zinc finger were required for the enhancement of secretion by Rabphilin3 (23, 27). In fact, N-terminal Rabphilin3 constructs terminating after the zinc finger inhibit rather than enhance secretion. The enhancement of secretion by Rim1 requires only the zinc finger region and not the C-terminal C2 or other identified domains. In addition, Rim1 constructs revealed that nuclear localization was unnecessary for enhanced secretion from permeabilized cells. These differences are likely to reflect different mechanisms underlying the enhancements of secretion by these two Rab3a-binding proteins.

The enhancements of secretion by Rim1 and Rabphilin3 are similar in that neither requires interaction with Rab3a. The main function of the interaction of Rabphilin3 and Rab3a is to stabilize Rabphilin3 in chromaffin cells (23). Rim1 constructs that were unable to bind Rab3a were well expressed in chromaffin cells (e.g. Rim(51–399), Rim(71–399)). There was no evidence that interaction with Rab3a stabilized Rim1 in chromaffin cells.

Other Rim Family Members—Rim1 belongs to a family of proteins, each of which has many possible splice variants. Rim2 is highly homologous to Rim1 (19). Although we did not investigate Rim2, it is likely that its N terminus functions similarly to that of Rim1. Amino acids 19–50 of Rim1 constitute the minimal sequence necessary to bind Rabphilin3—similarly enhanced secretion.
region that is 79% homologous to the smallest Rim1 construct that enhanced secretion. This region contains a zinc finger whose integrity is required for the enhancement of secretion. The zinc finger domains in Rim1 and Rim2 are 90% identical. 

Nim2 and Nim3 have been also identified as part of the Rim family (19). Both these smaller proteins contain a C2 domain and flanking domains that are highly homologous to the C2B and adjacent regions in Rim1 (19). Neither contains zinc finger domains. Nim3 enhances elevated K⁺-induced secretion in PC12 cells (19). It is possible that C2B region as well as the zinc finger region in Rim1 can stimulate secretion. Thus, the protein may contain two regions that enhance the secretory response.

Several Rim-binding proteins have been identified. Two of them bind through SH3 domains to a specific region in the C terminus of Rim (19). Their function in secretion has not been investigated. Another protein, cAMP-GEFII, binds to a region C-terminal to the zinc finger domain and N-terminal to the first C2 domain (C2A) in Rim2 (26). CAMP-GEFII enhances secretion from PC12 and insulin-secreting cells through a mechanism that requires both interaction with Rim2 and the direct binding of cAMP. None of these Rim-binding proteins are likely to play a role in the enhancement of secretion caused by the zinc finger of Rim1, which does not contain the specific binding sites for these various proteins.

In summary, our investigation together with other recent studies highlights the importance of Rim as a regulator of exocytosis. Its many distinct domains are likely to reflect a role of the protein in integrating cellular signals, including those that modulate secretion. Although chromaffin cells do not normally express Rim1, we suspect that the activity of the transiently expressed protein in chromaffin cells reflects aspects of the function of the endogenous protein. We have identified the zinc finger domain as being sufficient for the enhancement of secretion. The interaction with Rab3a-GTP, although not necessary for the enhancement of secretion, is likely to serve another function, perhaps allowing the proper transport of the protein in the neuron.
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