Direct Interaction of the Rab3 Effector RIM with Ca\textsuperscript{2+} Channels, SNAP-25, and Synaptotagmin\*  

Received for publication, January 31, 2001, and in revised form, June 13, 2001  
Published, JBC Papers in Press, July 3, 2001, DOI 10.1074/jbc.M100929200

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To define the role of the Rab3-interacting molecule RIM in exocytosis we searched for additional binding partners of the protein. We found that the two C\textsubscript{2} domains of RIM display properties analogous to those of the C\textsubscript{2}B domain of synaptotagmin-I. Thus, RIM-C\textsubscript{2}A and RIM-C\textsubscript{2}B bind in a Ca\textsuperscript{2+}-independent manner to αB, the pore-forming subunit of N-type Ca\textsuperscript{2+} channels (EC\textsubscript{50} = 20 nM). They also weakly interact with the αC but not the αD subunit of L-type Ca\textsuperscript{2+} channels. In addition, the C\textsubscript{2} domains of RIM associate with SNAP-25 and synaptotagmin-I. The binding affinities for these two proteins are 203 and 24 nM, respectively, for RIM-C\textsubscript{2}A and 224 and 16 nM for RIM-C\textsubscript{2}B. The interactions of the C\textsubscript{2} domains of RIM with SNAP-25 and synaptotagmin-I are modulated by Ca\textsuperscript{2+}. Thus, in the presence of Ca\textsuperscript{2+} (EC\textsubscript{50} = 75 μM), the interaction with synaptotagmin-I is increased, whereas SNAP-25 binding is reduced. Synaptotagmin-I binding is abolished by mutations in two positively charged amino acids in the C\textsubscript{2} domains of RIM and by the addition of inositol polyphosphates. We propose that the Rab3 effector RIM is a scaffold protein that participates through its multiple binding partners in the docking and fusion of secretory vesicles at the release sites.

Secretion of neurotransmitters, polypeptide hormones, and a variety of other proteins occurs by exocytosis, a multistage process including targeting, docking, and, finally, fusion of secretory vesicles with the plasma membrane. During the last few years, a combination of genetic and biochemical approaches has led to the identification of several proteins involved in this complex cascade of events. Most of these proteins turned out to be specialized components of the evolutionary conserved machinery that governs intracellular vesicular trafficking in eukaryotic cells (1). Exocytosis was found to necessitate the assembly of a ternary complex between the vesicular SNARE\textsuperscript{1} VAMP, associated with the secretory vesicle, and the target SNAREs syntaxin-1 and SNAP-25, localized at the plasma membrane (2). The SNARE complex was initially proposed to ensure the docking of secretory vesicles at the plasma membrane (3). However, it is unlikely that SNARE assembly constitutes the sole determinant for the targeting of secretory vesicles at the release sites because SNARE pairing is rather promiscuous (4, 5), and the localization of syntaxin-1 and SNAP-25 is not restricted to active zones (6). A current hypothesis, supported by biochemical and structural data, proposes that the assembly of the heterotrimeric complex between VAMP, SNAP-25, and syntaxin-1 provides the driving force for membrane fusion (7).

In most secretory systems, the exocytotic process is initiated by an increase in the intracellular Ca\textsuperscript{2+} concentration. In some cells, such as neurons, the elevation of Ca\textsuperscript{2+} ions is due to opening of voltage-gated calcium channels that are clustered at the release sites, whereas in others, Ca\textsuperscript{2+} ions are mobilized from intracellular stores. Biochemical and genetic studies indicate that synaptotagmins constitute the main Ca\textsuperscript{2+} sensors for regulated exocytosis (8). Synaptotagmin (Syt)-I, the best characterized member of the synaptotagmin family, is localized on synaptic vesicles and is essential for the fast, Ca\textsuperscript{2+}-dependent component of neurotransmitter release (9, 10). Synaptotagmins possess a short N-terminal tail that resides inside secretory vesicles, a single membrane-spanning region, and a large cytoplasmic sequence containing two so-called C\textsubscript{2} domains designated C\textsubscript{A} and C\textsubscript{B}. These two domains cooperate to mediate the binding of synaptotagmins with several proteins and phospholipids. Although most of these interactions are influenced by both C\textsubscript{2} domains, Ca\textsuperscript{2+}-dependent binding to syntaxin-1 and anionic phospholipids is predominantly associated with C\textsubscript{A} (11–13). In contrast, Ca\textsuperscript{2+}-dependent oligomerization of synaptotagmins and several Ca\textsuperscript{2+}-independent interactions such as the binding to the II-III cytoplasmic loop or “synprint” (synaptic protein interaction) motif of the pore-forming subunit of Ca\textsuperscript{2+} channels (14) to inositol polyphosphates (15) and to SNAP-25 (16) are mediated predominantly by C\textsubscript{B}.

Ras-like GTPases of the Ras family represent additional components of the machinery involved in vesicle trafficking. Rab GTPases are associated with distinct cellular compartments and control different steps in the secretory pathway (17). The four isoforms of Rab3 (Rab3A, -B, -C, and -D) are associated with secretory vesicles of neuronal, endocrine, and exocrine cells and regulate exocytosis (18). It is still unclear whether Rab3 proteins participate in vesicle docking, membrane fusion, or both processes. Constitutively active mutants of Rab3 diminish regulated secretion (19–22), and in Rab3A-deficient mice, the number of synaptic vesicle fusions elicited by a nerve impulse is increased (23), suggesting that Rab3

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\* This work was supported by Swiss National Science Foundation Grants 31-05064.97 and 32-061400.00 (to R. R.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EBI Data Bank with accession number(s) AF262305-AF292310.

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§ The abbreviations used are: SNARE, SNAP receptor; Syt, synaptotagmin; GST, glutathione S-transferase; IP6, inositol-1,2,3,4,5,6-hexakisphosphate; RT-PCR, reverse transcription-polymerase chain reaction; SDS-PAGE, SDS-polyacrylamide gel electrophoresis.
functions as a negative modulator of exocytosis. However, amylase secretion is enhanced in transgenic mice displaying mild overexpression of Rab3D in pancreatic acinar cells (24), and inhibition of Rab3B expression in anterior pituitary cells by antisense oligonucleotides results in a decrease in Ca\(^{2+}\)-induced secretion (25). These contradictory observations could be due to differences in the cell systems, or they may reflect the existence of positive and negative regulatory functions of Rab3 proteins differentially highlighted by the experimental approach used. RabS have been suggested to contribute an additional layer of specificity to vesicular transport by controlling the assembly of the SNARE complex. Ypt1, a Rab protein implicated in the transport between endoplasmic reticulum and Golgi in yeast, has been proposed to favor SNARE assembly by associating with the target SNARE Sed5 (26). However, the binding of other members of the Rab family to target SNAREs was found to be inefficient and nonspecific (4). Rab6, Rab3, and Rab4 have also been shown to bind to the N-ethylmaleimide-sensitive fusion protein, but the physiological meaning of this interaction remains to be established (27).

Recently, the Rab5 effector EEA1 has been reported to interact directly with the target SNAREs syntaxin-6 and syntaxin-13 (28, 29), indicating that some RabS may rely on their effectors for controlling the assembly of the SNARE complex.

RIM is a putative effector of Rab3 that associates selectively with the active form of the GTPase (30). RIM contains an N-terminal domain that interacts with Rab3 and Munc13-1 (31) and two C\(_2\) domains located at the C terminus. In rat, the distance between the two C\(_2\) regions of RIM is determined by alternatively spliced sequences that generate a large set of different isoforms (30, 32). RIM is concentrated in presynaptic active zones, and overexpression of the Rab3-binding domain of RIM in PC12 and insulin-secreting cells enhances exocytosis elicited by secretagogues (30, 33). RIM was also found to bind to the cAMP sensor cAMP-GEFII and to mediate cAMP-induced secretion (34).

In this study, we attempted to elucidate the role of this putative Rab3 effector by searching for possible binding partners of the protein. We found that the C\(_2\) domains of RIM mediate the interaction of this Rab3 target with the pore-forming subunit of Ca\(^{2+}\) channels and with Syt-I and SNAP-25. This indicates that RIM constitutes a scaffold protein that binds to different components of the exocytotic machinery and suggests that it coordinates the docking and fusion of secretory vesicles at release sites.

**EXPERIMENTAL PROCEDURES**

**Materials**—The plasmids encoding the cytoplasmic domain of syntaxin-1A and a fusion protein between GST and the cytoplasmic domain of rat Syt-I (residues 80–421) were kindly provided by Dr. R. Scheller (Stanford University, Stanford, CA). The human brain cDNA clone KIA0340 (GenBank\textsuperscript{TM} accession number AB002338) was obtained from the Kazusa DNA Research Institute. The antibodies against Syt-I and the α1B subunit of Ca\(^{2+}\) channels were purchased from Chemicon (Temecula, CA). The antibodies against SNAP-25—

**Interaction of the C\(_2\) Domains of RIM with Synaptotagmins and SNAP-25**—Rat brain detergent extracts were prepared as described previously (11) in HEPES-buffered saline (50 mM HEPES, pH 7.4, 100 mM NaCl, and 1% Triton X-100). 1-ml aliquots of the brain extract (about 1 mg of protein) were preincubated for 30 min at 4 °C in the presence of 2 mM EGTA or 500 μM Ca\(^{2+}\) and then incubated in the same buffer with immobilized GST fusion proteins for 2 h at 4 °C. The proteins remaining attached to the beads after several washes were detected by Western blotting using the indicated antibodies. To investigate the effect of different Ca\(^{2+}\) concentrations on the binding of RIM C\(_2\) domains to Syt-I, the preincubations and incubations were performed in Ca\(^{2+}\)/EGTA buffers (40). The final concentration of EGTA in the buffers was 2 mM. The fraction of Syt-I bound to the GST affinity column was estimated by analyzing the films with Scion Image software. The same quantification method was used for saturation experiments.

**Site-directed Mutagenesis**—Site-directed mutagenesis was performed using the Quickchange kit (Stratagene) and verified by sequencing the insert of the plasmids.

**RESULTS**

**Cloning of the C Terminus of Six Different Isoforms of RIM from Human Brain**—To shed light on the role of the Rab3-interacting molecule RIM, we searched for additional binding partners interacting with the C terminus of the protein. For this purpose, the C\(_2\) region of RIM encompassing the two C\(_2\) domains was amplified by RT-PCR from human brain mRNA. Experiments performed on first-strand cDNA generated several heterogeneous DNA fragments with a size between 1600 and 2300 base pairs. The sequences of six DNA fragments have been submitted to GenBank (GenBank\textsuperscript{TM} accession numbers AF263305-AF263310). The sequences encoded by the DNA fragments display a high degree of homology with the corresponding region of rat RIM. Alignment of the sequences indicates that the RT-PCR fragments result from a combination of different splice variants of human RIM mRNA that diverge in two domains corresponding to the splicing site B and C described in rat RIM (30, 32). Site B appears to be a hypervariable region because each of the six clones displays a different...
sequence. These observations demonstrate that human brain contains several RIM isoforms that differ from each other in the distance between the two C2 domains.

Direct comparison of the C2 domains of RIM with the C2 domains of Syt-I (Fig. 1) reveals that the overall homology between the sequences is rather low (RIM-C2A has 20% and 19% homology with Syt-I C2A and C2B; RIM-C2B has 16% and 22% homology with Syt-I C2A and C2B). However, both C2 domains of RIM contain a cluster of four amino acids homologous to the KKKT sequence of Syt-I C2B. The KKKT sequence is conserved in RIM-C2B, whereas in RIM-C2A, two lysines are replaced with two arginines (KKRT) (Fig. 1). The KKKT sequence is known to be required for the interaction with the synprint motif of Ca2+ channels (14, 41), for Syt-I oligomerization, and for binding to the adaptor protein AP-2 (41, 42). More recently, this particular sequence has been postulated to be the oligomerization interface for Syt-I (43).

Binding of the C2 Domains of RIM to α1 Subunits of Ca2+ Channels—The presence of the KKKT sequence prompted us to investigate whether the C2 domains of RIM interact with known partners of Syt-I. We first tested the ability of the C2 domains of RIM to bind to the α1 subunits of N- and L-type Ca2+ channels. To address this issue, GST fusion proteins containing the cytoplasmic domain of Syt-I, RIM-C2A, or RIM-C2B were immobilized on glutathione-agarose beads. They were then incubated with 35S-labeled synprint loops of α1B (amino acids 713–933), α1C (amino acids 754–899), and α1D (amino acids 773–907). An aliquot (10%) of each in vitro translation product used for the reactions is shown for comparison (IVT). The proteins remaining associated with the GST affinity columns were resolved by SDS-PAGE and detected by autoradiography. The autoradiography film shown for α1B was exposed for 1 day, whereas the films for α1C and α1D were exposed for 11 days. B, measurement of the affinity of the different C2 domains for α1B. Increasing concentrations of the radioactively labeled α1B synprint fragment were incubated with 1 mg of the indicated GST fusion proteins. The material retained on the affinity columns was analyzed by SDS-PAGE. The radioactive protein band was quantified using Molecular Imager FX (Bio-Rad). The results are expressed as a percentage of α1B maximal binding. The calculated EC50 values were 14 nM for RIM-C2A, 18 nM for RIM-C2B, and 25 nM for Syt-C2B.

GST fusion proteins and variable amounts of in vitro-translated α1B (Fig. 2B). Using this approach, we estimated that half-maximal binding of α1B synprint to Syt-I is obtained at about 25 nM. This value is in good agreement with the Kd of 25 nm measured previously using a solid phase assay (14). The affinity of α1B for RIM-C2A and RIM-C2B (14 and 18 nM, respectively) was almost identical to that of α1B for Syt-C2B. These results suggest that in neurons, the interaction between the C2 domain of RIM and the N-type calcium channel could contribute to ensure a tight structural and functional association of the exocytotic machinery with the sites of calcium entry.

Syt-I has been reported to bind to syntaxin-1. This interaction is modulated by Ca2+ and is mediated mainly by the C2A domain of Syt-I (11, 44). As expected, we observed a strong binding of in vitro-translated syntaxin-1 with the cytoplasmic domain of Syt-I in the presence of calcium and a weaker binding in the absence of calcium (Fig. 3). In contrast, under the same conditions, syntaxin-1 was unable to interact efficiently with the C2 domains of RIM or with GST alone.

Interaction of the C2 Domains of RIM with Syt-I and SNAP25—We next tested the ability of the C2 domains of RIM to associate with Syt-I and SNAP-25. In this case, GST fusion proteins containing Syt-I C2B, RIM-C2A, or RIM-C2B were incubated with brain extracts in the presence of either EGTA or 500 μM Ca2+. In the absence of Ca2+, binding of rat brain Syt-I to the GST affinity columns was barely detectable (Fig. 4). However, in the presence of Ca2+, the binding to RIM-C2A and RIM-C2B and the oligomerization with Syt-I were dramatically increased. Similar results were obtained with an antibody directed against Syt-II, indicating that the C2 domains can interact in a Ca2+-dependent manner with different Syt isoforms (data not shown). Ca2+-dependent binding was also observed in
autoradiography. An aliquot (10%) of the columns was analyzed by SDS-PAGE, and syntaxin-1A was detected by used for the reactions is shown on a separate lane (fig. 3).

Thus, Ca$^{2+}$ was removed. The figure shows one of four representative experiments. The calculated EC$_{50}$ values were 24 nM for RIM-C$_{2A}$, 16 nM for RIM-C$_{2B}$, and 12 nM for Syt-I C$_{2B}$. Measurement of the affinity of Syt-I C$_{2B}$, RIM-C$_{2A}$, and RIM-C$_{2B}$ for native Syt-I and SNAP-25. A rat brain detergent extract was incubated with the indicated GST fusion proteins immobilized on glutathione-agarose beads in the presence of 500 mM Ca$^{2+}$-buffers. The amount of Syt-I that remained associated with the affinity columns was quantified by Western blotting using Scion Image software. The figure shows one of three representative experiments. The calculated EC$_{50}$ values were 203 nM for RIM-C$_{2A}$, 224 nM for RIM-C$_{2B}$, and 253 nM for Syt-I C$_{2B}$.

**Definition of the Binding Interface of the C$_{2}$ Domains of RIM**

Rab3 is thought to control docking and fusion of secretory vesicles at the plasma membrane. In this study, we provide reduced. To determine the affinity of the different C$_{2}$ domains for SNAP-25, we performed dose-response experiments in the absence of Ca$^{2+}$. SNAP-25 in our rat brain extract was estimated to represent about 0.35% of the protein content. Using this estimated value, under our experimental conditions, the EC$_{50}$ of the interaction of SNAP-25 with Syt-I C$_{2B}$, RIM-C$_{2A}$, and RIM-C$_{2B}$ was ~253, 203, and 224 nM, respectively (fig. 5B).

The Binding of the C$_{2}$ Domains of RIM to Syt-I Is Sensitive to Inositol Polyphosphates—Synaptotagmins are known to bind inositol polyphosphates (15). The binding site has been shown to overlap the domain required for self-oligomerization and for the binding of the protein to the Ca$^{2+}$ channel (41). To assess whether the interaction of RIM with its partners occurs by a similar mechanism, the two positively charged amino acids in the middle of the KRRT and KKKT sequence of Syt-I are known to be required for self-oligomerization (45). Thus, Ca$^{2+}$ triggers not only the self-association of synaptotagmins but also promotes the hetero-oligomerization of distinct C$_{2}$ domains with properties analogous to those of Syt-I C$_{2B}$.

To test whether the C$_{2}$ domains of RIM are able to associate with SNAP-25, an aliquot of the proteins remaining attached to the affinity columns was analyzed by Western blotting, using an antibody against SNAP-25. As shown in fig. 4, in the absence of Ca$^{2+}$, RIM-C$_{2A}$ and RIM-C$_{2B}$ were able to fix SNAP-25. In the presence of Ca$^{2+}$ (500 nM), the amount of SNAP-25 associated with the affinity columns was strongly
evidence that RIM, a putative effector of Rab3, constitutes a functional link between the GTPase and the machinery for exocytosis. The association of RIM with the components of the secretory apparatus is mediated by two C2 domains located at the C terminus of the protein. C2 domains are widespread motifs that provide Ca\(^{2+}\)/H1001 and phospholipid binding activity to many of their parent molecules (47). The various C2 domains display distinct features that enable them to participate in multiple biological functions. The two C2 domains of Syt-I are endowed with unique binding properties that render the protein ideally suited to respond, in a millisecond time scale, to variations in the intracellular Ca\(^{2+}\)/H1001 concentration (10). Both C2 domains of Syt-I cooperate to perform Ca\(^{2+}\)/H1001-dependent interactions. However, Ca\(^{2+}\)/H1001-dependent binding to phospholipids...
and syntaxin-1 depends more on the C2A domain, whereas the C2B domain mediates synaptotagmin multimerization (42). C2B is also involved in several Ca2+-independent interactions including binding to Ca2+-channels (14), inositol polyphosphates (48), and SNAP-25 (16). Genetic studies have demonstrated that synaptotagmin-C2B plays a critical role in exocytosis-secretion coupling and that C2B-mediated oligomerization of different synaptotagmin isoforms modulates the strength of synaptic transmission (43, 49). The overall homology between the C2 domains of RIM and the C2B domain of Syt-I is relatively low. Despite this, RIM-C2A and RIM-C2B possess properties that are remarkably similar to those of the C2B domain of Syt-I. Thus, the C2 domains of RIM interact with the pore-forming subunit of N-type Ca2+ channels and with SNAP-25 and Syt-I with affinities almost identical to the one displayed by the C2B domain of Syt-I. The presence of two “C2B-like” domains within the same molecule could permit simultaneous interaction with multiple binding partners, placing the Rab3 effector in an ideal position to coordinate docking and fusion of secretory vesicles. In rats (30, 32) and humans (the present study), the distance between the two C2 domains varies considerably between RIM isoforms. This may provide each isoform with specific properties, permitting a selective tuning of the secretory process in different parts of the brain. The “C2B-like” properties of the C2 domains of RIM are provided by the presence of the amino acid motif KK(R)K(R)T. In fact, alterations of the KK(R)K(R)T motif abolished the capacity of RIM C2 domains to associate with its potential partners.

In neurons, positioning of synaptic vesicles in the immediate proximity of Ca2+-channels is critical for rapid and efficient synaptic transmission. A tight association of RIM with the α1 subunits of the N-type Ca2+-channel could contribute to guide the docking of secretory vesicles carrying Rab3 close to Ca2+-entry sites. The C2 domains of RIM are also able to interact weakly with the α1C (L-type subtype) but not the α1D (L-type subtype) subunit of Ca2+ channels. This suggests that close apposition of RIM to the sites of Ca2+-influx may be restricted to secretory systems specialized for fast release.

The binding of RIM to the components of the fusion machinery is differentially modulated by Ca2+. The association with SNAP-25 does not require the presence of Ca2+ and could therefore pre-exist on the plasma membrane of resting cells. In contrast, the complex with Syt-I is formed at micromolar Ca2+ concentrations and is likely to occur exclusively under stimulatory conditions. The hetero-oligomeric interaction is most probably driven by Ca2+-triggered changes in the electrostatic potential of the C2B domain of Syt-I. In fact, the C2 domains of RIM are unlikely to bind Ca2+ because they lack the aspartic residues required for the coordination of the cation (see Fig. 1). RIM is therefore probably not a Ca2+ sensor but rather an acceptor for Ca2+-activated Syts. The C2 domains of RIM (in particular, RIM-C2B) display a very high affinity for Syt-I, and RIM is quite abundant in the brain (we estimated that it represents about 1–2% of the proteins associated with synaptosomal membranes). For these reasons, the interaction of the C2 domains of RIM with Syt-I is likely to play an important role in the exocytotic process. Our findings would be consistent with a model in which the Ca2+-dependent hetero-oligomerization of the C2B domain of Syt-I with the C2 domains of RIM liberates SNAP-25 and permits the assembly of the SNARE complex.

Inositol polyphosphates, such as IP6, reduce Ca2+-triggered vesicular fusion by a mechanism that is not yet fully elucidated (12, 50). They have been proposed to act by preventing the oligomerization of synaptotagmins, by blocking the binding of Syt-I with phospholipids, or by interfering with the association of Syt-I with plasma membrane proteins (12). Here, we demonstrate that IP6 precludes the formation of the Ca2+-dependent complex between Syt-I and the C2 domains of RIM. Thus, in view of these results, it is possible that at least part of the effects of inositol polyphosphates on exocytosis reported in the literature are due to the disruption of the interaction between Syt-I and RIM.

The properties of the C2 domains of RIM described in this study are linked to the presence of two positively charged amino acids. The amino acid motif KK(R)K(R)T, which plays a critical role in the interaction of RIM with the components of the secretory machinery, is also found in the C2 domains of other proteins concentrated in active zones such as Piccolo and Azconin (51, 52). Future studies will have to determine whether these proteins are also endowed with properties analogous to those of RIM. If this is the case, the array of protein-protein interactions taking place in active zones could turn out to be much more complex than previously supposed.

In conclusion, we have found that the C2 domains of RIM display previously unsuspected binding properties that could favor the targeting and docking of secretory vesicles near Ca2+ channels. In addition, the C2 domains of RIM are high-affinity binding sites for the Ca2+-bound form of Syt-I, suggesting that this Rb3-binding protein is also involved in the control of vesicle fusion. Thus, our data indicate that through its multiple binding partners, RIM is able to coordinate different stages of the secretory process.

Acknowledgments—We thank Dr. R. Scheller for providing the plasmids encoding syntaxin-1A and GST-Syt-I and Drs. D. Laverty, G. Knott, and H. Hirling for critical reading of the manuscript.

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RIM Binds to Ca$^{2+}$ Channels, SNAP-25, and Synaptotagmin

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*J. Biol. Chem. 2001, 276:32756-32762.*

doi: 10.1074/jbc.M100929200 originally published online July 3, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M100929200

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