The RIM/NIM Family of Neuronal C2 Domain Proteins
INTERACTIONS WITH Rab3 AND A NEW CLASS OF Src HOMOLOGY 3 DOMAIN PROTEINS*

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RIM1 is a putative effector protein for Rab3, synaptic GTP-binding proteins. RIM1 is localized close to the active zone at the synapse, where it interacts in a GTP-dependent manner with Rab3 located on synaptic vesicles. We now describe a second RIM protein, called RIM2, that is highly homologous to RIM1 and also expressed primarily in brain. Like RIM1, RIM2 contains an N-terminal zinc finger domain that binds to Rab3 as a function of GTP, a central PDZ domain, and two C-terminal C2 domains that are separated by long alternatively spliced sequences. Unexpectedly, the 3'-end of the RIM2 gene produces an independent mRNA that encodes a smaller protein referred to as NIM2. NIM2 is composed of a unique N-terminal sequence followed by the C-terminal part of RIM2. Data bank searches identified a third RIM/NIM-related gene, which encodes a NIM isoform referred to as NIM3; no RIM transcript from this gene was detected. To test if NIMs, like RIMs, may function in secretion, we investigated the effect of NIM3 on calcium-triggered exocytosis in PC12 cells. NIM3 induced a dramatic increase in calcium-evoked exocytosis (50%), with no significant effect on base-line release, suggesting that NIMs, like RIMs, regulate exocytosis. The combination of conserved and variable sequences in RIMs and NIMs indicates that the individual domains of these proteins provide binding sites for interacting molecules during exocytosis, as shown for the zinc finger domain of RIM, which binds to GTP-bound Rab3. To search for additional interacting proteins for RIMs, we employed yeast two-hybrid screens with the C-terminal half of RIM1. Two members of a new family of homologous brain proteins, referred to as RIM-binding proteins (RIM-BPs), were identified. RIM-BPs bind to RIM in yeast two-hybrid and GST pull-down assays, suggesting a specific interaction. In RIMs, the binding site for RIM-BPs consists of a conserved proline-rich sequence between the two C2 domains, N-terminal to the beginning of NIMs. RIM-BPs are composed of multiple domains, including three fibronectin type III-domains and three Src homology 3 domains, of which the second Src homology 3 domain binds to RIMs. With the RIM-BPs, we have identified a partner for RIMs that may bind to RIMs at the synapse in addition to Rab3.

Rab proteins are GTP-binding proteins that are generally believed to be essential components of the membrane trafficking machinery of eukaryotic cells (reviewed in Refs. 1–4). In brain, a family of Rab proteins collectively referred to as Rab3s is particularly abundant. Four Rab3 isoforms are known (Rab3A, -3B, 3C, and -3D). Of these, Rab3A and Rab3C are concentrated on synaptic vesicles (5, 6), while the localization of Rab3B and Rab3D in brain is less clear (7–9). Rab3A is the best characterized and most abundant Rab protein in the brain, accounting for approximately 25% of total GTP binding by Rab3 in brain. Rab3A and Rab3C are attached to synaptic vesicles via a C-terminal lipid modification. Both coordinately dissociate from synaptic vesicles during or after exocytosis, and reassociate after endocytosis (6). In chromaffin cells, exogenous Rab3A inhibits exocytosis, suggesting a regulatory role in exocytosis (10, 11). Knockout experiments in mice showed that Rab3A, although the most abundant Rab3 isoform, is not essential for exocytosis or for brain function (12). However, Rab3A performs an important role in regulating the extent of neurotransmitter release in response to Ca2+ (13). In addition, Rab3A is essential for some forms of long term potentiation and long term depression (14, 15). Other Rab isoforms probably perform similar regulatory roles in synaptic membrane fusion, but their localizations and functions have not been determined.

As GTP-binding proteins, it is likely that Rab3s perform their functions by binding to an effector in a GTP-dependent manner. Two putative effectors for Rab3s have been identified: rabphilin and RIM (16–18). Rabphilin is a synaptic vesicle protein that is recruited to the vesicles by Rab3 and dissociates from the vesicles together with Rab3 (19). The N-terminal half of rabphilin contains a zinc finger domain that binds Rab3 (17); the crystal structure of this zinc finger domain complexed with Rab3A revealed an unusual fold in which there are multiple contacts between this domain and Rab3 (20). The C-terminal half of rabphilin is composed of two C2 domains that are similar to the C2 domains of synaptotagmin and probably also bind Ca2+ (21, 22). These findings suggested that rabphilin may couple a Ca2+-dependent aspect of synaptic vesicle traffic to Rab3 activation by GTP. The second putative Rab3 effector, RIM, is similar to rabphilin in that it also contains an N-terminal zinc finger domain and two C-terminal C2 domains (18). The zinc finger domain of RIM is homologous to that of rabphilin; both specifically bind to Rab3s in the GTP-complexed form. The C2 domains, however, are quite different in that the RIM C2 domains do not contain the consensus calcium binding sites that were defined in the synaptotagmin C2 domains and that are present in the rabphilin C2 domains (21). Other properties of RIM are even more strikingly distinct from those of rabphilin. RIM is much larger than rabphilin, is subject to extensive alternatively splicing, and contains multiple additional domains that are absent from rabphilin, most

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notably a central PDZ domain (18). Furthermore, RIM is not localized to synaptic vesicles but instead associated with the synaptic active zone (18). Biochemically, native RIM in brain is a component of the matrix of the active zone; it is virtually insoluble in all detergents except under denaturing conditions. Thus, the two Rab3 effectors interact with Rab3 via homologous N-terminal domains but differ in their subcellular localizations, biochemical properties, and domain structures.

The discovery of two distinct putative effectors for Rab3 at the synapse was somewhat surprising. It raised the question of whether the two effectors mediate different Rab3 functions, whether they cooperate in the same Rab3 function, or whether one of them modifies the function of the other by competing with it. The distinct localizations of rabphilin and RIM suggest different functions; rabphilin would be in an ideal position to mediate a Ca$^{2+}$-dependent effect that requires GTP-Rab3 but occurs on free vesicles in the backfield of the synapase, while RIM can only interact with Rab3 when synaptic vesicles are attached to the active zone. In fact, the localization of RIM to the active zone suggests that it could function to recruit vesicles to the active zone in a “tethering” reaction, a function that is similar to what has been proposed for other Rab proteins (3).

The precise respective functions of RIM and rabphilin are currently unclear. A large body of work on rabphilin suggests a variety of possible functions (reviewed in Ref. 23) that involve processes ranging from endocytosis and recycling over assembly of the cytoskeleton to exocytosis. The best controlled studies on rabphilin are probably experiments in chromaffin cells, which demonstrated that rabphilin directly participates in exocytosis, although the mechanism of action remained unclear (24, 25). However, rabphilin is not an essential component of the exocytic machinery in brain, since mice lacking rabphilin exhibit no major phenotype (26). Strikingly, mice without rabphilin are viable and fertile without apparent morbidity and exhibit no measurable changes in neurotransmitter release or in the regulation of synaptic transmission (26). This result suggests that RIM may be the more important effector for Rab3, in agreement with its localization to the active zone of the synapase. As a potential effector for Rab3 in regulating neurotransmitter release, RIM in turn presumably binds to downstream target proteins. Although a large number of such proteins have been described for rabphilin, no interacting protein for RIM is known. The large size of RIM and its molecular architecture with multiple separate domains suggest that it binds to several targets in performing its function and that its function is not restricted to an interaction with Rab3. However, it is unclear at this point what these interactions and functions are.

In the current study, we have identified a novel gene for a protein related to RIM, and now refer to the protein products of the two genes as RIM1 and RIM2. Both RIMs are unusually polymorphic due to extensive alternative splicing. Furthermore, we uncovered a second gene product of the RIM2 gene, called NIM2, that is transcribed from the 3′-end of the RIM2 gene and composed of only the C-terminal parts of RIM2, including the C2B domain. We describe a third related gene, which encodes an analogous protein, called NIM3, and demonstrate that NIM3 also functions in exocytosis as suggested by its homology to RIMs. Finally, we use yeast two-hybrid screens to identify a new class of proteins interacting with RIMs. These proteins are modular SH3 domain proteins that we refer to as RIM-BPs. Although it is likely that many additional interacting partners for RIMs remain to be discovered, this is the first step toward elucidating a mechanism of action of RIMs. Together, our data describe a large family of related proteins, the RIMs and NIMs, which function in regulating exocytosis presumably by distinct binding interactions that are partially shared between them and partially specific for the each class of proteins.

**EXPERIMENTAL PROCEDURES**

**Materials and Vectors**

All chemicals and enzymes used in the current study were obtained commercially and were of the highest purity available. The following vectors, partly derived from the cDNA clones whose isolation is described below, were constructed using standard procedures (Ref. 27–30; see Figs. 1 and 7).

**Mammalian Expression Vectors (all in pCMV5)—**These clones were as follows: A, pCMV5-Rim1 (full-length Rim1 cloned into the EcoRI/SalI sites); contains a full-insert in splice sites 1 and 2, no insert in splice sites 3 and 5, and a partial insert in splice site 4 (lacking residues 1054–1177; see Fig. 1); B, pCMVMyc-Rim1C2A (residues 726–905 of Rim1 preceded by a Myc epitope cloned into the EcoRI/HindIII sites); C, pCMVMyc-Rim1C,B (residues 890–1614 of Rim1 with the same splice sites as pCMV5-Rim1 preceded by a Myc epitope cloned into the EcoRI/HindIII sites); D, pCMV5-Rim2 (full-length Rim2 cloned in the EcoRI/SalI sites); contains no inserts in splice sites 1 and 3, the “A” insert in splice site 2 (see Fig. 1B), a short insert (residues 1013–1034; Fig. 1) at splice site 2, and a partial insert at splice site 5 (residues 1146–1318, but lacking residues 1132–1145); E, pCMV5-Rim2-Zn finger domain (residues 1–466 cloned into the EcoRISalI sites); F, pCMV5-Rim2 PDZ (residues 474–987 cloned into BglII/HindIII sites; contains “A” insert in splice site 2 and no insert in splice site 3); G, pCMV5-Rim2 N terminus (residues 159–319 cloned into the EcoRISalI sites); H, pCMV5-NIM (full-length NIM3 cloned into the EcoRISalI sites).

**Bacterial Expression Vectors—**All bait vectors are in pLexN, and all prey vectors are in pVPV16–3 (28, 29); all splice variants correspond to the pCMV5-Rim1 and -Rim2 vectors. These clones are as follows: A, pLexNRim1-PDZ (residues 492–772 in the Smal site); B, pLexNRim1-C2 (residues 713–1588 in the Smal site); C, pLexNRim1-C2A (residues 713–1588 in the Smal site); E, pLexNRim2C (residues 1147–1280 in the SalI sites); F, pLexNRim2D (residues 1084–1184 in the EcoRISalI sites); G, pLexNRim2E (residues 944–999 in the EcoRISalI sites); H, pLexNRim2F (residues 1096–1168 in the EcoRISalI sites); I, pLexNRim2G (residues 929–1184 in the EcoRISalI sites); J, pLexNRim2H (residues 929–1184 in the EcoRISalI sites); K, pLexNRim2I (residues 944–1188 in the EcoRISalI sites); L, pLexNRim2J (residues 944–1188 in the EcoRISalI sites); M, pLexNRim2K (residues 929–1189 in the EcoRISalI sites); N, pLexNRim2L (residues 662–1555 in the XbaI site); O, pPreyC80 (residues 602–1686 (original RimBP1 prey clone)); P, pPreyC16 (1–265 (original Rim-BP2 prey clone)); Q, pVP16RimBP1 (residues 652–12170 of RIM-BP1; R, pVP16RimBP2 (residues 1271–1625 of RIM-BP1).

**Bacterial Expression Vectors—**All GST fusion proteins were produced in pGEX-KG (30), and all maltose-binding protein fusion proteins were produced in pMal-C2 (New England Biolabs); all splice variants correspond to those of pCMV5-Rim1 and -Rim2. These clones are as follows: A, pMalRim1-C2A (residues 726–905 in SalI/HindIII sites); B, pMalRim1-C,B (residues 890–1614 in SalI/HindIII sites); C, pGexKG-Rim2 zinc finger (residues 1–466 in EcoRISalI); D, pGexKG-Rim2 N terminus (residues 159–319 in EcoRISalI); E, pGexKG-Rim2 PDZ (residues 461–987 in Smal); F, pGexKG-Rim2-C2 (residues 662–1555 in XbaINcoI).
Quantitations were then performed for selected bait/prey pairs with a protein without the initiator methionine. Brain fractionations were carried out as described (33) and analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting with antibodies to RIM1 (Q703), RIM2 (U952), NIM3 (U2132), synaptophysin (C17.2), and Rab3A/C (C142.1) as described (34, 35).

**GST Fusion Protein Affinity Chromatography**

We homogenized frozen rat brains (obtained from Pelfreeze) in 0.5% Triton X-100, 1 mM EDTA, 0.1 mM NaCl, protease inhibitors, and 50 mM Hepes-NaOH, pH 7.4, and obtained a total brain homogenate after pelleting insoluble material by centrifugation (36). To examine the binding of Rab3 to RIM2, glutathione-agarose columns containing either GST alone or N-terminal RIM2-GST fusion proteins with the zinc finger domain (residues 1–466) or without the zinc finger domain (residues 159–319) were incubated with the brain homogenate at 4 °C overnight in the presence of either 0.5 mM GDP/GS or GTP/S. Samples were washed three times in the same buffer without nucleotides before analysis by SDS-polyacrylamide gel electrophoresis and immunoblotting with C142.1 antibody against Rab3 and antibody C7.2 against synaptophysin. To test the binding of RIM-BP to RIMs, glutathione-agarose columns containing GST alone or GST-RIM-BP fusion proteins with the N-terminal (residues 650–1270) or C-terminal region of RIM-BP (residues 1271–1625) were used for pull-downs. Since RIMs are almost completely insoluble in brain homogenates with non-denaturing detergents (18), RIMs first had to be solubilized with 1% SDS from the homogenates. The SDS-containing brain extract was then diluted with binding buffer containing Triton X-100 to quench the SDS, resulting in final concentrations of 0.16% SDS and 1% Triton X-100. This brain extract was used in the pull-down experiments with GST-RIM-BP fusion proteins as described above for Rab3 binding, except that no nucleotides were added.

**RNA Blotting Experiments**

RNA blotting experiments were performed using multiple tissue blots purchased from CLONTECH. Northern blots were hybridized at high stringency with a DNA segment encoding residues 159–319 of RIM2, the entire coding region of NIM3, residues 1271–1625 of RIM-BP, or a ubiquitously expressed control protein (VAP33).

**RESULTS**

**Identification and Molecular Cloning of RIM2: The Conserved Molecular Architecture of a Family of Rab3-interacting Molecules**—Data bank searches uncovered several EST sequences that were homologous to, but distinct from, the RIM protein that we had originally isolated (data not shown). To determine if these sequences originated from a RIM-related protein, we used EST clones as probes to isolate overlapping cDNA clones from a rat brain library. Assembly of the sequences of these clones revealed that they encode a large protein of 1705 residues that is closely related to RIM (Fig. 1A). The new protein is similar to RIM over its entire length, suggesting that it represents a novel RIM homolog. This prompted us to refer to the old and new proteins as RIM1 and RIM2, respectively. Partial human sequences for RIM1 and RIM2 were deposited in GenBank as random cDNA sequences (KIAA0340 and KIAA751, respectively). Their translated sequences are highly homologous to the rat RIM1 and RIM2 sequences reported here, suggesting that both RIMs are evolutionarily conserved in vertebrates (data not shown).

The two RIMs exhibit the same overall domain architecture consisting of an N-terminal zinc-finger module, a single central PDZ domain, and two C-terminal C2 domains (Fig. 2). These modules are highly conserved between RIMs and are connected by sequences of variable length and conservation. Some of the connecting sequences are very homologous between RIMs, while others diverge (Fig. 1A). For example, the coupling of a PDZ domain with a C2 domain is characteristic of RIMs (Fig. 2), and the short sequences that couple these domains are almost identical between the RIMs, indicating that the connection between the two domains is functionally important. Furthermore, the sequence preceding the PDZ domain is also highly conserved among RIMs. In contrast, the more N-terminal region between the zinc finger and PDZ domains contains islands of similarity separated by stretches of variable sequences (Fig.
Some of the conserved sequences outside of the identified domains may represent novel domains that have not yet been defined; future studies will have to determine if these sequences represent independently folding modules. The fact that only some of the connecting sequences are conserved while others are variable suggests that the conserved sequences are functionally meaningful.

Alternative Splicing of RIMs—Analysis of multiple independent RIM2 cDNA clones revealed heterogeneity between clones at several positions, suggesting that RIM2 is extensively alternatively spliced. Similar alternative splicing was previously observed for RIM1 (18). We analyzed the alternative splicing of RIM2 by sequencing multiple independent cDNA clones, and reanalyzed RIM1 alternative splicing by a further characterization of cDNA clones for this protein. In these analyses, we accepted as bona fide alternative splicing only events that could be reproduced in multiple independent cDNA clones or independently observed in sequences reported in GenBank™ or detected in both RIM1 and RIM2. Of the five sites of alternative splicing that were identified in this manner, one site was only found in RIM1 and two only in RIM2 (all three in the N-terminal half of RIMs), while the remaining two sites of alternative splicing were present in both RIM1 and RIM2 (in the C-terminal half of RIMs) (Figs. 1 and 2). To facilitate discussion of the sites of alternative splicing, we number the sites consecutively from the N to the C terminus for both RIMs, although the N-terminal sites of alternative splicing were not shown to be present in both RIMs (Fig. 1A). As described below, the presence of multiple types and combinations of inserts in these sites generates a large diversity of RIM proteins with potentially more than 100 isoforms, suggesting that RIMs are highly polymorphic neuronal proteins.

Fig. 1. Primary structures and alternative splicing of RIM1, RIM2, N1M2, and NIM3. A, the rat sequences of RIM2, RIM1, N1M2, and NIM3 as deduced by cDNA cloning are aligned for maximal homology in single-letter amino acid code. Sequences are identified on the left and numbered on the right. The numbering corresponds to the sequences shown, since the actual number of amino acids differs among the various splice forms. Shared residues are highlighted in a color code corresponding to protein domains as follows: green, the zinc finger domain that binds to Rab3s; blue, the PDZ domain; red, the two C2 domains; yellow, identical residues outside of defined domains. Cysteine residues in the Rab3-interacting domain that are coordinating the two zinc atoms are shown on a black background. Dashes indicate gaps. Alternatively, spliced sequences are shown in italic cyan-blue type (see “Experimental Procedures”). Sites of alternative splicing are numbered 1–5 above the sequences, with the same numbering for both RIMs, although not all of the sites have been demonstrated to be alternatively spliced in both genes. B, sequence variants at splice site 2 N-terminal to the PDZ domain of RIM2. Two variants were observed in rat cDNAs, referred to as variants a and b. Variant a is similar to rat and human RIM1 (KIAA0340; GenBank™ accession no. AB002338), and variant b is similar to the human RIM2 sequence encoded by KIAA0751 (hRIM2b; GenBank™ accession no. AB018294), suggesting that the two variants are not cloning artifacts.
Splice site 1 is in the N-terminal Rab3-interacting domain. Here RIM1 displays a variable sequence that disrupts the homology with RIM2 and with the Rab3-binding domain of rabphilin (shown in green in Fig. 1A). Previous cDNA cloning identified variants lacking residues 83–105 and 83–106 at this site (18). In addition, we observed a human EST sequence (GenBank™ accession no. AA774730) that lacks residues 57–105. Thus, there are at least four variants of RIM1 in this site, although none have yet been identified for RIM2. In RIM2, two different inserts were observed at splice site 2 immediately before the PDZ domain (residues 544–564 in Fig. 1A; aligned in Fig. 1B). The shorter variant A (20 residues) is homologous to the corresponding sequence observed in RIM1. The longer variant B (68 residues) exhibits no homology to RIM1. The human RIM2 sequence in the random cDNA clone KIAA0751 also contains this insert, suggesting that it does not represent a cloning artifact. In splice site 3 at the beginning of the first C2 domain, residues 733–748 were either present or absent in multiple RIM2 cDNA clones. No alternative splicing in sites 2 and 3 was observed for RIM1 at this point.

Probably the most interesting alternative splicing of RIMs occurs in the region between the two C2 domains at the C terminus at splice sites 4 and 5. This region contains two sites of alternative splicing that are separated by only 38 and 37 residues in RIM1 and RIM2, respectively. These sites of alternative splicing are composed of the largest number variants, contain the longest inserts (up to 187 amino acid residues), and are conserved between RIM1 and RIM2.

In RIM2, three variants were observed in splice site 4, the first of the two C-terminal splice sites: a variant containing residues 1013–1034 but lacking residues 1035–1094, a variant containing only the second sequence (residues 1037–1097), and a variant containing both sequences (residues 1015–1097). It seems likely that a variant lacking all inserts also exists. Analysis of new cDNA clones revealed that RIM1 is expressed in at least four variants at this splice site: a variant lacking all inserts that joins residue 981 to residue 1118 and variants containing the full insert (residues 1032–1167) or different partial inserts (residues 1083–1167 and 1107–1167). A picture of a modular design of alternatively spliced sequences emerges from these observations, with as many as four blocks of sequences that can be variably inserted into, or omitted from, the RIM sequences at this position. These four blocks of sequences correspond to residues 1032–1053, 1054–1082, 1083–1106, and 1007–1167 in RIM1. Of these blocks, the splice sites following residues 1031, 1106, and 1176 were observed in both RIMs. Splice site 5 of RIM2 was also subject to complex alternative splicing in that either the whole region or only residues 1132–1145 or 1145–1318 were spliced out. Here, RIM1 exhibits only two variants (18). Again the alternatively spliced inserts are highly homologous between the two RIMs. In the analysis of the limited number of RIM1 and RIM2 cDNA clones performed in our studies, we observed no evidence that alternative splicing of RIMs at the different sites is interdependent. If the different splice sites are used independently, combinatorial mixing of different inserts in the various sites would result in more than 100 distinct RIM proteins that differ by as much as 30 kDa.

GTP-dependent Interaction of RIM2 with Rab3s—RIM1 was initially identified because of the GTP-dependent binding of its N-terminal zinc finger domain to Rab5A and Rab3C. The sequence homology between RIM1 and RIM2 in the N-terminal zinc finger domain suggests that RIM2 may have a similar activity. To test this, we performed GST-pull-down experiments with the N-terminal region of RIM2 (Fig. 3). The N-terminal zinc finger domain of RIM2 specifically captured both RIM2 and Rab3A, but not synaptophysin used as a negative control, from rat brain homogenates. Binding was GTP-dependent, since only GTPyS and not GDPyS allowed binding. The full-length zinc-finger domain (residues 1–1466) was required for binding, while GST alone or a fragment of the zinc finger domain (residues 159–319) exhibited no background binding of Rab3s (Fig. 3). Thus, RIM2 specifically binds Rab3s, similar to RIM1, in a GTP-dependent interaction.

Cloning and Characterization of NIMs—In addition to the various splice variants, sequencing of RIM2 cDNA clones uncovered two overlapping cDNA clones from the C terminus of RIM2 that differed from the other cDNA clones. These two clones contained all of the sequences 3’ of splice site 5 of RIM2 but diverged N-terminally from the RIM2 sequence at the 3’ junction of splice site 5. The 5′-end of the inserts of the two clones encoded a unique sequence not found in the other RIM cDNA clones; this sequence included a consensus initiator methionine and was preceded by a stop codon in the clone with the largest insert. This result suggested that in addition to full-length RIM2, a separate shorter mRNA is transcribed from the RIM2 gene, probably by an independent promoter. This shorter mRNA encodes a protein composed of a unique N-terminal sequence followed by the entire sequence of RIM2 from splice site 5 onwards, including the C-terminal C2B domain (Fig. 1A). Because this protein lacks a Rab3-interacting domain and thus cannot bind to Rab3 (see Fig. 3) but is nevertheless derived from the RIM2 gene, we called it NIM2.

Interestingly, further database searches identified EST sequences and a random human cDNA clone (KIAA0237; GenBank™ accession no. D87074) that were highly homologous to, but distinct from, the C-terminal parts of RIM1 or RIM2. Similar to NIM2, these human sequences N-terminally diverged from the RIM structures at splice site 5 and also contained in-frame stop codons at the 5′-end, suggesting that these entries correspond to a novel NIM3 protein. This finding raised the question of whether a corresponding RIM3 protein exists for NIM3, since there is a RIM2 protein for NIM2. To test this possibility, we performed extensive cDNA screening experi-
not only the C2B domain but also the sequences preceding and subsequent to the C2B domain domain preceded by a relatively short sequence. As in NIM2, the homology covers the C-terminal sequence of NIM2, the RIM transcript (Fig. 2).

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Fig. 4. RNA blot analysis of the tissue distribution of RIM2 and NIM3 mRNAs. Rat multitissue RNA blots were hybridized at high stringency with cDNA probes from the 5’-end of RIM2, from the entire coding region of NIM3, and from a ubiquitously distributed control mRNA. Positions of molecular size markers are identified on the left.

Fig. 5. Analysis of NIM3 antibodies in transfected COS cells. COS cells transfected with salmon sperm DNA (COS/SS) or a NIM3 expression vector (COS/NIM) and rat brain homogenates (BRAIN) were analyzed by immunoblotting with an antiserum against a peptide derived from the N terminus of NIM3. The asterisk identifies a cross-hybridizing band that is probably artifactual. Numbers on the left indicate positions of molecular mass markers.
addition to the major band, several minor bands were observed. We think that these minor bands are probably due to cross-reactivity with unrelated proteins and not to alternative NIM3 transcripts, because our extensive cDNA cloning and the RNA blotting experiments failed to reveal any evidence for alternative transcripts. We then analyzed the immunoblotting signals for RIMs and NIM3 in different brain regions (Fig. 6). In all brain regions, RIM1 and RIM2 were detected as multiple bands consistent with extensive alternative splicing. RIMs were differentially distributed in the various brain regions in a pattern distinct from that of synaptophysin and Rab3A, two synaptic vesicle proteins. Whereas synaptophysin was similarly present in all brain regions, RIMs were more abundant in evolutionarily new rostral brain regions (cortex, cerebellum, olfactory bulb) than in evolutionarily old caudal brain regions (midbrain, hind brain, spinal cord). This difference in expression levels was even more pronounced for NIM3 (Fig. 6). Significant levels of NIM3 could only be detected in the rostral brain regions; we observed no signal in spinal cord, hind brain, or midbrain.

**NIM3 Enhances Ca\(^{2+}\)-dependent Secretion in Transfected PC12 Cells**—The homology of NIMs to RIMs and their derivation from the same or similar genes (Figs. 1A and 2) suggest that NIMs, like RIMs, may be involved in exocytosis. Such a function would have to be mediated, however, via a different mechanism, since NIMs, in contrast to RIMs, do not bind to Rab3s. To test a possible involvement of NIMs in exocytosis, we measured the effect of overexpression of NIM3 on Ca\(^{2+}\)-dependent secretion in transfected PC12 cells (Fig. 7). For this purpose, we used a system that we and others have extensively employed previously (e.g. see Refs. 18, 24, 26, 32, and 37), namely co-transfection of hGH as a reporter gene with the protein that is being investigated. We co-transfected hGH into PC12 cells with either an empty expression vector or the NIM3 expression vector, stimulated the cells by KCl depolarization, and determined the amount of hGH release under control or stimulation conditions. In previous studies we had shown that under the conditions used, KCl depolarization triggers Ca\(^{2+}\)-dependent exocytosis that is inhibited by tetanus toxin (32). Measurement of hGH secretion as a function of NIM3 expression revealed that NIM3 had no significant effect on base-line hGH release from unstimulated cells but dramatically enhanced Ca\(^{2+}\)-dependent exocytosis stimulated with KCl (Fig. 7). This was a surprising result, because in previous studies, we identified several proteins that inhibit release significantly (e.g. truncated syntaxin 1, synaptogyrins, and synaptophysins (32, 37)), whereas proteins that enhance secretion have been much less frequently observed. These results suggest that NIM3 functions in exocytosis.

**Identification of RIM-BPs Using Yeast Two-hybrid Screens**—The modular domain structure of RIMs suggests a potential role as a scaffolding molecule that functionally connects Rab3 to other synaptic proteins. To identify proteins that interact with RIMs, we performed yeast two-hybrid screens. In these experiments, we chose as a bait the C-terminal half of RIM1 that includes the C\(_2\) domains, because this part of RIMs is the most highly conserved (Fig. 1). We screened a rat brain library with this bait and tested all positive clones by retransformation and sequencing. Among 55 positive clones isolated, we observed two homologous SH3 domain proteins, which we named RIM-BP1 and RIM-BP2. RIM-BP1, the protein encoded by the prey vector with the larger insert, was chosen for further study because it interacted with RIM1 and with RIM2, was obtained in multiple independent isolates in the screens, and was expressed at high levels only in brain (see below). Since the initial RIM-BP1 prey clone contained only part of the coding sequence (residues 602–1686), we isolated additional overlapping cDNA clones from a rat brain library that together cover almost the complete coding region (Fig. 8). During the course of this study, a sequence related to RIM-BPs was published as that of PRAX1, a protein interacting with the mitochondrial peripheral benzodiazepine receptor (38). In addition, data banks contain a second protein sequence related to RIM-BPs, KIAA0318 (GenBank accession no. AB002316), which is from a random human cDNA clone. PRAX1 is more homologous to RIM-BP1.
than to RIM-BP2, and KIAA0318 is more homologous to RIM-BP2 than RIM-BP1, suggesting that they may be the human orthologs of RIM-BP1 and RIM-BP2, respectively.

Sequence analyses revealed that RIM-BPs, PRAX1, and KIAA0318 contain three dispersed SH3 domains and three contiguous fibronectin type III repeats (shown on red and blue backgrounds, respectively, in Fig. 8). These domains are the most closely related sequences in RIM-BPs, PRAX1, and KIAA0318 (Fig. 8) and are flanked by highly charged sequences that are less well conserved. The sequences of the SH3 and fibronectin type III domains are rather atypical, which is probably the reason why they were overlooked in the initial analysis of the PRAX1 sequence (38). The sequences outside of the SH3 domains and fibronectin type III repeats are rich in arginine, lysine, and glutamic acid; these sequences contain strings of charged residues with up to 13 consecutive glutamic acid residues.

Although RIM-BP1 and PRAX1 are very similar, there are stretches of significant sequence divergence between the two proteins (e.g. residues 1166–1241 in PRAX1; see Fig. 8). The sequence differences between RIM-BP1 and PRAX1 exceed those we have typically observed between the rat and human homologs of many brain proteins (e.g. see synaptophysin (39)), raising the question of whether RIM-BP and PRAX are true orthologs or just homologs. Analysis of multiple rat cDNA clones encoding RIM-BP1 identified a 61-residue sequence that is alternatively present or absent in three independent cDNA clones each, suggesting that it is differentially spliced (Fig. 8).

The human gene for PRAX1 was sequenced during the genome project (GenBank™ accession no. AC004687) and shown to be a relatively small gene (approximately 25 kilobases) on chromosome 17. Curiously, the gene sequence reveals that the 63-residue sequence that is alternatively spliced in RIM-BP1 is in the middle of a large exon. This raises the question of how this sequence can be alternatively spliced if it is in the middle of an exon. In contrast, a random human cDNA sequence that also reports the PRAX1 sequence (KIAA0612; GenBank™ accession no. AB014512), lacks residues 191–250. Here the variable sequence is precisely encoded in a single exon, indicating alternative splicing. Together with the limited sequence homology between RIM-BP1 and PRAX1, these findings raise the possibility that RIM-BP1 and PRAX1 may be closely related but do not actually represent orthologs. Furthermore, RIM-BP1 is highly expressed only in the brain (see below), while the peripheral benzodiazepine receptor is a ubiquitously functioning receptor with up to 13 consecutive glutamic acid residues.

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RIM-BP1 is specifically expressed in the brain—We performed RNA blotting experiments to determine which tissues express RIM-BP1 (Fig. 9). A single large mRNA was found that was expressed at high levels only in brain. In addition, a smaller weaker signal was found in testis, which we interpret as an artifact because we have observed a similar signal with a number of unrelated probes (data not shown).

The second SH3 domains of RIM-BPs bind to a proline-rich sequence in RIM—We identified RIM-BPs as RIM-binding proteins by yeast two-hybrid screens. To confirm this interaction, we used GST pull-down experiments (Fig. 10). Rat brain homogenates were dissolved in SDS and then quenched in Triton X-100 in order to solubilize and renature RIMs because RIMs are not solubilized from the active zone in the absence of denaturing detergents. The homogenate was then incubated with GST-RIM-BP1 fusion proteins containing either the highly charged sequence between the third fibronectin III domain and the second SH3 domain or the second SH3 domain (Fig. 8). Immunoblotting of the bound proteins with antibodies to RIMs showed that only the SH3 domain was capable of capturing RIMs from brain homogenates, thereby confirming the interaction observed in the yeast two-hybrid system (Fig. 10).

We then turned to yeast two-hybrid assays to identify which sequence in RIMs binds to RIM-BP1. Comparison of the insert sequences of the RIM-BP1 and RIM-BP2 prey clones showed that they overlap only in the highly conserved second SH3 domain, suggesting that the interaction of this domain with RIMs led to the isolation of RIM-BPs in the yeast two-hybrid screens (Fig. 8). To confirm this, we first analyzed the interaction of the RIM-BP1 prey vector with a series of prey constructs encoding the C terminus of RIM2. Assays using the nonquantitative β-galactosidase filter assay identified a short sequence in RIM2 (residues 1097–1149) that was involved in the interaction (Table I; Fig. 1A). Quantitative liquid β-galactosidase assays confirmed that this sequence is sufficient for binding RIM-BP1 (Table II). Inspection of the RIM sequences in this area reveals that it contains a single conserved classical SH3-binding motif (sequence RQLPQ(L/V)P), suggesting that RIM-BP1 binds to RIMs at this position. It is interesting that this sequence is present in the short region between the two alternatively spliced sequences in the C terminus of the RIMs and is the only conserved sequence in this short region (Fig. 1A).

Discussion

Neurotransmitter release is one of the most tightly regulated processes in biology, because the precise control of synaptic signaling is fundamentally important for information processing in the brain. Rab3 is a GTP-binding protein of synaptic vesicles that regulates neurotransmitter release at the synapse (reviewed in Ref. 41). Mechanistically, Rab3 is thought to function by interacting with effector proteins in a GTP-dependent manner. Two such effectors are known, RIM1 and rabphilin (16–18). Current data suggest that RIM1 is the more important of these two effectors because it appears to be essential for synaptic function, while rabphilin is not (26),2 and because the strategic localization of RIM1 at the active zone would allow RIM to mediate a GTP-dependent tethering of synaptic vesicles at the active zone (18). We now demonstrate that RIM1 belongs to a larger family of proteins composed of RIM1 and RIM2 and the related NIM2 and NIM3, and we identify a family of RIM-interacting proteins called RIM-BPs. Furthermore, we show that RIM2, similar to RIM1, specifically binds to Rab3 in a GTP-dependent manner, while NIMs do not; nevertheless, NIMs are probable regulators of exocytosis, since NIM3 overexpression greatly facilitates exocytosis. Together, these results expand our view of the components and complexity of the Rab3-dependent regulation of neurotransmitter release.

The present study describes the identification and molecular characterization of a family of novel proteins related to RIM, RIM2, NIM2, and NIM3, and of a separate class of brain proteins that bind to RIMs, named RIM-BPs. These data show that the RIM/NIM family contains at least three closely related genes: RIM1, RIM2, and NIM3, which produce transcripts encoding at least four proteins (RIM1, RIM2, NIM2, and NIM3). Analysis of human genomic sequences containing the RIM1 and NIM3 genes failed to identify coding sequences for NIM1 and RIM3 proteins, respectively, which would suggest that only the RIM2/NIM2 gene encodes both variants. Rabphilin and associated proteins (DOC2, NOCR) are more distantly related members of this gene family because they also have zinc finger and/or C2 domains; however, these domains are less homologous to those of RIMs and NIMs than they are to each other.

2 S. Schoch, T. Joh, and T. C. Südhof, unpublished observation.
FIG. 8. Primary structure of RIM-BPs: Comparison with PRAX1 and KIAA0318. The partial sequences of rat RIM-BP1 and RIM-BP2 (rRBP1 and rRBP2) are aligned in single letter amino acid code with those of the human peripheral benzodiazepine receptor-interacting protein PRAX1 (hPRAX, GenBank™ accession no. 4104812), and the random human cDNA sequence KIAA0318 (GenBank™ accession no. AB002316). RIM-BPs are composed of three dispersed SH3 domains (highlighted in red) and three consecutive fibronectin III repeat domains (highlighted in blue) with highly charged flanking and connecting sequences that do not correspond to currently identified modular domains (highlighted in yellow). Residues that shared among RIM-BP1 and PRAX1 or between either of these proteins and RIM-BP2 or KIAA0318 are highlighted. Residues that are identical between RIM-BP2 and KIAA0318 but differ from the residues observed in RIM-BP1/PRAX at the same position are highlighted.
outside of the brain except for testis, where a smaller mRNA is detected overexposure to illustrate that there are no cross-hybridizing bands bottom expressed control probe (top). The RIM-BP1 blot is shown as an cDNA probe from the C terminus of RIM-BP1 (top). A rat multitissue RNA blot was hybridized at high stringency with a

Rab3s.

NIMs and DOC2s) they are present without any connection to i.e. RIMs and rabphilin), while in other family members (i.e. members of this family to Rab3-interacting zinc fingers (i.e.

The alternatively spliced sequences are often strategically located next to identified domains (e.g. splice site 2 immediately N-terminal of the PDZ domain, or splice sites 4 and 5, which flank the binding site for RIM-BPs). More importantly, the position and inserts of splice sites 4 and 5 are conserved between RIM1 and RIM2, with inserts that can be very large (almost 200 residues). As a result, this alternative splicing is expected to change the proteins produced considerably. A further interesting feature of RIMs is their interaction with RIM-BPs. The modular domains structure of RIMs and NIMs indicates a possible interaction with multiple other proteins in addition to Rab3s in the case of RIMs. With the RIM-BPs, our study identifies the first of such potential interacting partners. Two related but distinct RIM-BPs were independently isolated in yeast two-hybrid screens, and the interaction of RIM-BP with RIM-BPs was verified in GST pull-down experiments and quantitative yeast two-hybrid assays. The fact that RIM-BPs use an SH3 domain to bind to RIMs and that the binding site for RIM-BPs is located in the small island of constant inactive zone.

residues 1021–1052 in RIM-BP1 (shown in italic cyan-blue type schematically shown in Fig. 2). Thus, RIMs, NIMs, rabphilin, DOCs, and NOCs form a superfamily of proteins composed of combinations of Rab3-interacting zinc finger domains and C2 domains. Overall, the RIM/rabphilin protein family is surprising in the way in which C2 domains are coupled in some members of this family to Rab3-interacting zinc fingers (i.e. in RIMs and rabphilin), while in other family members (i.e. in NIMs and DOC2s) they are present without any connection to Rab3s.

An interesting feature of RIMs is their conserved extensive alternative splicing with a large number of variants (as opposed to a single splice variant in rabphilin; see Refs. 17 and 42). This suggests that RIMs are not only diversified in the expression of two genes but that the exact shape of these isoforms can differ. Although we have not investigated whether alternative splicing of RIMs is regulated in brain and whether it results in functionally different proteins, it is striking that

highlighted in green. Gaps are indicated by hyphens. Residues 1021–1052 in RIM-BP1 (shown in italic cyan-blue type) were variably present or absent in multiple cDNA clones, suggesting that they are alternatively spliced. The yeast two-hybrid prey clones isolated in the RIM screen encoded residues 602–1686 of RIM-BP1, and 1–265 of RIM-BP2.
A function of RIMs in exocytosis is likely because these proteins interact with Rab3 in a regulated manner. However, no such supposition can be made for NIMs. Here the only clue to a possible function in neurotransmitter release is their brain-specific expression, similarity to RIMs outside of the Rab3-binding domains, and content of a C2 domain that is often found in exocytotic proteins. To address the question of whether or not NIMs are functionally related to exocytosis, we examined the effect of NIM3 overexpression on Ca2+-triggered secretion in transfected PC12 cells (Fig. 7). We found that NIM3 induces a dramatic enhancement of secretion, confirming a function for this protein as well in exocytosis. Thus, it appears likely that all members of the RIM/NIM superfamily is involved in the control of exocytosis.

Our results also raise a number of questions. On an immediate, more technical level, one wonders if the RIM1 gene also produces a NIM1 product, if the NIMs are generated by independent promoters within the RIM genes, and whether there is a spatially differentiated transcription of RIMs and NIMs in brain; i.e. are their promoters differentially regulated? Another more technical question is whether the interaction of RIM-BP with RIM is specific, since many SH3 domain interactions are known to be quite promiscuous. Second, the distribution of RIM-BP1, which suggests a high degree of specificity and argues against a promiscuous interaction. Second, the distribution of RIM-BP1, which is primarily expressed in the brain, resembles that of RIMs much more than that of the peripheral benzodiazepine receptor, which, as indicated by its name, is not brain-specific. Furthermore, the modular structure of RIM-BPs and SH3 and fibronectin type III repeat domains would fit well into a role as an organizer of the active zone. Third, we confirmed an interaction of RIM-BP1 with RIM biochemically and localized the binding site in RIMs by quantitative yeast two-hybrid assays. The fact that an SH3 domain of RIM-BPs binds to a conserved PXXP sequence in RIMs gives credence to the interaction, P

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The RIM/NIM Family of Neuronal C2 Domain Proteins: INTERACTIONS WITH Rab3 AND A NEW CLASS OF Src HOMOLOGY 3 DOMAIN PROTEINS

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