Binding to Rab3A-interacting Molecule RIM Regulates the Presynaptic Recruitment of Munc13-1 and ubMunc13-2*

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Transmitter release at synapses between nerve cells is spatially restricted to active zones, where synaptic vesicle docking, priming, and Ca\(^{2+}\)-dependent fusion take place in a temporally highly coordinated manner. Munc13s are essential for priming synaptic vesicles to a fusion competent state, and their specific active zone localization contributes to the active zone restriction of transmitter release and the speed of excitation-secretion coupling. However, the molecular mechanism of the active zone recruitment of Munc13s is not known. We show here that the active zone recruitment of Munc13 isoforms Munc13-1 and ubMunc13-2 is regulated by their binding to the Rab3A-interacting molecule RIM1\(\alpha\), a key determinant of long term potentiation of synaptic transmission at mossy fiber synapses in the hippocampus. We identify a single point mutation in Munc13-1 and ubMunc13-2 (I121N) that, depending on the type of assay used, strongly perturbs or abolishes RIM1\(\alpha\) binding in vitro and in cultured fibroblasts, and we demonstrate that RIM1\(\alpha\) binding-deficient ubMunc13-2\(^{1211}\) is not efficiently recruited to synapses. Moreover, the levels of Munc13-1 and ubMunc13-2 levels are decreased in RIM1\(\alpha\)-deficient brain, and Munc13-1 is not properly enriched at active zones of mossy fiber terminals of the mouse hippocampus if RIM1\(\alpha\) is absent. We conclude that one function of the Munc13/RIM1\(\alpha\) interaction is the active zone recruitment of Munc13-1 and ubMunc13-2.

The release of neurotransmitters at chemical synapses between nerve cells is spatially restricted to specialized compartments of the axonal plasma membrane. These so-called active zones, at which exocytosis takes place in a temporally highly coordinated manner, are typically localized in axon terminals, face the postsynaptic signal reception apparatus, and consist of electron-dense, insoluble proteinaceous material (1–3).

Most presynaptic axon terminals contain hundreds of vesicles that are clustered in close proximity of the active zone. Usually, only a fraction of vesicles docked at the active zone are in a fusion-competent primed state, and only these primed vesicles, which are referred to as the readily releasable pool, can fuse with the plasma membrane in response to an elevation of the intracellular Ca\(^{2+}\) concentration. The readily releasable vesicle pool represents a reservoir that allows a synapse to repetitively release neurotransmitters during bursts of action potentials, and its size determines synaptic release probability and signaling capacity (1, 4).

Given their central role in late exocytotic steps of the synaptic vesicle cycle, active zones must harbor the specific protein machinery that is responsible for vesicle tethering, priming, and fusion. Indeed, several large non-membrane proteins are specifically enriched at active zones in the mammalian central nervous system. They include (i) Piccolo/Accozin (5, 6) and Bassoon (7), two very large multidomain proteins that play a role in the organization and function of active zones (8–13); (ii) ERC/CASTs (glutamate-leucine-lysine-serine-rich protein/Rab6-interacting protein/cytomatrix of the active zone-associated structural protein) (14–18), coiled-coil domain proteins that act as scaffolding proteins in the active zone (9, 15–17); (iii) αRIMs (19–21), Rab3 effector proteins that regulate synaptic transmitter release and long term synaptic plasticity (22–25); (iv) α-Liprins (26), multidomain coiled-coil proteins that may act as anchoring proteins and regulators of other active zone components (22, 27); and (v) Munc13s (28–30), which are essential synaptic vesicle priming proteins (31, 32).

All known active zone-specific proteins interact with each other in multiple combinations (9, 15, 17, 22, 27, 30) and thus form a complex proteinaceous network. These interactions between active zone components are likely to serve functions beyond mere recruitment and anchoring of the active zone protein complement. Rather, the active zone protein network is thought to be responsible for the temporal coordination of synaptic vesicle docking, priming, and fusion, and for the spatial restriction of these processes to the active zone subdomain of the presynaptic plasma membrane (1).

The molecular processes that are mediated by individual active zone-specific proteins are in most cases still unknown. An exception are the members of the Munc13 family of essential synaptic vesicle priming proteins, Munc13-1, ubMunc13-2, bMunc13-2, and Munc13-3. Studies on deletion mutant mice showed that in the absence of Munc13s, synaptic vesicle priming is abolished, resulting in a complete block of spontaneous and evoked synaptic transmission (31, 32). The same phenotype is observed in Caenorhabditis elegans and Drosophila mutants lacking the corresponding invertebrate homologues, Unc-13 and Dunc-13 (33, 34). Munc13s and their invertebrate homologues are thought to exert their priming function by binding syntaxin 1 and stabilizing it in an open conformation.

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that is able to form SNARE (soluble N-ethylmaleimide-sensitive fusion factor attachment protein receptor) complexes (35–37). In addition, Syntaxin binding by Munc13-like proteins may regulate subsequent steps in the transmitter release process (38). Given that Munc13-mediated synaptic vesicle priming is absolutely essential for synaptic transmission, the active zone-specific localization of Munc13s is a key contributor to the spatial restriction of transmitter release to active zones and to the speed of excitation secretion coupling at synapses (1).

The Munc13 isoforms Munc13-1 and ubMunc13-2 bind to the Zn$^{2+}$ finger region of αRIMs via their evolutionarily conserved N-terminal region (30, 39), allowing the formation of tripartite Munc13-αRIM-Rab3 complexes (39). RIM1α plays a crucial role in the control of synaptic transmitter release and presynaptic forms of synaptic long term potentiation. Hippocampal neurons lacking RIM1α exhibit reduced synaptic vesicle priming, altered short term synaptic plasticity, and changes in evoked asynchronous transmitter release (25), a phenotype that is similar to that of Munc13-deficient neurons but less severe (29, 32). Specific interference with the interaction between Munc13-1 and αRIMs inhibits synaptic vesicle priming (30, 39), indicating that Munc13/αRIM interaction has a regulatory function in synaptic vesicle priming in vivo, and that the two proteins act in the same presynaptic regulatory pathway. This notion is supported by the findings that Munc13-1 levels are dramatically reduced in the brains of RIM1α-deficient mice (22), and that in C. elegans the phenotypes of loss of function mutations in both, the unc-13 and unc-10 (RIM) genes, are partially rescued by the overexpression of a mutant Syntaxin that preferentially adopts an open conformation (36, 40). In addition to its role in basal synaptic transmitter release, RIM1α is essential for long term potentiation (LTP)$^2$ of synaptic transmission at mossy fiber synapses in the hippocampus and at parallel fiber synapses in the cerebellum (23, 24). Both types of LTP are expressed presynaptically and require the activity of protein kinase A, and protein kinase A-mediated phosphorylation of RIM1α is necessary for the expression of mossy fiber LTP (24). This role of RIM1α in mossy fiber LTP appears to be critical for cognitive functions as RIM1α-deficient mice exhibit severely impaired learning and memory (41).

Whereas the evidence for a functional interaction of Munc13s and αRIMs in vivo is extensive, the exact role of this interaction is not known. The currently available data are compatible with a role of αRIMs in both, anchoring and direct regulation of Munc13-1 and ubMunc13-2. In the present study, we determined the role of RIM1α in the synaptic recruitment of Munc13-1 and ubMunc13-2. Our data show that RIM1α regulates the active zone recruitment of Munc13-1 and ubMunc13-2.

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**MATERIALS AND METHODS**

Identification of RIM Binding-deficient ubMunc13-2 Mutants—αRIM binding-deficient ubMunc13-2 constructs were generated by random mutagenesis and reverse yeast two-hybrid screening using a LexA/VP16 reporter system with L40 as the yeast reporter strain (30, 42). For that purpose, the αRIM-binding yeast two-hybrid bait construct pLexN-ubMunc13-2(1–181), which encodes LexA in-frame with residues 1–181 of ubMunc13-2 and represents the minimal αRIM binding region of ubMunc13-2 (30), was used as a template for error-prone PCR (GeneMorph II, Stratagene). PCR products were subcloned into pLexN to generate a library of mutant pLexN-ubMunc13-2(1–181)$^{\text{mut}}$ constructs in-frame with N-terminal LexA. This library was used in a reverse yeast two-hybrid screen with pPrey-RIM1α(1–344), which encodes VP16 in-frame with residues 1–344 of RIM1α and contains the binding site for Munc13-1 and ubMunc13-2 (30). αRIM binding-deficient pLexN-ubMunc13-2(1–181)$^{\text{mut}}$ variants were identified by β-galactosidase screens of L40 yeast colonies. pLexN-ubMunc13-2(1–181)$^{\text{mut}}$ bait plasmids from colonies that lacked β-galactosidase activity, which were assumed to encode αRIM binding-deficient ubMunc13-1(1–181)$^{\text{mut}}$, were isolated and sequenced. Using this approach, we isolated 26 RIM1α binding-deficient pLexN-ubMunc13-2(1–181)$^{\text{mut}}$ constructs, 18 of which contained nonsense mutations. In addition to these nonsense mutants, we identified eight αRIM binding-deficient pLexN-ubMunc13-2(1–181)$^{\text{mut}}$ constructs that carried one or several missense mutations (Fig. 1C). In cases of constructs carrying multiple missense mutations, individual mutations were introduced into pLexN-ubMunc13-2(1–181) using QuikChange (Stratagene) and analyzed separately for their effects on RIM1α binding in yeast two-hybrid screens. Using this approach, we identified a total of six missense mutations (pLexN-ubMunc13-2(1–181)$^{T22I}$, pLexN-ubMunc13-2(1–181)$^{T22A}$, pLexN-ubMunc13-2(1–181)$^{H119R}$, pLexN-ubMunc13-2(1–181)$^{H119R}$, pLexN-ubMunc13-2(1–181)$^{T121N}$, and pLexN-ubMunc13-2(1–181)$^{T121N}$), that abolished binding of LexN-ubMunc13-2(1–181)$^{\text{mut}}$ to VP16-RIM1α(1–344) (Fig. 1, C and D). As all affected residues are conserved in the αRIM binding regions of ubMunc13-2 and Munc13-1 (Fig. 1B), the second αRIM binding Munc13 isoform, 4 homologous mutations were introduced individually into a previously characterized (30) RIM1α binding bait construct of Munc13-1 (pLexN-Munc13-1(1–150)$^{T22I}$, pLexN-Munc13-1(1–150)$^{T22N}$, pLexN-Munc13-1(1–150)$^{H119R}$, and pLexN-Munc13-1(1–150)$^{H119R}$), as was the case with the corresponding ubMunc13-2 constructs, all these LexN-Munc13-1(1–150)$^{mut}$ constructs failed to bind VP16-RIM1α(1–344) in yeast two-hybrid assays (Fig. 1E). The Munc13-1 mutations T22I, H119R, I121N, and P168L were further analyzed in biochemical binding assays, whereas the consequences of the I121N mutation were further analyzed in the context of full-length Munc13-1 and ubMunc13-2 function. All DNA manipulations were verified by sequencing.

Expression of Recombinant Proteins—Bacterial expression vectors encoding GST in-frame with the RIM1α binding N ter-

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$^2$ The abbreviations used are: LTP, long term potentiation; EGFP, enhanced green fluorescent protein; EYFP, enhanced yellow fluorescent protein; HEK, human embryonic kidney; NMDAR, N-methyl-D-aspartate receptor; RIM, Rab3A-interacting molecule; GST, glutathione S-transferase.
αRIMs Recruit Munc13-1 and ubMunc13-2 to Active Zones

mini of Munc13-1 and ubMunc13-2 were constructed in pGex-KG (pGEX-Munc13-1(3–317) and pGEX-ubMunc13-2(3–320)) (43). Mutant variants (pGEX-Munc13-1(3–317)T22I, pGEX-Munc13-1(3–317)I121N, pGEX-Munc13-1(3–317)H119R, pGEX-ubMunc13-2(3–320)T22I, pGEX-ubMunc13-2(3–320)I121N, and pGEX-ubMunc13-2(3–320)H119R) were generated using QuikChange (Stratagene). The Munc13 binding Zn$^{2+}$ finger region of RIM1α was expressed as a His-tagged bacterial fusion protein from a pET32c expression vector (Novagen) (pET-RIM1α(131–214)). Mammalian expression vectors encoding full-length Munc13-1, Munc13-1I121N, ubMunc13-2, and ubMunc13-2H119R with C-terminal enhanced green fluorescent protein (EGFP) tags were constructed in pEGFP-N1 (Clontech) using a combination of previously published cDNA fragments (28, 30) (GenBank™ accession numbers U24070 and U24071) and engineered PCR fragments that ensured reading frame conservation. The I121N mutation in the full-length pEGFP-N1 expression constructs was generated by replacement of part of the wild-type sequence with mutated cDNA fragments from the corresponding pGEX-KG vectors. Lentivirus expression vectors encoding ubMunc13-2 variants in-frame with a C-terminal EYFP tag (ubMunc13-2-EYFP and ubMunc13-2-I121N-EYFP) were constructed in pRRLsinPPT-CMV-WPRE (44). All DNA manipulations were verified by sequencing.

**Cosedimentation Assays**—For cosedimentation experiments using synaptosomal extracts, crude synaptosomes from rat brain were solubilized at a protein concentration of 2 mg/ml in 50 mM Tris (pH 8), 150 mM NaCl, 1 mM EGTA, 1% sodium deoxycholate, 0.2 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 0.5 μg/ml leupeptin. After stirring on ice, insoluble material was removed by centrifugation (60 min at 250,000 × gmax). The equivalent of 4 mg of total extracted protein was then incubated overnight at 4 °C with 20 μg of GST-Munc13 fusion protein, immobilized on glutathione-agarose beads. Beads were then washed three times with solubilization buffer containing 0.1% sodium deoxycholate. Bound proteins were eluted with 100 μl of SDS-PAGE sample buffer and analyzed by SDS-PAGE and immunoblotting. Immunoblots for native αRIMs were performed using a monoclonal anti-αRIM antibody (Transduction Laboratories). For cosedimentation assays using purely recombinant His-RIM1α(131–214), the His-tagged proteins were purified on nickel-nitrilotriacetic acid beads. The assay procedure was as described above for native RIM1α from crude synaptosomes, with the exception that 1% Triton X-100 was used for solubilization. Western blotting was performed essentially as described previously (48). They were designated as follows: H, homogenate; P1, nuclear pellet; P2, crude synaptosomal pellet; P3, light membrane pellet; S3, cyto-
solic fraction; LP1, lysed synaptosomal membranes; LP2, crude synaptic vesicle fraction; LS2, cytosolic synaptosomal fraction; S1, supernatant after synaptosome sedimentation; and LS1, supernatant after LP1 sedimentation. For the separation of soluble and particulate material from mouse cortex, individual cortices were homogenized by Ultra-Turrax (IKA) and soluble and particulate fractions were separated by ultracentrifugation (300,000 × g, 20 min, 4 °C).

**Immunohistochemistry**—Animals were deeply anesthetized with tribromethanol and perfused transcardially with 200 ml of NaCl solution (9 g/liter) containing sodium nitrite (1 g/liter) for 10 min. The brains were quickly and carefully removed and frozen in isopentane at −40 °C. Sagittal 12-μm frozen cryostat sections were collected and post-fixed with either cold 4% paraformaldehyde in phosphate-buffered saline (pH 7.4) or methanol (−20 °C). The slices were blocked in phosphate-buffered saline containing 2% normal goat serum and 0.25% Triton X-100 for 30 min at room temperature. Thereafter, sections were incubated with polyclonal antibodies to Munc13-1 (1:10,000) (49) and monoclonal antibodies to synaptophysin (Synaptic Systems; 1:1,000) at 4 °C for 16 h. The antibodies were diluted in phosphate-buffered saline that contained 5% normal goat serum and 0.1% Triton X-100. After three rinses in phosphate-buffered saline, the sections were incubated with Alexa 488- or Alexa 568-labeled goat anti-rabbit or anti-mouse IgG secondary antibodies (Molecular Probes) at room temperature. Coverslips were mounted using Mowiol (Calbiochem) and viewed using an Olympus BX61 microscope and AnalySIS imaging program (Olympus Corp.). Single plane images of cultured neurons were acquired with an Axiovert 200 LSM 510 confocal laser scanning microscope (Zeiss). Relative fluorescence intensity levels were analyzed with a web-based image processing and analysis program in Java, Image J 1.32.

**Protein Analysis in RIM1Δ Mutant Mice**—Munc13-1 and ubMunc13-2 protein levels were assessed by Western blotting using polyclonal antibodies to Munc13-1 (49) and ubMunc13-2. Levels of NMDAR1 and GDP dissociation inhibitor were determined as loading controls using monoclonal anti-NMDAR1 (50) and polyclonal rabbit anti-GDP dissociation inhibitor antibodies (provided by Dr. Reinhard Jahn, Göttingen). Secondary antibodies were horseradish peroxidase-coupled goat anti-rabbit antibodies (Bio-Rad) or Alexa Fluor 680-coupled goat anti-rabbit antibodies for Odyssey (Molecular Probes). The respective stainings were visualized by enhanced chemiluminescence (Amersham Biosciences) and quantified with the Odyssey imaging system (LI-COR).

**RESULTS**

Identification of RIM1Δ Binding-deficient Munc13-1 and ubMunc13-2 Mutants in Reverse Yeast Two-hybrid Assays—An N-terminal truncated Munc13-1 variant that lacks its entire L region including the αRIM binding domain (Munc13-1(1520–1736)-EGFP) exhibits reduced priming activity in neurons (30). This observation indicates that αRIM binding regulates active zone trafficking or priming activity of Munc13-1 and most likely also of ubMunc13-2. However, the elimination of the entire L region of Munc13-1 may also have functional consequences that are not related to the αRIM/Munc13-1 interaction.

To develop more suitable molecular tools for the functional analysis of Munc13/αRIM interactions, we used a reverse yeast two-hybrid approach for the identification of missense mutations in Munc13-1 and ubMunc13-2 that disrupt their interaction with αRIM while leaving other interactions unaffected. The N terminus of ubMunc13-2–2 (LexA-ubMunc13-2(1–181)), which is highly homologous to the N-terminal region of Munc13-1 and contains the minimal αRIM binding region (Fig. 1, A and B), was used as a model protein in this screen in combination with VP16-RIM1Δ (1–344). Of the RIM1Δ binding-deficient constructs isolated, the majority carried nonsense mutations that caused premature truncation of the minimum αRIM binding region (data not shown). This finding confirms earlier studies, which showed that residues 1–181 of ubMunc13-2 indeed represent the minimal αRIM binding site whose further truncation abolished binding (30). In addition to these nonsense mutants, we identified 8 αRIM binding-deficient pLexN-ubMunc13-2(1–181)mut constructs that carried one or several missense mutations (Fig. 1C). In cases of constructs carrying multiple missense mutations, individual mutations were introduced into pLexN-ubMunc13-2(1–181) and analyzed separately for their effects on RIM1Δ binding in yeast two-hybrid screens. Using this approach, we identified a total of 6 missense mutations (pLexN-ubMunc13-2(1–181)T22I, pLexN-ubMunc13-2(1–181)T22A, pLexN-ubMunc13-2(1–181)Y23N, pLexN-ubMunc13-2(1–181)I121N, pLexN-ubMunc13-2(1–181)P169L) that abolished binding of LexA-ubMunc13-2(1–181)mut to VP16-RIM1Δ (1–344) (Fig. 1, C and D). As all affected residues are conserved in the αRIM binding regions of ubMunc13-2 and Munc13-1 (Fig. 1B), the second αRIM binding Munc13 isoform, 4 homologous mutations were introduced individually into a previously characterized (30) αRIM binding bait construct of Munc13-1 (pLexN-Munc13-1(1–150)T22I, pLexN-Munc13-1(1–150)T22A, pLexN-Munc13-1(1–150)Y23N, pLexN-Munc13-1(1–150)I121N, and pLexN-Munc13-1(1–150)P169L) that abolished binding of LexA-ubMunc13-2(1–181)mut to VP16-RIM1Δ (1–344) (Fig. 1, C and D). All these LexN-Munc13-1(1–150)mut constructs failed to bind VP16-RIM1Δ (1–344) in yeast two-hybrid assays (Fig. 1E).

Analysis of αRIM Binding-deficient Mutants of Munc13-1 and ubMunc13-2 in in Vitro Binding Assays and Living Cells— To examine the αRIM binding-deficient mutants of ubMunc13-2 and Munc13-1, which we had identified in reverse yeast two-hybrid screens, in biochemical assays, we performed cosedimentation experiments. GST fusion proteins of wild-type and mutant N termini of ubMunc13-2 (GST-ubMunc13-2(3–320)), GST-ubMunc13-2(3–320)T22I, GST-ubMunc13-2(3–320)T22A, GST-ubMunc13-2(3–320)I121N, GST-ubMunc13-2(3–320)P169L, and Munc13-1 (GST-Munc13-1(3–317)), GST-Munc13-1(3–317)T22I, GST-Munc13-1(3–317)T22A, GST-Munc13-1(3–317)I121N, and GST-Munc13-1(3–317)P169L) were expressed in bacteria, immobilized on glutathione-agarose beads, and first tested for binding of native RIM1Δ using rat brain synaptosome extracts. All tested mutant GST-ubMunc13-2(3–320) constructs showed either no interaction with RIM1Δ or the binding was significantly reduced (not shown). However, in the case of GST-
Munc13-1 constructs only GST-Munc13-1(3–317) showed significantly reduced binding to RIM1α in cosedimentation assays, whereas all other constructs bound to RIM1α (not shown). Effectively, the I121N point mutation was the only modification that robustly and reproducibly interfered with RIM1α binding of the Munc13-1 and ubMunc13-2 N termini.

FIGURE 1. Reverse yeast two-hybrid screen for RIM1α binding-deficient variants of Munc13-1 and ubMunc13-2. A, the domain structure of C. elegans Unc-13-LR, r-Munc13-1, and r-ubMunc13-2. aa, amino acid; C1, C1 domain; C2, C2 domain; ce, C. elegans; r, Rattus norvegicus. B, amino acid residues 1–180 of Munc13-1 and 1–181 of ubMunc13-2. Residues that are identical in the two sequences are shown on a black background. C, RIM1α binding-deficient variants of the ubMunc13-2 bait construct LexA-ubMunc13-2(1–181) as identified in reverse yeast two-hybrid screens. D, RIM1α binding-deficient variants of the ubMunc13-2 bait construct LexA-ubMunc13-2(1–181) containing single point mutations that were generated with site-directed mutagenesis. E, RIM1α binding-deficient variants of the Munc13-1 bait construct LexA-Munc13-1(1–150) that were generated with site-directed mutagenesis. Amino acids are given in single letter code.

FIGURE 2. Analysis of αRIM binding-deficient variants of Munc13-1 and ubMunc13-2 in cosedimentation assays. Identical amounts of the indicated wild-type or mutant GST fusion proteins or GST alone were immobilized on glutathione-agarose beads and used in cosedimentation assays with RIM1α from rat brain synaptosome extract or recombinant His-RIM1α(131–214). Proteins that bound to the immobilized GST fusion proteins were analyzed by SDS-PAGE and immunoblotting with antibodies specific to αRIM1α. Amino acids are given in single letter code. Data are representative of at least three independent experiments with identical results. M13–1, Munc13-1; ubM13-2, ubMunc13-2. A, Western blot (top panel) for cosedimentation experiments with wild-type and I121N mutant GST-Munc13-1(3–317) and GST-ubMunc13-2(3–320) for direct comparison of αRIM binding characteristics. B, Western blot (top panel) of cosedimentation assays with purified recombinant wild-type and I121N mutant GST-Munc13-1(3–317) and GST-ubMunc13-2(3–320) and His-RIM1α(131–214). The respective lower panels in A and B show Coomassie Brilliant Blue-stained SDS-PAGE gels loaded with the same amounts of proteins as were used for the Western blots shown in the corresponding top panels. Note that similar amounts of the GST-Munc13-1(3–317) and GST-ubMunc13-2(3–320) variants were used for the different cosedimentation assays.

Munc13-1(3–317) constructs only GST-Munc13-1(3–317)I121N and GST-ubMunc13-2(3–320)I121N (Fig. 24). We therefore concentrated on this mutation and used it for all subsequent experiments analyzing Munc13-1/αRIM interactions.

In a second set of in vitro cosedimentation experiments, wild type and I121N mutant constructs, i.e. GST-Munc13-1(3–317), GST-Munc13-1(3–317)I121N, GST-ubMunc13-2(3–320), and GST-ubMunc13-2(3–320)I121N were tested for binding to the bacterially expressed, recombinant His-tagged N terminus of RIM1α (His-RIM1α(131–214)). In these experiments the respective wild-type Munc13 constructs bound robustly to His-RIM1α(131–214), whereas the I121N mutants did not bind at all (Fig. 2B). Taken together, these data indicate that the I121N...
mutation abolishes αRIM binding of Munc13-1 and ubMunc13-2, at least when only recombinant proteins are used for the binding assay, and thus can be used as a model to study the Munc13/αRIM interaction in further detail.

To analyze the effect of the I121N mutation on αRIM binding in the context of living cells, eukaryotic expression vectors encoding full-length recombinant versions of wild-type and mutant Munc13-1 with a C-terminal attached EGFP tag were generated in pEGFP-N1 (Munc13-1-EGFP and Munc13-1I121N-EGFP). These EGFP-tagged Munc13-1 variants and full-length RIM1α were then overexpressed in HEK293 cells either alone or in combination. When expressed singly in HEK293 cells, Munc13-1-EGFP and Munc13-1I121N-EGFP formed large aggregates in addition to a diffuse cytoplasmic pool of protein (Fig. 3). In contrast, overexpressed RIM1α was found in the nucleus as well as diffusely distributed in the cytoplasm (Fig. 3). When Munc13-1-EGFP and RIM1α were coexpressed, Munc13-1 again formed large aggregates and RIM1α was invariably coenriched with Munc13-1 in these aggregates (Fig. 3A). On the other hand, when Munc13-1I121N-EGFP and RIM1α were coexpressed in HEK293 cells, RIM1α was never recruited into Munc13-1I121N-EGFP aggregates. Rather, the cellular RIM1α distribution remained diffuse and partially nuclear as was the case when it was expressed in the absence of Munc13-1-EGFP (Fig. 3B). Due to the presence of a C1 domain, Munc13-1 as well as Munc13-2 and Munc13-3 bind diacylglycerol and phorbol esters and translocate to the plasma membrane of overexpressing fibroblasts and chromaffin cells in response to phorbol ester binding (51, 52). We took advantage of this phorbol ester-dependent translocation of Munc13-1 and tested if the binding to Munc13-1 causes the cotranslocation of RIM1α in coexpressing cells. We found that Munc13-1-EGFP and RIM1α cotranslocated to the plasma membrane of coexpressing HEK293 cells upon treatment with 100 nM 4β-12-O-tetradecanoylphorbol-13-acetate (Fig. 3C). In contrast, RIM1α expressed alone or in combination with Munc13-1I121N-EGFP did not translocate to the plasma membrane in response to phorbol ester treatment (Fig. 3D). Taken together, our coexpression studies in HEK293 cells show that Munc13-1 binds RIM1α in a cellular environment and that the I121N mutation is sufficient to abolish the Munc13-1/αRIM interaction, without altering phorbol ester sensitivity and translocation properties of the Munc13-1 protein. Given the high sequence homology and very similar RIM1α binding characteristics of Munc13-1 and ubMunc13-2 (Figs. 1 and 2), ubMunc13-2 is likely to behave identically.

**Analysis of Intracellular Trafficking of ubMunc13-2 and ubMunc13-2I121N in Cultured Primary Hippocampal Neurons**—To study the role of αRIM binding in targeting of Munc13cs, we expressed ubMunc13-2-EYFP and ubMunc13-2I121N-EYFP as model proteins in primary hippocampal cultures from Munc13-1/2 double deficient neurons (32) using lentiviral constructs, and studied their subcellular localization. Neurons expressing ubMunc13-2-EYFP exhibited a punctate ubMunc13-2-EYFP expression that perfectly colocalized with Bassoon (Fig. 4A). In contrast, ubMunc13-2I121N-EYFP was always distributed diffusely (Fig. 4B). We quantified the degree of presynaptic active zone targeting of ubMunc13-2-EYFP and ubMunc13-2I121N-EYFP by determining the number of Bassooon-positive active zones that also contain ubMunc13-2-EYFP or ubMunc13-2I121N-EYFP in traceable axons of individual transfected neurons (Fig. 4C). We found that in axons of neurons expressing ubMunc13-2-EYFP, the vast majority of Bassoon-positive active zones (76 ± 15%); n = 3 neurons) also contained ubMunc13-2-EYFP, whereas in axons of neurons...
expressing ubMunc13-2I121N-EYFP, hardly any of the Bassoon-positive active zones (6 ± 4%; n = 3 neurons) also contained ubMunc13-2I121N-EYFP. These observations show that, like wild-type endogenous Munc13-1 (49), the full-length wild-type ubMunc13-2-EYFP fusion protein is recruited to presynaptic active zones, whereas ubMunc13-2I121N-EYFP is not. This indicates that αRIMs contribute to the recruitment of ubMunc13-2 and, by deduction, of Munc13-1 to active zones or to their anchoring within the proteinaceous network at active zones.

Analysis of the Subcellular Distribution of Munc13-1 and ubMunc13-2 in RIM1α Knock-out Mice—To further analyze the role of αRIMs in the presynaptic targeting and retention of Munc13, RIM1α knock-out mice (22) were analyzed. Previously published studies on these mutants showed that Munc13-1 expression levels are strongly reduced in the absence of RIM1α (22). We quantified the levels of Munc13-1 and both splice variants of Munc13-2, i.e. bMunc13-2, which does not bind αRIMs, and ubMunc13-2, which has been shown to bind RIM1α (30, 39), in wild-type and littermate RIM1α-deficient adult mice using the levels of NMDAR1 as a loading control.

We found that the expression levels of both, Munc13-1 and ubMunc13-2, to be reduced by ~60% in RIM1α knock-out animals (Fig. 5), whereas the levels of bMunc13-2 were not altered (data not shown).
We determined whether the Munc13-1 and ubMunc13-2 fractions that remain in the absence of RIM1α are distributed abnormally. For that purpose, we performed subcellular fractionation experiments on cerebral cortex homogenates from wild-type and littermate RIM1α-deficient mice and examined the distribution of Munc13-1 and ubMunc13-2 in the various subcellular fractions obtained. In agreement with previously published data (29), we found Munc13-1 to be enriched in synaptosomal (P2) and synaptic membrane fractions (LP1), but also in soluble fractions (S1, S3, and LS1). A similar subcellular distribution was observed for ubMunc13-2 (Fig. 6A). This subcellular distribution of Munc13-1 and ubMunc13-2 was not altered in RIM1α-deficient brains, with the notable exception of ubMunc13-2 levels in fraction S1, which arises after removal of crude synaptosomes from total homogenates. The levels of ubMunc13-2 in this fraction appeared to be increased in the case of RIM1α-deficient brains as compared with wild-type controls (Fig. 6B), indicating that ubMunc13-2 is less efficiently anchored within the presynaptic protein network in the absence of RIM1α.

To analyze the effect of RIM1α deficiency on the subcellular distribution of Munc13-1 and ubMunc13-2 in more detail, soluble and membrane-bound protein fractions were separated by ultra-thorax homogenization and high-speed centrifugation. The levels of membrane-bound and soluble Munc13 proteins were determined by quantitative Western blotting using NMDAR1 and GDP dissociation inhibitor levels as loading controls, and the ratio between insoluble and soluble protein levels were calculated. We found that in the absence of RIM1α, the relative amounts of soluble Munc13-1 and ubMunc13-2 are significantly increased as compared with the wild-type situation (Fig. 6C). Taken together, our analyses of Munc13 protein levels in subcellular fractions from wild-type mice demonstrate that RIM1α is necessary for the normal distribution of Munc13-1 and ubMunc13-2 in the presynaptic terminal.
and RIM1α-deficient brains show that both RIM1α binding partners, Munc13-1 and ubMunc13-2, are less efficiently anchored to synaptic membranes and, presumably as a consequence, exhibit a higher turnover rate and lower total expression levels in the absence of RIM1α. These findings further support the notion that binding of Munc13-1 and ubMunc13-2 to αRIMs is critical for their proper recruitment to and anchoring at active zones.

**Distribution of Munc13-1 in the Hippocampus of Wild-type and RIM1α Knock-out Mice**—So far, all our experimental data indicated that αRIMs are involved in the presynaptic targeting and/or anchoring of Munc13-1 and ubMunc13-2. To further analyze such a role of αRIMs in the intact brain, we studied the distribution of Munc13-1 in wild-type and RIM1α-deficient hippocampus by immunofluorescence staining. Similar experiments on ubMunc13-2 were not possible because of the lack of suitable antibodies. In wild-type hippocampus, Munc13-1 was found to be strongly enriched in the granule cell mossy fiber terminals, which terminate within the stratum lucidum of the CA3 region, and much less abundant in the surrounding neuropil and cell body layers (Fig. 7A). In contrast, hippocampal sections from RIM1α-deficient brains showed no evidence of a relative accumulation of Munc13-1 in mossy fiber terminals of the stratum lucidum (Fig. 7B), although the presynaptic marker synaptophysin was distributed normally. This misdistribution of Munc13-1 in RIM1α-deficient hippocampus was most apparent upon analysis of the Munc13-1 immunofluorescence intensity across the neuropil and cell body layers of the CA3 region. In wild-type sections, Munc13-1 immunofluorescence intensity increased sharply at the interface between the cell body layer and stratum lucidum and decreased sharply at the interface between stratum lucidum and the adjacent neuropil layer of the stratum radiatum. In RIM1α-deficient hippocampal sections, on the other hand, Munc13-1 immunofluorescence intensity increased only moderately at the interface between the cell body layer and stratum lucidum and remained constant at the interface between stratum lucidum and stratum radiatum. We quantified the degree of enrichment of Munc13-1 and synaptophysin in mossy fiber terminals of wild-type and RIM1α-deficient hippocampal sections by determining the Munc13-1 and synaptophysin immunofluorescence intensity in the stratum lucidum and stratum radiatum of individual sections and calculating the percent fluorescence intensity increase from stratum radiatum to stratum lucidum (Fig. 7C). The average immunofluorescence intensity increase for synaptophysin in stratum lucidum over stratum radiatum was similar for wild-type and RIM1α-deficient hippocampal sections (13 ± 6%, wild-type, 20 ± 8%, RIM1α-deficient, n = 4), which indicates that synaptophysin is enriched in mossy fiber terminals irrespective of the presence of RIM1α. In contrast, an immunofluorescence intensity increase for Munc13-1 in stratum lucidum over stratum radiatum was only detectable in wild-type hippocampal sections (27 ± 6%, n = 4) but not in RIM1α-deficient sections (1 ± 4%, n = 4), indicating that mossy fiber terminal enrichment of Munc13-1 depends on the presence of RIM1α. Taken together, these findings provide in vivo evidence for a role of αRIMs in presynaptic targeting and/or anchoring of Munc13-1 at active zones.

**FIGURE 7. Analysis of the synaptic enrichment of Munc13-1 in hippocampal mossy fiber terminals of RIM1α deletion mutant mice.** A, wild-type hippocampal section in the CA3 region. B, RIM1α deletion mutant hippocampus section in the CA3 region. Left panels, hippocampal sections were double immunostained for Munc13-1 (green) and synaptophysin 1 (red) and viewed using an Olympus BX61 microscope. Right panels, relative fluorescence intensity levels. Data are representative of four independent experiments that yielded identical results (see C). Arrows indicate the transition between the respective strata of the hippocampal CA3 region. Scale bars, 80 μm; or, stratum oriens; py, stratum pyramidale; lu, stratum lucidum; rad, stratum radiatum. C, quantitative analysis of Munc13-1 and synaptophysin immunofluorescence intensity in stratum lucidum (lu) as compared with stratum radiatum (rad), as a measure for Munc13-1 and synaptophysin enrichment in mossy fiber terminals. For each section and each antigen, the average fluorescence intensity was calculated for the entire stratum lucidum (lu) and for the first 20 μm of the adjacent stratum radiatum (rad), using fluorescence intensity scans like the ones shown in A and B. The bar diagram expresses the mean percent increase of immunofluorescence intensity in stratum lucidum (lu) as compared with the adjacent stratum radiatum (rad, set to 100%) (n = 4 mice per genotype and antigen). Error bars represent S.D. The asterisk indicates a statistically significant difference (p < 0.05) from the wild-type control.
In contrast to the effects of RIM1α deficiency on the expression and localization of Munc13-1 and ubMunc13-2, the expression level (32) and distribution (Fig. 8) of RIM1α are not altered in the absence of Munc13-1 and ubMunc13-2. Rather RIM1α and Bassoon were found to be perfectly colocalized in both, wild-type and Munc13-1/2 double deficient hippocampal neurons (Fig. 8).

**DISCUSSION**

Munc13-like proteins contain two modules that have evolved differentially. The mammalian Unc-13 homologs, Munc13-1, bMunc13-2, ubMunc13-2, and Munc13-3, and the corresponding C. elegans N-terminal alternative splice variants, Unc-13-MR and Unc-13-LR are very similar in their C-terminal R-region, which contains a tandem C1/C2 domain, two Munc13 homology domains and a C-terminal C3 domain (30). The Unc-13-LR splice variant contains an N terminus that is highly homologous to that of Munc13-1 and ubMunc13-2, whereas the N-terminal regions of bMunc13-2, Munc13-3, and Unc-13-MR are completely unrelated. These differentially evolved Munc13 modules have distinct functional roles. The conserved C-terminal R-region is necessary and sufficient for secretory vesicle priming (30, 37, 38, 53, 54). The N-terminal αRIM binding L-region of Munc13-1 and ubMunc13-2, on the other hand, was shown to convey a synapse-specific function that is not directly involved in the priming reaction but influences it indirectly (30, 39). αRIM binding was postulated to either mediate synaptic interaction or anchoring of Munc13-1 and ubMunc13-2 to the active zone protein network. However, RIM1α appears to be the main active zone recruitment protein for Munc13-1 and ubMunc13-2. The Munc13-1 proteins that remain in RIM1α-deficient brains are still mostly localized in the synaptic neuropil (Fig. 7) and large fractions of both, Munc13-1 and ubMunc13-2, remain associated with membrane fractions in the absence of RIM1α (Fig. 6), indicating that RIM2α or other active zone components contribute to the anchoring of Munc13-1 and ubMunc13-2 to the active zone protein network. However, RIM1α appears to be the main active zone recruitment protein for Munc13-1 and ubMunc13-2 because overall Munc13-1 and ubMunc13-2 levels are reduced by 60% in RIM1α-deficient brains (Fig. 5) and the synaptic recruitment of the αRIM binding-deficient ubMunc13-2I121N-EYFP is severely perturbed (Fig. 4). The reduced expression levels of Munc13-1 and ubMunc13-2 in RIM1α-deficient brains are most likely due to increased turnover and degradation of the proteins in the cytosol as compared with the active zone-associated fraction.

All currently available functional data on the Munc13/αRIM interaction are compatible with a role of αRIMs in the accumulation of Munc13-1 and ubMunc13-2 at active zones. In hippocampal neurons (30) and in the calyx of Held (39), selective disruption of the Munc13/αRIM interaction leads to decreased synaptic vesicle priming. These effects can be explained by inefficient active zone recruitment of the essential priming proteins Munc13-1 and ubMunc13-2 upon perturbation of their interaction with αRIMs.

Apart from their relevance for our understanding of the regulation of basal synaptic transmission, the present data are also of importance with regard to the interpretation of the role of
αRIMs in mossy fiber LTP. αRIMs contain binding sites for Rab3s and Munc13s (39), and both, RIM1α and Rab3A, are essential for mossy fiber LTP (23, 55). Given the central and essential role of Munc13s in determining the transmitter release efficacy of synapses, it is possible that Munc13-1 and ubMunc13-2 are involved in the final execution of Rab3/αRIM-mediated mossy fiber LTP. At first glance, this hypothesis is contradicted by the finding that the heterozygous Munc13-1 deletion mutant mice, whose Munc13-1 are reduced by about 50%, show normal mossy fiber LTP, whereas in RIM1α deletion mutants, in which Munc13-1 levels are reduced by 60%, mossy fiber LTP is abolished (23). However, the absence of RIM1α does not only cause reduced Munc13-1 expression but also a comparable reduction of ubMunc13-2 levels (Fig. 5) and a mis-distribution of Munc13-1 with strongly impaired synaptic accumulation in mossy fiber terminals (Fig. 7). Thus, the loss of RIM1α is likely to have more deleterious effects on the Munc13 levels in mossy fiber terminals than the elimination of a single Munc13-1 allele, where active zone recruitment of Munc13s is not perturbed. The deficiency in Munc13 accumulation at mossy fiber terminals in RIM1α deletion mutant mice may then well be one cause of the observed mossy fiber LTP deficiency.

Clearly, additional studies are needed to determine as to whether Munc13-1 and ubMunc13-2 are really effectors of Rab3/αRIM-dependent mossy fiber LTP. For example, future experiments will have to determine whether phosphorylation of RIM1α, which is essential for mossy fiber LTP (24), regulates the Rab3/RIM1α/Munc13 interaction and increases the levels or activities of Munc13-1 and ubMunc13-2 in mossy fiber terminals. Ultimately, mouse genetic experiments involving knock-in mutants that express αRIM binding-deficient Munc13-1 and ubMunc13-2 or Munc13 binding-deficient RIM1α instead of the respective wild-type proteins are necessary to determine the functional significance of Munc13-αRIM complexes in mossy fiber LTP. In this regard, the present study makes an important contribution. Given that only limited structural information on the Munc13-1-αRIM complex is currently available (39), we had to resort to random mutagenesis and reverse yeast two-hybrid screening to identify subtle mutations in Munc13-1 and ubMunc13-2 that interfere with αRIM binding (Fig. 1). This approach led to the identification of a point mutation, I121N, that depending on the assay used, strongly perturbs or abolishes αRIM binding of Munc13-1 and ubMunc13-2 (Figs. 1–3) without affecting their expression or phorbol ester sensitivity (Fig. 3), indicating that the mutation does not interfere with overall protein folding. Knock-in mutant mice expressing Munc13-1I121N and ubMunc13-2I121N instead of the corresponding wild-type proteins could serve as useful tools for the dissection of the molecular processes that mediate mossy fiber LTP.

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