Rim1 and Rabphilin-3 Bind Rab3-GTP by Composite Determinants Partially Related through N-terminal α-Helix Motifs*

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Rim1 is a protein of the presynaptic active zone, the area of the plasma membrane specialized for neuro-transmitter exocytosis, and interacts with Rab3, a small GTPase implicated in neurotransmitter vesicle dynamics. Here, we have studied the molecular determinants of Rim1 that are responsible for Rab3 binding, employing surface plasmon resonance and recombinant, bacterially expressed Rab3 and Rim1 proteins. A site that binds GTP- but not GDP-saturated Rab3 was localized to a short α-helical sequence near the Rim1 N terminus (amino acids 19–55). Rab3 isoforms A, C, and D were bound with similar affinities ($K_d = 1–2 \mu M$). Low affinity binding of Rab6A-GTP was also observed ($K_d = 16 \mu M$), whereas Rab1B, -5, -7, -8, or -11A did not bind. Adjacent sequences up to amino acid 387, encompassing differentially spliced sequences, the zinc finger module, and the SGAWFF motif of Rim1, did not significantly contribute to the strength or the specificity of Rab3 binding, whereas a point mutation within the helix (R33G) abolished binding. This Rab3-binding site of Rim1 is reminiscent of the N-terminal α-helix that is part of the Rab5-binding region of rabphilin-3, and indeed we observed low affinity, specific binding of Rab3A ($K_d$ on the order of magnitude of 10–100 μM) to this region of rabphilin-3 alone (amino acids 40–88), whereas additional sequences up to amino acid 178 are needed for high affinity Rab3A binding to rabphilin-3 ($K_d = 10–20 \text{ nM}$). In contrast, an N-terminal α-helix motif in aczonin, with sequence similarity to the Rab3-binding site of Rim1, did not bind Rab3A, -C, or -D or several other Rab proteins. These results were qualitatively confirmed in pull-down experiments with native, prenylated Rab3 from brain lysate in Triton X-100. Munc13 bound to the zinc finger domain of Rim1 but not to the rabphilin-3 or aczonin zinc fingers. Pull-down experiments from brain lysate in the presence of cholate as detergent detected aczonin zinc fingers. Pull-down experiments from brain lysate in the presence of cholate as detergent detected aczonin zinc fingers.

Small GTPases of the Rab family are important for intracellular membrane traffic and are thought to convey directionality and contribute to the targeting specificity of vesicle transport (reviewed in Refs. 1–3). Rab3 is implicated in regulated exocytosis. Its four known isoforms are found in many exocytotically active cell types including various endo- and exocrine cells, adipocytes, egg cells, mammary epithelial cells, and osteoclasts, in association with secretory granules and other intracellular membranes. Rab3A and Rab3C, in particular, are highly expressed in neurons and are associated with the membranes of synaptic neurotransmitter vesicles. In isolated nerve terminals (synaptosomes), Rab3A and Rab3C undergo cycles of GTP/GDP conversion and of membrane association/dissociation concurrent with the life cycle of synaptic vesicles (4–6). Different types of experiments in a number of endocrine and neuronal cell types indicate that Rab3 acts as a GTP-dependent negative regulator of Ca2+-stimulated exocytosis (see Ref. 7 and references therein). Rab3A knockout mice display electrophysiological phenomena that also speak for a negatively regulatory role in synaptic vesicle release (8–11). The Rab3A knockout phenotype is relatively subtle, suggesting that Rab3A is not essential for neurotransmitter exocytosis, but it is possible that the B, C, or D isoforms partially compensate for the lack of Rab3A.

The molecular mechanisms of action of Rab3 are poorly understood, but they are thought to involve effector proteins that interact specifically with GTP-saturated Rab3. At least two such proteins have been identified: rabphilin-3 and Rim. Rabphilin-3 dissociates from synaptic vesicles concurrently with Rab3A (12), Rab3A knockout mice display cytosolic mislocalization and reduced abundance of rabphilin-3 (8, 13), and rabphilin-3 stimulates Ca2+-dependent exocytosis (14, 15). These observations are in support of a functional interaction between the two proteins. However, mutations either in Rab3A or in rabphilin-3 that abolish direct binding between the two proteins do not impair the inhibition of exocytosis by Rab3A (7, 16) or the stimulation of exocytosis by rabphilin-3 (17), respectively. Moreover, rabphilin-3 knockout mice display no detectable phenotype; in particular, they do not exhibit the phenomenon observed in Rab3A knockout mice (18). Therefore, the mechanistic role of rabphilin-3 in the neurotransmitter vesicle life cycle and particularly in relation to Rab3 remains unclear.

Rim, with two known isoforms, Rim1 (19) and Rim2 (20), is firmly associated with the release site of neurotransmitter vesicles, the active zone of the synapse. Its N-terminal region binds GTP-complexed Rab3A and Rab3C, so that Rim may target and tether synaptic vesicles to their release sites through Rab3. Rims are large (1250–1600-amino acid) multidomain proteins containing zinc finger, PDZ, and C2 modules and are subject to extensive structural variation by differential splicing and alternative promoter choice (20). Related domain architectures with N-terminal double zinc fingers and two C-
terminal C2 domains are also found in rabphilin-3 and in another active zone-associated protein named aczonin (21), alias piccolo (22). Aczonin, like Rim, also possesses a PDZ domain upstream of the C2 domains.

To understand how these putative effector proteins act in concert with Rab3 in the orchestration of neurotransmitter vesicle dynamics, it is necessary to define their functional domains and the interactions of these domains within each protein and with Rab3 and other binding partners. Large multidomain proteins anchored at the active zone, like Rim and aczonin, probably function as scaffolds for the ordered assembly of multiprotein complexes involved in vesicle trafficking. In rabphilin-3, a sequence region surrounding the zinc finger (amino acids 45–161) has been defined as the Rab3A-GTP binding domain. The rabphilin-3 zinc finger module itself does not contact Rab3A in the crystal structure (23), but it seems to be necessary to uphold the binding configuration because mutations of zinc-coordinating cysteine residues severely impair Rab3A binding (12, 24). In the present study, we have investigated which parts of the N-terminal region of Rim1 are involved in the GTP-stimulated binding of Rab3. We find that a short α-helical sequence near the Rim1 N terminus, distantly similar to an α-helical sequence in the Rab3A-binding domain of rabphilin-3, is sufficient for high affinity, GTP-dependent, direct and specific binding of Rab3.

### Experimental Procedures

**Recombinant Proteins**—Full-length coding sequences of Rab proteins, from codon 2 to the stop codon, were amplified by reverse transcription-polymerase chain reaction from mouse brain RNA and inserted into the Smal site of the His tag vector pQE32 (Qiagen). The following reference sequences were used: Rab3A (accession number NM_009005), Rab3B (AF263365), Rab7 (NM_017382), Rab11A (NM_017382), all from mouse. The mouse Rab3C cDNA sequence was newly determined after reverse transcription-polymerase chain reaction using an N-terminal primer based on the rat Rab3C DNA (D78197) and a C-terminal primer based on a mouse EST (AI850886). Expression constructs of rat Rab5 (AF072935) with an N-terminal His tag in pET-11d and of the human endosomal autoantigen EEAL (amino acids 1257–1411; L01457) fused to glutathione S-transferase (GST)3 were the gift of M. Zerial (EMBL, Heidelberg). Constructs Rab1B (X14842) and human Rab8 (L19333) with N-terminal His6-Myc tags in pQE9 were the gift of T. Weide (Department for Experimental Tumor Biology, University of Munich, Germany). Recombinant Rab proteins were expressed and purified under non-denaturing conditions following Qiagen protocols, with the addition of protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 2 μg/ml pepstatin A, 2 μg/ml leupeptin) to the lysis buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 10 mM imidazole). Sequence regions of Rim1, rabphilin-3 (reference sequence, accession number D29965), and aczonin (Y19185) were amplified from mouse brain RNA or from earlier subclones of these proteins and inserted into the Smal site of the GST fusion vector pGEX-4T-2 (Amersham Pharmacia Biotech). The mouse Rim1 partial cDNA sequence (codons 2–387) was newly determined after amplification with primers derived from the rat sequence (AF072836). GST fusion proteins were expressed in DH5α or 2921 cells and purified essentially according to Amersham Pharmacia Biotech protocols. To optimize the integrity of zinc finger domains, in a number of purifications, 50 μM ZnCl2 was added to bacterial culture media, and the standard phosphate-buffered saline buffer (140 mM NaCl, 2.7 mM KCl, 8 mM Na2HPO4, 1.5 mM KH2PO4) was replaced by Tris-buffered saline plus DTT (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM DTT, 0.25 mM phenylmethylsulfonyl fluoride, 2 μg/ml pepstatin A, 2 μg/ml leupeptin) for lysis and glutathione affinity column washing. Subsequent analysis showed that recombinant zinc finger proteins prepared in either way did not differ in zinc content or Rab3 binding behavior. GST fusion proteins were eluted with 15 mM glutathione in 50 mM Tris pH 8.0, 1 mM DTT, and dialyzed against phosphate-buffered saline or Tris-buffered saline plus DTT, respectively. Protein concentrations were determined according to Bradford. All expression clones were authenticated by full-length sequencing of the inserts. The R33G mutation arose spontaneously during polymerase chain reaction/subcloning of the Rim-9 (9–35) sequence.

**Zinc Stoichiometry Determinations**—The zinc assay was performed essentially as described (25). 700 μl of GST fusion proteins at 1 μM were mixed with 7 μl of 10 mM 4-(2-pyridylazo)-resorcinol, a Zn2+ binding dye, and titrated with successive 0.7-M aliquots of 10 mM p-hydroxymercuriphenylsulfonate, a cysteine-modifying reagent, with 20-min incubations at room temperature between additions until a constant absorbance was reached. The protein buffer samples (phosphate-buffered saline or Tris-buffered saline plus DTT) were treated in the same way as blanks, and standard curves were prepared with ZnCl2 in the same buffer. As negative controls, samples of zinc fingerless constructs Rim-(2–55) and Rim-(18–55) were also analyzed, yielding only background values of <0.01 μmol/ml. Zn2+ standard curves were also prepared in solutions of these control GST fusion proteins but did not differ in steepness from standard curves in buffer only.

**Surface Plasmon Resonance (SPR) Binding Measurements**—SPR experiments were performed using a Biacore 2000 instrument (Biacore AB, Uppsala, Sweden). One binding partner, referred to as the ligand, is immobilized on a sensor chip, and the interaction with a binding partner in free solution flowing over this surface, the analyte, is detected. Changes in surface concentration of the analyte are proportional to changes in the refractive index of the surface, measured as a shift in the frequency of the SPR signal, plotted as response units (RU’s). 1000 RU’s corresponds to a surface concentration of 1 ng/ml.

Recombinant Rab proteins at concentrations of 20 μM were preincubated for 3.5 h at room temperature in buffer A (20 mM MOPS, pH 7.0, 150 mM KCl, 0.005% Tween 20) including 1 mM EDTA and either 0.5 mM GTP-S or 1 mM GDP. A control preincubation time course was performed, showing that saturation of Rab3A with GTP-S (i.e. maximal SPR signal intensity of Rab3A binding to Rim-(2–55)) was reached after 1 h. MgCl2 was then added to a total concentration of 3 mM, and incubation continued for 30 min. Dilution series of Rab proteins, typically in the concentration range from 20–40 nM to 5–10 μM were then prepared in buffer A with 3 mM MgCl2 and either 0.5 mM GTP-S or 1 mM GDP. The Rab3A dilution series was in the concentration range 78 nM to 10 μM. GST fusion proteins with Rim1, aczonin, and rabphilin-3 partial sequences were immobilized at surface densities of 500–800 RU’s on an anti-GST antibody-coated surface, and the interaction with Rab proteins in rising concentration order was analyzed at 20 °C and at a flow rate of 30 μl/min in association and dissociation, again in buffer A. Both GST-MgCl2 and 0.5 mM GTP-S or 1 mM GDP. After Rab protein concentration, the antibody surface was flushed with 10 mM glycine, pH 2.2, to remove the whole complex and was recharged with GST fusion proteins with Rim1, aczonin, and rabphilin-3 at the same surface density before a new Rab protein concentration was measured. Rab samples first passed through a sensor chip with a blank antibody surface, in series with the fusion protein-charged sensor chip, and the unspecific signal of the control channel deducted from the total signals of the specific binding (unless stated otherwise, binding data were evaluated by steady-state analysis, plotting the plateau of the SPR signal versus the analyte concentration, and fitted by nonlinear regression analysis with the Prism software package (GraphPad). In selected cases, association and dissociation rate constants were deduced from the initial signal courses of the association and dissociation phases, assuming Langmuir 1:1 binding and employing the BIAevaluation 3.0 software (Biacore). Additional zinc finger-protecting measures in control experiments (inclusion of 10 μM ZnCl2, and 5 mM DTT in the preincubation and dilution buffers) did not enhance the Rab3A binding of Rim1 zinc finger constructs.

**Pull-down Binding Experiments**—Rat brains were homogenized in a glass-Teflon homogenizer in lysis buffer and centrifuged for 30 min at 120,000 × g at 4 °C. The standard lysis buffer was 50 mM Tris, pH 7.4, 150 mM NaCl, 2 mM MgCl2, 0.5 mM EDTA, 1 mM DTT, 0.5% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride, and 2 μg/ml each of pepstatin A, leupeptin, and aprotinin. Alternatively, 25 mM Hepes/ KOH, pH 7.4, 100 mM NaCl, 2 mM EGTA, 1% sodium cholate, plus protease inhibitors as above, was used (“synaptosome extraction buffer”, Ref. 26). Supernatants were preclarified by incubation with 1/10 volume of glutathione-agarose for 4 h and adjusted to a protein concentration of 10 mg/ml with lysis buffer. Glutathione-agarose beads (Sigma) were loaded with GST or with GST fusion proteins (20 μg of protein/20 μl of beads), preblocked with 3% BSA in lysis buffer for 2 h at 4 °C, and washed with 1 ml of lysis buffer. 20-μl samples of loaded beads were incubated with 250-μl aliquots of cleared brain lysate supplemented with 2 mM GDP or with 0.5 mM GTP-S, at 4 °C overnight.

1 The abbreviations used are: GST, glutathione S-transferase; RU, response unit; SPR, surface plasmon resonance; mAb, monoclonal antibody; MOPS, 4-morpholinepropanesulfonic acid; GTP-S, guanosine 5′-3′-O-thio-triphosphate.
Rab3 Binding Determinants of Rim1 and Rabphilin-3

RESULTS

Recombinant mouse Rim1 sequences encompassing various parts of the N-terminal 387 amino acids (Fig. 1) were fused to GST and expressed in bacteria, and their interaction with bacterially expressed, His<sub>6</sub>-tagged recombinant Rab3A was studied by SPR (Fig. 2, Table I). A binding site for GTP<sub>S</sub>-saturated Rab3A was localized within a short sequence of high α-helix potential near the N terminus (amino acids 19–55; MQL . . . MLK), with a K<sub>d</sub> of 1.3 μM (mean of values determined with constructs Rim-(19–55) and Rim-(2–55)). GDP-saturated Rab3A at 20 μM concentration gave no binding signals.

Sequences immediately adjacent to this Rab3 binding site of Rim1 are subject to differential splicing (deletion of amino acids 56–62 (CVR . . . PPL), 83–105 (NIF . . . RNW), or 56–105). In RNA from 5-month-old mouse brain, we found all four possible variants to occur in similar abundances, judged from the relative band intensities of reverse transcription-polymerase chain reaction products in gel electrophoresis and from the recovery of cDNA subclones. Rab3 binding, however, was not significantly affected by differential splicing in this region (Fig. 1, Table I). Sequences further downstream, extending to amino acid 228 (. . . EVP) or 387 (. . . RYP), did not bind Rab3A on their own; nor did they affect significantly the Rab3A affinity of the Rim1 N-terminal α-helix (Fig. 1, Table I). These downstream sequences include the zinc fingers and the SGAWFF motif, whose counterparts are important for Rab3A binding to rabphilin-3 (12, 23, 24). In contrast, a point mutation (R33G) in the α-helical motif virtually abolished Rab3A binding of long and short Rim1 constructs (Fig. 1, Table I).

The N-terminal α-helix motif of Rim1 was found to contain sufficient information not only for the affinity but also for the Rab protein specificity of binding. The Rab3 isoforms, Rab3C and Rab3D, bound with affinities indistinguishable from Rab3A and similarly to long, intermediate, and short Rim1 constructs (Table I). Rab6A bound at 2.5 or 5 μM to Rim1 constructs representing all sizes and different splice variants (Rim-(2–55), Rim-(2–228, Δ56–82), Rim-(9–387, Δ56–82), Rim-(9–387, Δ83–105), and Rim-(9–387, Δ56–105)). A K<sub>d</sub> of 16 μM was determined for Rab6A binding to Rim-(9–387, Δ56–82) (Table I). Like Rab3, Rab6A bound only if saturated with GTP<sub>S</sub>, not with GDP. In contrast, GTP<sub>S</sub> complexes of several other Rab proteins at concentrations of 5 μM (Rab1B, Rab5, Rab7, Rab8, and Rab11A) did not bind to Rim-(2–55) or to Rim-(2–228, Δ56–82) (not shown).

Rabphilin-3 also contains an α-helical motif in its N-terminal region, which has distant sequence similarity to the Rab3 binding site of Rim1 (Fig. 3). In the crystal structure (23), this α-helix constitutes the main interface of rabphilin-3 with Rab3A, contacting switch I and switch II of the GTPase. However, pull-down and crystallization experiments have shown that additional downstream sequences including the zinc finger and SGAWFF motifs are important for Rab3A binding to rabphilin-3 (12, 23, 24). To compare the contributions of these different determinants to the Rab3 binding behaviors of Rim1 versus rabphilin-3 in our experimental system, we also analyzed two selected rabphilin-3 constructs by SPR, rabphilin-(40–88) and rabphilin-(40–178) (Fig. 2). The first construct, containing the N-terminal α-helix of rabphilin-3 but not the zinc finger motif indeed also bound Rab3A-GTP<sub>S</sub> but not Rab3A-GDP, with rapid association and dissociation kinetics and low affinity. K<sub>d</sub> values of 16 and 97 μM were estimated in two independent measurements like that shown in Fig. 2, with concentration series up to 10 μM. The longer construct, rabphilin-(40–178), was endowed by the additional downstream sequences with a complex binding behavior that seemed to be composed of a slow, high affinity and a fast, low affinity component (Fig. 2). Kinetic fits at low Rab3A concentrations (2–78 nM and 156–625 nM in four measurements with two different rabphilin-(40–178) protein preparations) resulted in K<sub>d</sub> values for the high affinity component of 10–20 nM (i.e. 2 orders of magnitude lower than the Rim1 constructs). For the fast low affinity component, K<sub>d</sub> values of 5 and 7 μM were determined in two measurements by steady-state analysis after subtraction of the 156 nM signals. This K<sub>d</sub> is similar to that of the isolated N-terminal helix. Both rabphilin-3 constructs also bound Rab3D, but they did not bind Rab1B, Rab4, Rab5, Rab6, Rab7, Rab8, or Rab11A at 5 μM concentrations in the presence of GTP<sub>S</sub> (not shown).

Since the zinc finger domain of rabphilin-3 is critical for Rab3A binding (12, 24) whereas the Rim1 zinc finger did not
contribute to Rab3A binding in our SPR measurements, we wanted to confirm that it indeed exists in an intact, zinc-coordinating conformation in our recombinant protein preparations. We therefore determined the zinc stoichiometries of most zinc finger constructs used in our measurements, employing a complexometric technique after liberation of zinc ions by covalent modification of cysteines (25). All five middle-sized Rim1 constructs and Rim-(9–387, Δ56–82) were analyzed (eight preparations of six constructs), giving stoichiometries between 0.84 and 1.78 mol of Zn2+ per mol of protein. Eight independent preparations of rabphilin-3-(40–178) gave values of 0.65–1.00 mol/mol. Aczonin-(374–654) and aczonin-(863–1115), one preparation each, had Zn2+ stoichiometries of 0.94 and 0.55 mol/mol, respectively. As a positive control, the Cys4 "FYVE finger" region (amino acids 1257–1411) of the early endosomal autoantigen EEA1 (two preparations) gave values of 0.80 and 0.85. Stoichiometries were not influenced by specific measures to protect the zinc fingers (i.e., the addition of Zn2+ and DTT during expression and purification (see "Experimental Procedures")).

FIG. 2. Representative SPR tracings of short, middle-sized, and long Rim1 and rabphilin-3 constructs binding to Rab3A. In the association phase, Rab3A solution flows over the sensor surface preloaded with the Rim1 or rabphilin-3 constructs, followed in the dissociation phase by buffer only. The short Rim1 construct displays fast high affinity binding, onto which a minor slow, low affinity component is superimposed in the middle-sized and long constructs. The short rabphilin-3 construct exhibits fast, low affinity binding to which a slow, high affinity component adds itself in the long construct. For rabphilin-(40–178), two separately measured Rab3A concentration series in a high (156 nM to 10 μM) and in a low (2–78 nM; inset) concentration range are shown.

Regardless of whether these stoichiometries are true or an underestimation of the actual value, the rabphilin-3 zinc finger domain did confer high affinity Rab3A binding to rabphilin-(40–178), although this construct had on the average a lower apparent zinc stoichiometry than the Rim1 constructs, and our Rim1 zinc finger constructs also bound Munc13 (see below).

The N-terminal region of aczonin contains a predicted α-helix with a sequence motif (amino acids 41–59) of significant similarity to amino acids 24–42 in the Rab3-binding site of Rim1. Among the conserved residues is arginine 33, which is critical for the Rab3A binding of Rim1 (Fig. 3). However, aczonin constructs encompassing amino acids 2–80 or 2–218 did not bind Rab3A, -C, or -D nor Rab1B, Rab4, Rab5, Rab6A, Rab7, Rab8, or Rab11A at 5 μM. Nor could we detect Rab3A binding to the aczonin zinc finger regions (amino acids 374–654 and 863–1115) by SPR, in confirmation of previous blot overlay experiments (21) (data not shown).

Selected Rim1, rabphilin-3, and aczonin constructs were coupled to glutathione-agarose and analyzed for their ability to precipitate native, prenylated Rab3 from brain lysate (Fig. 4A). The results fully confirmed the observations made by SPR with bacterially expressed, unprenylated Rab3. GTP-dependent binding was found with the Rim-(2–55), Rim-(2–228, Δ56–82), and Rim-(9–387, Δ56–82) constructs, irrespective of the presence or absence of the zinc finger and SGAWFF region, but it was abolished by deletion of the acidic cluster (amino acids 45–51) in the C-terminal part of the helix (construct Rim-(2–42)) or by the R33G mutation (Rim-(9–387, R33G)). The low
affinity binding of Rab3 to the short rabphilin-3-(40–88) construct and of Rab6A to Rim1 constructs seen by SPR were not detectable by the pull-down method (Fig. 4A, and data not shown). The long rabphilin-3-(40–178) construct bound much more Rab3 than equal or higher quantities of the Rim1 constructs did, in two independent experiments with different fusion protein preparations (Fig. 4, A and B). This is in agreement with the higher Rab3 affinity of rabphilin-3-(40–178) measured by SPR. There was no detectable Rab3 binding to the aczonin N terminus (positions 2–50) and zinc finger (374–654 and 863–1115) regions (not shown). The zinc finger region of Rim1 binds Munc13 (27), independent of GDP or GTPγS (Fig. 4A) or of detergent choice (Fig. 4, A and D; see below). Munc13 binding to our Rim1 constructs confirms the integrity of their zinc finger structures, which have been shown to be crucial for Munc13-Rim1 binding as mutations of their cysteine residues abolish Munc13 binding (27). The double Cys5 zinc fingers of bassoon, aczonin, Rim, rabphilin-3, and Noc2 constitute a distinct family of zinc fingers ("BARRN fingers") defined by a characteristic cysteine spacing and sequence signature (19, 21, 22). In our pull-down experiments, however, the zinc fingers of rabphilin-3-(40–178) (Fig. 4A), aczonin-(374–654) (Fig. 4D), and aczonin-(863–1115) (not shown), unlike Rim1, did not bind Rab3A (this study), whereas Rim1-(1–50) does (28), suggesting that the acidic cluster beginning at amino acid 45 is an important determinant of the Rab3-binding site of Rim1. Rabphilin-3 sequences that are in close contact with Rab3A in the crystal structure are underlined below the Noc2 sequence. The eight cysteine residues of the double zinc fingers are marked by diamonds.

The GTP-dependent binding of Rab3 to all Rim1 constructs carrying the N-terminal α-helix was sensitive to the choice of detergent in the pull-down assay. It was abolished if 0.5% Triton X-100 was replaced by 1% sodium cholate. In contrast, the strong Rab3 binding to the long rabphilin-3-(40–178) construct was indifferent to detergent choice (Fig. 4, A and B).

In the presence of cholate instead of Triton X-100 and if no guanine nucleotides were added to the lysate, we instead detected binding of Rab3 to all zinc-finger-containing Rim1 constructs, as observed by Betz et al. (27), including Rim-(56–228), which lacks the N-terminal α-helix, and Rim-(9–387, R33G) with the mutated helix, whereas Rim-(2–55) and aczonin-(374–654) did not bind Rab3 under these conditions (Fig. 4C). However, this binding of Rab3 to the Rim1 zinc finger region was not GTP-stimulated, but it was instead inhibited by the addition of either GDP or GTPγS (Fig. 4, B and C). Moreover, Rab6

**TABLE I**

| Rim1 construct | Rab3C binding to Rim1 | Rab3D binding to Rim1 | Rab6A binding to Rim1 |
|----------------|----------------------|----------------------|----------------------|
| Rim-(4–55)     | 1.7 ± 0.3 μM         | 1.7 ± 0.5 μM         | 2.1 ± 0.3 μM         |
| Rim-(2–228, Δ83–105) | 2.0 ± 0.4 μM     | 2.0 ± 0.3 μM         | 2.0 ± 0.4 μM         |
| Rim-(2–228, Δ56–82) | 2.1 ± 0.2 μM     | 2.1 ± 0.2 μM         | 2.1 ± 0.2 μM         |
| Rim-(9–387, Δ56–82) | 1.7 ± 0.2 μM     | 1.7 ± 0.2 μM         | 1.7 ± 0.2 μM         |

\[ K_d = 0.3 \pm 0.2 \mu M \] for Rab3A binding to Rim1 and Rab6A binding to Rim1. The eight cysteine residues of the double zinc fingers are marked by diamonds.

**Fig. 3. Alignment of N-terminal sequence stretches from rat Rim1, mouse aczonin, rat rabphilin-3, and rat Noc2-2.** The alignment illustrates the pairwise sequence relationship between Rim1 and aczonin and between rabphilin-3 and Noc2, respectively. The upper alignment shows the helical regions, and the lower alignment shows the zinc finger regions. The rabphilin-3 and Noc2 sequences are contiguous, whereas Rim1 and aczonin have additional sequences between the two sequence stretches shown (see amino acid numbering on the left). The Rab3-binding site of Rim1 identified in this study (amino acids 19–55) is overlined, and arginine 33 is marked by an asterisk. Rim1-(2–42) does not bind Rab3A (this study), whereas Rim1-(1–50) does (28), suggesting that the acidic cluster beginning at amino acid 45 is an important determinant of the Rab3-binding site of Rim1. Rabphilin-3 sequences that are in close contact with Rab3A in the crystal structure are underlined below the Noc2 sequence. The eight cysteine residues of the double zinc fingers are marked by diamonds.

**Rab3 Binding Determinants of Rim1 and Rabphilin-3**

\[ K_d \text{ values} \]

For Rab3 binding to Rim1 sequences determined by SPR.

The K_d values are means from up to n = 10 independent measurements with Rab3 concentration series as shown in Fig. 2 ± S.D. In all cases with n > 1, measurements were performed with at least two independent preparations of each Rim1 fusion protein, with no significant differences in binding behavior between preparations. Several Rim1 constructs were assayed with two or more independent Rab3A preparations, also with similar results. K_d values of Rab3C, Rab3D, and Rab6A were determined from n = 1 concentration series each. Mutant Rim1 constructs are marked by an asterisk.
Rab3 Binding Determinants of Rim1 and Rabphilin-3

32485

**FIG. 4.** Pull-down of native Rab3 and other proteins from brain lysate with recombinant Rim1 and rabphilin-3 GST fusion proteins. **A**, GTPyS-dependent binding of Rab3 to all Rim1 constructs containing the unmutated N-terminal helix and guanine-nucleotide-independent binding of Munc13 to all Rim1 constructs containing the zinc finger. Rabphilin-(40–178) binds Rab3 much more strongly than do the Rim1 constructs, but it does not bind Munc13. The experiment was performed in standard lysis buffer with 0.5% Triton X-100. Rim1 sequences downstream from amino acid 55 do not bind Rab3 or syntaxin under these conditions. **B**, replacement of Triton X-100 by sodium cholate abolishes GTPyS-dependent binding of Rab3 to the N-terminal helix of Rim1. Rabphilin-(40–178) binds Rab3 in both detergents. Upper panel, lysates in synaptosome extraction buffer with 0.5% Triton X-100 instead of 1% sodium cholate; lower panel, lysate in standard lysis buffer with 1% sodium cholate instead of 0.5% Triton X-100. Comparing also with the result in standard lysis buffer with Triton X-100 shown in A, C, the Rim1 zinc finger region binds Rab3 from brain lysate if cholate is used as detergent; this binding is inhibited by either GDP or GTPyS. The aczonin-(374–654) zinc finger region does not bind Rab3 under these conditions. The experiment was performed in synaptosome extraction buffer. If no nucleotides are added, binding of Rab3 to the Rim1 zinc finger is also detectable in Triton X-100-containing buffers but weaker (not shown). In additional pull-down experiments not shown, free Ca$^{2+}$ and Mg$^{2+}$ concentrations were varied in the standard and synaptosome lysis buffers with little effect on the outcome. Either whole brain or crude synaptosomes lysed in synaptosome extraction buffer produced similar results. Guanine nucleotides and detergent choice were determined as the critical factors for Rab3 binding to the helix versus the zinc finger motifs. D, the same blots as in C were sequentially reprobed with additional antibodies, showing that in cholate buffer (no guanine nucleotides added) Munc13, syntaxin, and additional small G proteins including Ras also bind to the zinc finger-containing Rim1 constructs. Whereas Munc13 binds uniformly to all three zinc-finger constructs, the GTPases bind preferentially and syntaxin binds only to the Rim-(9–387, R33G) sequence. Binding of Ras, Rab5, and even the distant relative, Ras (Fig. 4D), were bound in similar nucleotide-inhibited fashion, although less efficiently than Rab3 in comparison with the respective concentrations in the lysate. Syntaxin was efficiently pulled down by the Rim-(9–387, R33G) construct in cholate buffer (Fig. 4D) but not in Triton buffer (Fig. 4A) and was uninfluenced by guanine nucleotides (not shown). Weak binding of synaptobrevin-2 to Rim-(9–387, R33G), presumably through syntaxin, was also observed (not shown). As a positive control, Munc13 was nucleotide-independently precipitated by all three Rim1 zinc finger constructs with similar efficiencies, whereas the negative controls γ-adaptin, synaptophysin (Fig. 4D), Na/K-ATPase β subunit, and cadherin (not shown) bound to none of the fusion proteins in cholate buffer.

**DISCUSSION**

Rab3-binding Determinants of Rim1 and Rabphilin-3: Specificities and Affinities—We have mapped a binding site on Rim1 for GTP-saturated Rab3A, -C, and -D to a short N-terminal sequence with high α-helix potential. Other sequences within the N-terminal 387 amino acids of Rim1, including the differentially spliced intervals, the zinc finger module, and the SGAWFF motif, did not significantly modify the binding properties of this site in our experimental systems, nor did they bind Rab3-GTP by themselves. Very recently, Sun et al. (28) have identified the same N-terminal motif (amino acids 19–50) as the minimal Rab3-GTP binding site of Rim1 by qualitative pull-down measurements with Rab3A expressed in eukaryotic cells.

In the SPR measurements, the longer Rim1 constructs did show additional, slow or irreversible low affinity components in association and dissociation that are absent from the short constructs with the N-terminal site only (compare the binding curve shapes of Rim-(19–55) versus Rim-(2–228, Δ56–105) and Rim-(9–387, Δ56–105) in Fig. 3). It is possible that the downstream Rim1 sequences subtly modify the Rab3 binding behavior of the N-terminal site. Perhaps more likely, however, these slow components reflect unspecific and partially irreversible interactions that occur under in vitro conditions after Rab3 has first been specifically recruited to the α-helical site. A residual low affinity component is probably responsible for the fact that the $K_d$ values determined on this basis for the long and middle-sized Rim1 constructs are ~ 1.5-fold higher than those of the short constructs, Rim-(19–55) and Rim-(2–55) (Table 1). Also, the inclusion of Rab3 concentrations up to 10 or 20 μM in the evaluation, however, did not influence the results substantially, enhancing $K_d$ values by another factor of 1.5.

This Rab3 binding site of Rim1 has distant sequence similarity with one of the two Rab3 binding determinants, also an N-terminal α-helix, of rabphilin-3. We find that this part of rabphilin-3 can indeed by itself specifically bind Rab3-GTPyS (not shown), Rab5, and even the distant relative, Ras (Fig. 4D), were bound in similar nucleotide-inhibited fashion, although less efficiently than Rab3 in comparison with the respective concentrations in the lysate. Syntaxin was efficiently pulled down by the Rim-(9–387, R33G) construct in cholate buffer (Fig. 4D) but not in Triton buffer (Fig. 4A) and was uninfluenced by guanine nucleotides (not shown). Weak binding of synaptobrevin-2 to Rim-(9–387, R33G), presumably through syntaxin, was also observed (not shown). As a positive control, Munc13 was nucleotide-independently precipitated by all three Rim1 zinc finger constructs with similar efficiencies, whereas the negative controls γ-adaptin, synaptophysin (Fig. 4D), Na/K-ATPase β subunit, and cadherin (not shown) bound to none of the fusion proteins in cholate buffer.
with low affinity ($K_d$ on the order of magnitude of 10–100 μM), detectable by SPR but apparently washed off in pull-down assays. However, the acidic cluster (amino acids 45–51) at the downstream end of the Rab3 binding site of Rim1 has no counterpart in rabphilin-3 (Fig. 3). Instead, for high affinity Rab3 binding, rabphilin-3 requires a second element more distant from the N-terminal α-helix (the SGAWFF motif) plus an intact double zinc finger in between, probably to position the whole arrangement properly. Hence, Rim1 and rabphilin-3 seem to achieve high affinity binding of Rab3-GTP through combinations of two structural motifs. The first determinant, an N-terminal α-helix, is similar in Rim1 and rabphilin-3, whereas the second is different in both proteins: an acidic cluster contiguous with the helical motif in Rim1, but a completely different motif centered around the SGAWFF consensus and separated by a long sequence interval from the N-terminal helix in rabphilin-3. It is remarkable that through these only distantly related binding determinants, Rim1 and rabphilin-3 achieve very similar binding specificities for Rab proteins.

The N-terminal region of aczonin harbors a sequence stretch that is strikingly similar to the Rab3 binding site of Rim1. However, like rabphilin-3, it lacks the acidic cluster, and no binding of Rab3-GTP or a number of other Rab proteins to this aczonin sequence and its surroundings (amino acids 2–80 and 2–218) could be detected in SPR and pull-down experiments. The sequence similarity of this aczonin motif to the Rab3 binding site of Rim1 remains intriguing. It may be needed for the binding of yet another protein at this site. It is also possible that the aczonin N terminus binds Rab3 with the contribution of a more distant second determinant, in analogy to rabphilin-3.

The sequence similarity of this aczonin motif to the Rab3 binding site of Rim1. Instead, for high affinity Rab3 binding, rabphilin-3 achieve very similar binding specificities for Rab proteins. Our SPR measurements are the first determinations of Rab3 interactions with putative effector proteins at near equilibrium and in real time on the time scale of seconds. Previous experiments either employed the two-hybrid system, a semiquantitative technique under undefined molecular conditions, or the pull-down method. The pull-down method is a nonequilibrium technique that measures residual analyte retention after washing of the solid phase, which carries the ligand, with analyte-free buffer. It may therefore miss interactions of low affinity or fast dissociation kinetics that are detected by SPR, like the binding of Rab6A to Rim1 or of Rab3A to rabphilin-3 (40–88) (Figs. 2 and 4A), and it may exaggerate nonspecific or irreversible components of binding. The SPR recordings of Fig. 2 illustrate that the binding components detected in pull-down experiments would correspond approximately to the residual signals at the right-hand ends of the SPR tracings, after 1 min or more of flushing with Rab3-free buffer. The strong binding of Rab3 to the short Rim (2–55) construct in our pull-down experiments (Fig. 4, A and B) was reduced notably by more extensive washing (not shown) and may additionally depend on low temperature (pull-down at 4 °C, SPR at 20 °C). A detailed mechanistic understanding of the interplay of the many protein-protein interactions in neurotransmitter vesicle trafficking clearly requires the quantitative determination of thermodynamic and kinetic parameters by techniques like SPR or fluorescence spectroscopy (31–33).

Compared with $K_d$ values of the binding of other small G proteins to their effectors determined in recent years, Rab3-Rim1 binding is at the lower end, and Rab3-rabphilin-3 binding is at the upper end of the range of affinities (Ha-Ras-Raf-1, $K_d$ = 160 nM (31); Cdc42-WASP, 70 nM (32); Ran-RanBP1, 0.6–3.5 nM (33)). As rate constants for the association and dissociation kinetics of Rab3A-GTP binding to the short Rim1 constructs, Rim-(2–55) and Rim-(19–55), we have determined the following: $k_{on}$, 1.3 ± 0.8 × 10^5 M^{-1} s^{-1}; $k_{off}$, 320 ± 130 × 10^{-3} s^{-1} ($n$ = 21; fits of three binding curves each from seven independent concentration series, five with Rim-(19–55) and two with Rim-(2–55). The on rate constant is similar to the values determined for the Cdc42-WASP and Ran-RanBP1 interactions, whereas the off rate constants are more diverse (Cdc42-WASP: $k_{on}$ = 1.9 × 10^6 M^{-1} s^{-1}; $k_{off}$ = 12–19 × 10^{-3} s^{-1} (32); Ran-RanBP1: $k_{on}$ = 1.5–8.5 × 10^6 M^{-1} s^{-1}; $k_{off}$ = 0.3–1.1 × 10^{-3} s^{-1} (33); Ha-Ras-Raf-1: $k_{on}$ = 450 × 10^5 M^{-1} s^{-1}, $k_{off}$ = 7400 × 10^{-3} s^{-1} (31)).

Complexity of the Rab3-Rabphilin-3 Interaction—The parameters of Rim1-Rab3 binding were well reproducible in our
SPR measurements, in many independent determinations employing multiple constructs and protein preparations (Table 1). In contrast, in five measurements using three different fusion protein preparations, the high affinity component of rabphilin-(40–178) binding to Rab3 was much more variable, ranging from a pronounced contribution as shown in Fig. 2 to virtual disappearance. In the latter case, low affinity binding ($K_d \approx 10 \mu M$) with rapid off rates similar to the rabphilin-(40–88) construct was observed. We suppose that the 10 nM component is representative of the Rab3 binding behavior of native rabphilin-3, in accordance with the other evidence for the high affinity of this interaction discussed above. The complicated folding of the α-helix, zinc finger, and SGAWFF motifs of rabphilin-3 around Rab3 that is required for optimal binding may be easily perturbed and achieved only by variable proportions of the bacterially expressed, recombinant rabphilin-3-(40–178) protein molecules, while the remaining molecules display only the low affinity component conferred by the helix motif alone. Because of its rapid dissociation kinetics, this low affinity component is detected only by a real time technique like SPR, whereas a pull-down assay visualizes only the high affinity component while the low affinity component is washed off. Pull-down conditions (low temperature, long time, the presence of additional proteins) may also permit more recombinant rabphilin-3 molecules to assume the high affinity fold around Rab3. In contrast, the simple, contiguous Rab3-binding determinant of Rim1 appears to be more robust in vitro.

Lability of the Rab3-binding region of rabphilin-3 under experimental conditions is also indicated by the observations of Ostermeier and Brünger (23), whose rabphilin-3 constructs were prone to aggregation and degradation, which was overcome by two-plasmid co-expression with Rab3A, leading to a stable, crystallizable complex. It may be better to express this part of rabphilin-3 and similarly Noc2 (see below) in eukaryotic cells for in vitro studies. However, data from Rab3A knockout mice show that even native rabphilin-3 in vivo is of reduced stability in the absence of Rab3A (8).

The delicacy of the rabphilin-3-type interaction with Rab3 may also explain the divergent observations regarding Rab3 binding to Noc2. Noc2 is a protein of endocrine cells that has high sequence similarity to the N-terminal region of rabphilin-3 (Fig. 3) but lacks the C2 domain region. Whereas two laboratories have failed to detect Rab3A binding to Noc2 (Refs. 16 and 34; no technical details were reported), Burgoyne and colleagues (35) recently described GTP-dependent binding of bacterially expressed Rab3A and of native Rab3A from tissue lysates to recombinant Noc2 in pull-down experiments, with a $K_d$ of $\approx 500 \text{ nM}$. This binding was completely abolished by mutation of the SGAWFF motif. The high sequence similarity of the Rab3-binding region of Noc2 to rabphilin-3, much higher than to Rim1 (Fig. 3), and the importance of the SGAWFF motif suggest that Noc2-Rab3 binding probably involves a similarly intricate and labile three-dimensional arrangement as rabphilin-3-Rab3 binding and is difficult to reproduce with bacterially expressed proteins in vitro.

The SPR data indicating that rabphilin-3 interaction with Rab3 is composed of a fast, low affinity component mediated by the N-terminal helix alone and a slow, high affinity component that in addition requires the zinc finger and SGAWFF motifs may be worth pursuing in more detail. They suggest a way by which binding of Rab3 triggers a conformational transition in rabphilin-3 that may be the next step in its effector mechanism and in which the zinc finger may play a critical role. Interestingly, a two-step mechanism involving an initial unstable complex has also been described for the interaction between Ha-Ras and Raf-1 (31).

Additional Rim1-binding Proteins—The GTP-dependent binding of Rab6A to Rim1 is a novel observation. Several other Rabs (Rab4B, Rab5, Rab7, Rab17, and Rab22) were previously tested for interaction with Rim1 in a two-hybrid assay with negative outcome, but Rab6A was not among them (19). Rab6A binding to Rim1 is 1 order of magnitude weaker than the binding of Rab3, and its biological relevance is unclear. From experiments in nonneuronal cell lines and membrane preparations, Rab6 is implicated in intra-Golgi and retrograde Golgi-to-ER membrane traffic events (Ref. 36 and references therein), but it has also been found in association with secretory granules of platelets and atrial myocytes (37, 38). In hypothalamic neurons in culture, Rab6 was found in association with synaptophysin-containing post-Golgi membranes, concentrated transiently in many axonal varicosities during an early stage of synapse formation, and was observed in association with synaptic vesicles in a small proportion of differentiated synapses also at later culture times (39). It is therefore conceivable that Rim1 functionally interacts with Rab6A, particularly during synaptogenesis and/or in extrasynaptic locations along the biosynthetic pathway. Rab6A did not bind to the rabphilin-3 and aczonin constructs in our SPR experiments.

Like rabphilin-3, the α-helical Rim-(19–55) site binds both bacterially expressed Rab3 and native Rab3 from brain lysate, indicating that prenylation of the C terminus of Rab3 is not critical for this interaction. However, Rab3 interactions with the Rim1 N-terminal region in brain lysate pull-down experiments were sensitive to detergent choice. Rab3-GTP bound well to the Rim1 helical site in the presence of Triton X-100 but not in the presence of cholate, whereas it bound well to rabphilin-3-(40–178) in the presence of either detergent (Fig. 4B). Also, Betz et al. (27) detected only marginal Rab3 binding to the N-terminal Rim1 helix in the presence of cholate. This observation is additional evidence that Rab3 binding to Rim1 is weaker than to rabphilin-3 and therefore more sensitive to interference and/or that Rab3 interaction with these two effector proteins involves partially distinct molecular determinants affected differentially by detergents.

In the presence of cholate, we instead observed binding of Rab3 from brain lysate to the Rim1 zinc finger region, as also found by Betz et al. (27), which was undetectable either in the presence of Triton X-100 or by SPR with unprenylated Rab3. Importantly, this Rab3 binding to the Rim1 zinc finger region was strongest if no guanine nucleotides were added to the brain lysate, whereas either GDP or GTPγS inhibited it (Fig. 4C). Moreover, also Rab5, Rab6, and even Ras bound to the Rim1 zinc finger region in the presence of cholate. This mode of binding is clearly distinct from the effector-type, GTP-activated, direct, Rab3-specific binding to the helical site. If not unspecific, it is probably prenylation-dependent; indirect or cooperative, requiring another protein from the brain lysate that is absent in the SPR measurements; or both. PRA1, for example, a binding partner of the aczonin zinc fingers (22), binds multiple Rab proteins including Rab3 only if they are prenylated and independently of GTP or GDP (40, 41). Inhibition by both GDP and GTP is characteristic for G protein binding to guanine nucleotide exchange factors, some of which also display partial G protein promiscuity and prenylation dependence (e.g. Refs. 42 and 43). Of note, a binding site for a cAMP-dependent guanine nucleotide exchange factor acting on Rap1A and -B but not on Rab3A has been localized to amino acids 530–806 of Rim1 (29).

Also syntaxin bound to recombinant Rim1 in cholate but not in Triton X-100 lysate. Even more than Rab3 but unlike Munc13, syntaxin preferred the longest construct, Rim-(9–387, R33G) over the shorter sequences (Fig. 4D). This suggests that
the differentially spliced and downstream Rim1 sequences contribute to the binding of Rab3 and syntaxin but not of Munc13 in this type of experiment and rules out that syntaxin binding to Rim-(9–387, R33G) is mediated exclusively by Munc13 (26). These observations of Fig. 4, C and D, binding of syntaxin and of native Rab3 not stimulated by GTP to Rim1 sequences downstream from amino acid 55 in cholate buffer, reach beyond the scope of the present study (i.e., the characterization of the GTP-dependent Rab3 binding domain of Rim1). Having discriminated Rab3 binding to the zinc finger region in cholate from the effector-type binding of Rab3 to the N-terminal α-helix, we did not pursue them further here, and we cannot yet decide whether they are functionally meaningful or unspecific. However, given the importance of both binding partners in synaptic vesicle dynamics, their apparent binding to the Rim1 zinc finger region may deserve further investigation.

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