Munc18-1 is a neuronal protein that interacts with syntaxin 1 and is required for synaptic vesicle exocytosis. We have now identified two Munc18-1-interacting proteins called Mint1 and Mint2 that may mediate the function of Munc18-1. Mint proteins are detectable only in brain and are composed of an N-terminal region that binds Munc18-1, a middle phosphotyrosine-binding domain, and two C-terminal PDZ domains thought to attract proteins to the plasma membrane. In brain, Mint proteins are part of a multimeric complex containing Munc18-1 and syntaxin that likely functions as an intermediate in synaptic vesicle docking/fusion. The phosphotyrosine-binding domain specifically binds to phosphatidylinositol phosphates known to be produced during vesicle exocytosis (Hay, J. C., Fisette, P. L., Jenkins, G. H., Fukami, K., Takonawa, T., Anderson, R. A., and Martin, T. F. J. (1985) Nature 374, 173–177). Our data suggest a model whereby local production of phosphatidylinositol phosphates may trigger the binding of vesicles to the active zone via the Mint-Munc18-1 complex in conjunction with syntaxin 1.

Exocytosis is a universal process in eukaryotes with many functions. At the synapse, exocytosis of synaptic vesicles releases neurotransmitters and constitutes the first step in synaptic transmission. Synaptic vesicle exocytosis starts with the docking of the vesicles at the active zone. Docked vesicles are then primed for Ca\(^{2+}\) in a complex reaction that may involve partial fusion of the vesicles. Finally, Ca\(^{2+}\) rapidly triggers the release of neurotransmitters with a latency of a few hundred microseconds. Although several proteins that function in synaptic vesicle exocytosis have been identified, the mechanisms of docking and fusion are still incompletely understood (for reviews, see Refs. 1–4).

Munc18-1 is a 65-kDa protein originally identified as the major brain protein that binds to syntaxin 1, a synaptic vesicle fusion protein (5). Munc18-1 belongs to a family of membrane trafficking proteins related to the yeast sec1, sly1, and sfp1 genes. Mammals express three highly homologous isoforms of Munc18. Munc18-1 is enriched in neurons, whereas Munc18-2 and Munc18-3 are expressed ubiquitously (alternative names: Munc18-1 = rbsec1 and nsec1, Munc18-2 = Munc18B, and Munc18-3 = Munc18c) (5–9). Knockouts of Munc18-1 demonstrated that it is essential for synaptic vesicle exocytosis. In addition to binding strongly to syntaxin 1, Munc18-1 binds to Doc2a and Doc2b, C2 domain proteins that are associated peripherally with synaptic vesicles (10). Since the interactions of Munc18-1 seem insufficient to explain its function, we have now searched for additional binding proteins for Munc18-1. We have identified a family of Munc18-interacting proteins named Mint proteins with unusual properties, suggesting a new model for the docking and priming reactions that initiate exocytosis.

**Materials and Methods**

**Yeast Two-hybrid Screens**—We screened a cDNA library constructed from poly(A)\(^{+}\)-enriched rat brain RNA from postnatal day 8 with a bait vector encoding full-length Munc18-1 fused to LexA (pBTMMunc18-1-1) (11, 12). Of >1000 positive clones obtained from 2.8 \(\times\) 10\(^6\) yeast colonies, 100 clones were rescued, transformed into fresh L40 yeast cells, and confirmed by growth on plates lacking histidine and by activation of \(\beta\)-galactosidase. 55 of the 100 clones were then further analyzed by cotransformation with an irrelevant bait and by sequencing. 5 clones were lost, 10 clones exhibited only weak activation of \(\beta\)-galactosidase and were not analyzed further, 22 clones encoded Mint1 and 7 clones encoded Mint2 (see Fig. 1; several clones were isolated multiple times independently), 3 clones encoded PSD95, 2 clones encoded syntaxin 5, 2 clones encoded a kinesin-related transcript, and 4 clones were not identified in the data banks.

**cDNA Cloning, Sequencing, and Sequence Analysis**—Rat brain cDNA libraries in AAZPII (Stratagene) were screened with random primed DNA probes as described (13). The following human Mint cDNAs were obtained as expressed sequence tag clones from Research Genetics (Huntsville, AL): human Mint1, 279624; and human Mint2, 183680, 328119, 188448, and 139567. DNA sequencing was performed by the dideoxy nucleotide chain termination method using fluorescently labeled primers and an ABI370A DNA sequencer. The nucleotide sequences of the cDNA clones were deposited in the GenBank\(^{TM}\)/EMBL Data Bank.

**Vector Construction and Protein Expression**—Plasmids were constructed in pVP16-3, pLexA, and pBTM116 for use as yeast two-hybrid prey and bait vectors, respectively; in pGEX-KG for expression as GST\(^2\) fusion proteins; in pCMV5 or pME18sf(\(^2\))-Mint1 (full-length) for expression in COS cells; and in pMAL-C2 for expression as maltose-binding protein fusion proteins. The following plasmids were used (listed by proteins; numbers indicate encoded residue numbers). Mint1: pGEX-Mint1/MID, residues 189–471; pGEX-Mint1 (full-length), residues 1–839; pGEX-Mint1/PTB, residues 450–620; pCMV-Mint1-N-term and pME18sf(\(^2\))-Mint1-N-term, residues 1–504; pCMV-Mint1 and pME18sf(\(^2\))-Mint1 (full-length), residues 1–839; pPreyMint1-1, residues 1–504; pPreyMint1-2, residues 26–551; pPreyMint1-3, residues 71–439; pPreyMint1-4, residues 93–836; pPreyMint1-5, residues 118–432; pPreyMint1-6, residues 129–314; pPreyMint1-7, residues 129–754; pPreyMint1-8, residues 185–839; pPreyMint1-9, residues 189–471; pPreyMint1-10, residues 192–615; pPreyMint1-11, residues 197–470; and pPreyMint1-12, residues 197–470.

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1 M. Verhage and T. C. Südhof, unpublished data.
2 The abbreviations used are: GST, glutathione S-transferase; PTB domain, phosphotyrosine-binding domain; CHAPS, 3-(3-cholamidopropyl)dimethylammonio-1-propanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; PIP, phosphatidylinositol phosphate; PIP\(_2\), phosphatidylinositol bisphosphate.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank\(^{TM}\)/EBI Data Bank with accession number(s)
AF029105–AF029108.

\(^{\dagger}\) Supported by postdoctoral fellowships from the HFSP, the TOYOBIO Biotechnology Foundation, and the Takeda Medical Foundation.

\(^{\S}\) To whom correspondence should be addressed. E-mail: TSudho@Mednet.SWMED.edu.
Mints as Munc18-binding Proteins—

Affinity Chromatography on Immobilized GST Fusion Proteins—Six frozen rat brains were homogenized in 15 ml of 10 mM HEPES-NaOH, pH 7.4, and 100 mg/10 ml phenylmethylsulfonyl fluoride. 15 ml of 10 mM HEPES-NaOH, pH 7.4, 0.2 mM NaCl, 2.5 mM MgCl2, and 5 mM EDTA were mixed in the appropriate buffer (50 mM Tris-HCl, pH 7.4, and 0.1 M NaCl; kept at 4 °C as a stock solution; and used within 2 weeks. For binding assays, 25 μg of GST fusion proteins (GST-Mint1/MID, 5 mg of GST-Mint2/MID, 5 mg of GST, or 7 mg of GST-Munc18-1) were pre-equilibrated with core buffer (50 mM Tris-HCl, pH 8.0, 0.1 mM NaCl, and 2.5 mM MgCl2) containing 2.5 mM CaCl2 and 0.25% Triton X-100. The total brain extract was solubilized with Triton X-100 to columns containing GST-Mint fusion proteins or control GST proteins. We applied total rat brain homogenates to immobilized GST-Mint fusion proteins or recombinant proteins to immobilized GST-Mint fusion proteins or control GST proteins. We applied total rat brain homogenates solubilized with Triton X-100 to columns containing GST-Mint1, GST-Mint2, and GST and eluted bound proteins with high salt after extensive washing. Analysis of the eluates by SDS-PAGE followed by Coomassie Blue staining and immunoblotting revealed that we purified Munc18-1 on both Mint columns as the major component and syntaxin 1 as a minor component (Fig. 2 and data not shown).}

Protein Binding of Mint Proteins and Munc18-1—Because yeast two-hybrid assays are prone to artifacts, we tested the interaction between Munc18-1 and Mint proteins by independent methods. For this purpose, we performed affinity chromatography experiments by binding rat brain proteins or recombinant proteins to immobilized GST-Mint fusion proteins or control GST proteins. We applied total rat brain homogenates solubilized with Triton X-100 to columns containing GST-Mint1, GST-Mint2, and GST and eluted bound proteins with high salt after extensive washing. Analysis of the eluates by SDS-PAGE followed by Coomassie Blue staining and immunoblotting revealed that we purified Munc18-1 on both Mint columns as the major component and syntaxin 1 as a minor component (Fig. 2 and data not shown). Control GST columns were unable to enrich either protein. To ensure that the column purification of Munc18-1 and syntaxin 1 on immobilized Mint proteins was specific, we analyzed the eluates by immunoblotting for a number of known synaptic proteins (data not shown).

RESULTS

Identification of Mint Proteins—We screened a rat brain cDNA library by yeast two-hybrid selection with full-length Munc18-1, a protein of 594 amino acids. Among 40 strongly positive clones, 29 clones were independent isolates of two closely related cDNAs (Fig. 1). The fact that we isolated multiple overlapping clones from two homologous proteins suggests that Munc18-1 specifically interacts with these proteins, which we therefore named Mint1 and Mint2. Two observations provided further support for the specificity of the Mint/Munc18-1 interaction. First, we did not isolate Mint clones in large yeast two-hybrid screens with several other baits. Second, we detected no interaction in yeast two-hybrid assays of Mint proteins with other proteins, such as Munc18-2, syntaxin, SNAP-25, synaptotagmin, and rabphilin (data not shown). Thus, Mint1 and Mint2 specifically bind to Munc18-1, but not to Munc18-2 or other trafficking proteins in yeast two-hybrid assays.

Since the yeast two-hybrid clones did not contain the complete coding sequence of Mint1 or Mint2, we isolated their full-length cDNAs. Their sequences revealed that Mint proteins have a multidomain structure composed of variable N-terminal and conserved C-terminal regions (Fig. 1F). Inspection of the different Mint prey clones localized the Munc18-1-interacting domain to the N-terminal region. The C-terminal sequences of Mint proteins are composed of a PTB domain and two PDZ domains. PTB domains from several proteins bind phosphotyrosine-containing peptides and PIPs (15–17). PDZ domains are found in peripheral proteins of the plasma membrane, where they often bind to the cytoplasmic tails of intrinsic membrane proteins (18, 19). Partial sequences of human Mint1 and mouse Mint2 were identified previously by positional cloning as candidate genes for Friedreich's ataxia, although they were later shown not to be involved in the disease (20, 21). The genes were designated as human X11 and mouse X11 with the notion that they represent orthologs. Our full-length sequences of both proteins from a single species, however, demonstrate that they are isoforms. Data bank searches revealed several Caenorhabditis elegans sequences corresponding to Mint proteins, suggesting that Mint proteins are conserved in invertebrates (data not shown).

Protein Binding of Mint Proteins and Munc18-1—Because yeast two-hybrid assays are prone to artifacts, we tested the interaction between Munc18-1 and Mint proteins by independent methods. For this purpose, we performed affinity chromatography experiments by binding rat brain proteins or recombinant proteins to immobilized GST-Munc18 fusion proteins or control GST proteins. We applied total rat brain homogenates solubilized with Triton X-100 to columns containing GST-Mint1, GST-Mint2, and GST and eluted bound proteins with high salt after extensive washing. Analysis of the eluates by SDS-PAGE followed by Coomassie Blue staining and immunoblotting revealed that we purified Munc18-1 on both Mint columns as the major component and syntaxin 1 as a minor component (Fig. 2 and data not shown). Control GST columns were unable to enrich either protein. To ensure that the column purification of Munc18-1 and syntaxin 1 on immobilized Mint proteins was specific, we analyzed the eluates by immunoblotting for a number of known synaptic proteins (data not shown).

Only Munc18-1 and syntaxin 1 were eluted. Control GST fusion proteins did not retain Munc18-1 or syntaxin 1 (data not shown). Thus, Munc18-1 and syntaxin 1 are purified on immobilized Mint proteins in a single step by a specific interaction. To ensure that Mint proteins and Munc18-1 also interact in brain, we performed immunoprecipitations with Mint1 and Mint2 antibodies from rat brain homogenates. Both revealed co-immunoprecipitation of Mint1 with Munc18-1 and syntaxin 1 (Fig. 3). Thus, three methods, yeast two-hybrid selection, affinity chromatography, and immunoprecipitation, show that Mint proteins are binding partners for Munc18-1.

These data demonstrate that syntaxin 1 and Munc18-1 are in a complex with Mint proteins, but do not tell us whether syntaxin 1 and Munc18-1 bind independently to Mint proteins or whether only one of the two proteins binds to Mint proteins...
and the other is purified piggyback on the first. To distinguish between these two possibilities, we expressed Munc18-1, syntapsin 1, and SNAP-25 individually in COS cells and tested their binding to Mint proteins singly or in combination (Fig. 4). Munc18-1 alone bound to Mint proteins. Syntaxin 1 bound only if Munc18-1 was also added, and SNAP-25 did not bind under any condition. These results agree well with the absence of an interaction of Mint proteins with syntaxin 1 in yeast two-hybrid assays (see above) and suggest that syntaxin 1 interacts indirectly with Mint proteins via Munc18-1.

Tissue Distribution and Subcellular Localization of Mint Proteins—We hybridized RNA blots from multiple rat tissues with Mint probes at high stringency. In agreement with in situ hybridization data on X11 (20), the mRNAs for both Mint proteins are shown on top and correspond to the following residues of Mint1: MID, Munc18-1-interacting domain, residues 226–314 of Mint1 as the minimal Munc18-1-interacting sequence tested; PTB, phosphotyrosine-binding domain, residues 451–622; and PDZa and PDZb, PDZ domains extending from residues 659–744 and residues 749–825 of Mint1, respectively. The locations of the prey clones form the Munc18-1 yeast two-hybrid screen are indicated below the domain structures.

Mints as Munc18-binding Proteins
proteins and Munc18-1 are not components of synaptic vesicles. Mint proteins were barely detectable. These data indicate that Mint proteins are primarily a plasma membrane protein, in addition to being presented on synaptic vesicles (22, 23). Thus, the Mint family of proteins may prefer PIP2, suggesting that Mint proteins bind to PIP2. To test this, we measured the binding of \(^{3}H\)-labeled liposomes composed of different phospholipids to GST-Mint1 and to control GST fusion proteins (Fig. 6). PIP and PIP2 bound specifically to the PTB domain of Mint1, but not to other membrane-trafficking proteins, Mint proteins have an unusual composition since they contain PTB and PDZ domains. Recent studies in permeabilized PC12 cells revealed that phosphatidylinositol-4-phosphate 5-kinase, an enzyme that generates PIP and PIP2, is required for exocytosis (24). Since the PTB domain of Shc binds PIP and PIP2 (25), we investigated the possibility that the PTB domain of Mint proteins may bind PIP2. To test this, we studied the binding of \(^{3}H\)-labeled liposomes composed of different phospholipids to GST-Mint1 and to control GST fusion proteins (Fig. 6). PIP and PIP2, but not other membrane-trafficking proteins, Mint proteins may prefer PIP2 in vivo.

**DISCUSSION**

Exocytosis of synaptic vesicles starts with the docking of vesicles, proceeds by priming the vesicles for \(Ca^{2+}\)-triggered release, and finishes with the release of neurotransmitters in a final \(Ca^{2+}\)-dependent reaction. A number of proteins are known to function in priming and/or \(Ca^{2+}\)-dependent release (e.g., CAPS, syntaxin 1, SNAP-25, synaptobrevin, synaptotagmin 1, Munc18-1, and Rab3), but few proteins involved in docking of vesicles have been described (1–4).

We have now identified a family of proteins called Mint proteins that interact with Munc18-1, a protein essential for synaptic vesicle exocytosis. The following evidence supports the notion that Mint proteins bind to Munc18-1 with high
affinity. 1) Mint1 and Mint2 are coexpressed with Munc18-1 and are present at significant levels only in neurons. 2) Mint proteins specifically interact only with Munc18-1 in yeast two-hybrid assays. 3) Affinity chromatography of rat brain proteins on immobilized Mint proteins leads to the purification of Munc18-1 to near homogeneity. 4) Immunoprecipitations show that Munc18-1 and Mint proteins are co-immunoprecipitated. Together, these data suggest that in brain, munc18-1 and Mint proteins form a complex. Since the affinity chromatography experiments and immunoprecipitations also purified syntaxin 1 that binds to Munc18-1 but not to Mint proteins, it is likely that brain contains a complex of Munc18-1, syntaxin 1, and Mint proteins. Although Doc2 proteins also interact with Munc18-1 (10), Doc2 proteins are not copurified with Munc18-1 in the affinity chromatography experiments because the Doc2 interaction is relatively weak compared with the high affinity interaction of Mint proteins. Since both syntaxin 1 and Munc18-1 are essential for vesicle exocytosis, it seems likely that Mint proteins also function in exocytosis.

Mint proteins have an unusual structure that includes domains not previously identified in vesicular trafficking proteins, suggesting a novel function. The PTB and PDZ domains in Mint proteins provide them with the potential to unite signal transduction (PTB domain), localization to intercellular junctions (PDZ domains), and vesicular membrane traffic (Munc18-1 binding). The presence of a PTB domain suggests a connection between neurotransmitter release and tyrosine phosphorylation and/or PIPs. Binding to phosphotyrosines could play a potential role in synaptic plasticity (26). We showed that the PTB domain of Mint1 binds to PIPs in a specific reaction, an activity that could be important in view of the transient generation of PIPs during exocytosis (24). Finally, the presence of PDZ domains in Mint proteins indicates a possible role for Mint proteins in connecting synaptic vesicles to the sites of synaptic intercellular junctions. PDZ domains are known in other proteins to localize peripheral proteins to intercellular junctions (17, 18) and may have an analogous function in Mint proteins.

Based on the properties of Mint proteins, we would like to propose a two-stage model for synaptic vesicle exocytosis that is meant as a framework for future experiments. In the first stage (docking), we propose that the Mint\textsuperscript{z}Munc18-1 complex provides a linkage between the plasma membrane and synaptic vesicles that docks the vesicles. Mint proteins could bind synaptic vesicles via their interaction with PIPs, and Munc18-1 could bind vesicles via its interaction with Doc2 proteins (10). The association of Mint proteins with the plasma membrane could be mediated by the binding of its PDZ domains to the cytoplasmic tails of membrane proteins (17, 18). In the second stage (priming), the Mint-Munc18-1 complex delivers the docked vesicles to the syntaxin 1-SNAP-25 complex by a direct interaction. As a result, synaptobrevin/vesicle-associated mem-

**FIG. 5.** Absence of the Mint-Munc18-1-syntaxin 1 complex in synaptic vesicles. Aliquots of total rat brain homogenate (left lanes) or of purified synaptic vesicles (right lanes) were immunoblotted for the indicated proteins. The lower molecular mass band in the Mint2 blot (asterisk) probably corresponds to a breakdown product of this proteolytically sensitive protein.

**FIG. 6.** Binding of PIP\textsubscript{2} to the PTB domain of Mint proteins. GST fusion proteins of the PTB domain of Mint1 (GST-PTB1), complex 1 (GST-CPX1), and SNAP-25 (GST-SNAP25) were immobilized on glutathione-agarose and incubated with radiolabeled liposomes in a Ca\textsuperscript{2+}-free buffer. Bound liposomes were quantified after extensive washing by scintillation counting. The composition of the liposomes is listed below the bar diagram in mole percent. Experiments were performed multiple times with similar results; the graph shows results ± S.E. from triplicate determinations. PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol.
brane protein on the vesicles binds to the syntaxin 1-SNAP-25 complex to form the core complex during membrane fusion.

The model that we propose is highly speculative at present, but would provide potential explanations for two intriguing observations. First, Hay et al. (24) demonstrated an essential role for PIPs in Ca$^{2+}$-regulated exocytosis from PC12 cells, suggesting that PIPs are important in exocytosis. A vesicle-docking mechanism mediated by binding of Mint proteins to PIPs is attractive because the transient production of PIPs could provide a highly localized signal for docking. Second, the so-called SNAREs synaptobrevin-vesicle-associated membrane protein, SNAP-25, and syntaxin were shown to be essential for exocytosis, but not for the attachment of vesicles to the active zone (docking) (27–29). Our model explains why docking does not require syntaxin because we propose that docking is mediated by the binding of Mint proteins to the plasma membrane probably via its PDZ domains and by the binding of Mint proteins and Munc18-1 to synaptic vesicles via PIPs and Doc2 proteins. The proposed model also offers a potential explanation for how membrane traffic could be targeted to intercellular junctions by suggesting a role for PDZ domains in exocytosis. In view of the widespread occurrence of Munc18 homologues in cell types other than neurons, the model may also be applicable, at least in part, to the general process of exocytosis in non-neuronal cells.

Acknowledgments—We thank I. Leznicki, E. Borowicz, and A. Roth for excellent technical assistance; Dr. Stan Hollenberg for plasmid pVP16, yeast strain L40, and invaluable advice; Drs. P. Bartel and S. FIELDS for plasmid pBTM116; Dr. R. Jahn for purified synaptic vesicles and antibodies; and Dr. R. H. SCHELLER for syntaxin cDNA clones.

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