Mints as Adaptors

DIRECT BINDING TO NEUREXINS AND RECRUITMENT OF Munc18*

Received for publication, September 19, 2000, and in revised form, October 11, 2000
Published, JBC Papers in Press, October 17, 2000,
DOI 10.1074/jbc.C000656200

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Mint1 (X11/human Lin-10) and Mint2 are neuronal adaptor proteins that bind to Munc18–1 (n/rb-secl), a protein essential for synaptic vesicle exocytosis. Mint1 has previously been characterized in a complex with CASK, another adaptor protein that in turn interacts with neurexins. Neurexins are neuron-specific cell surface proteins that act as receptors for the excitatory neurotoxin α-latrotoxin. Hence, one possible function for Mint1 is to mediate the recruitment of Munc18 to neurexins. In agreement with this hypothesis, we now show that the cytoplasmic tail of neurexins captures Munc18 via a multiprotein complex that involves Mint1. Furthermore, we demonstrate that both Mint1 and Mint2 can directly bind to neurexins in a PDZ domain-mediated interaction. Various Mint and/or CASK-containing complexes can be assembled on neurexins, and we demonstrate that Mint1 can bind to Munc18 and CASK simultaneously. Our data support a model whereby one of the functions of Mints is to localize the vesicle fusion protein Munc18 to those sites at the plasma membrane that are defined by neurexins, presumably in the vicinity of points of exocytosis.

Mints, CASKs, and Velis are multidomain proteins that probably function as molecular adaptors in a number of biological processes. They contain PDZ domains, which are protein-protein interaction modules that often bind to the extreme C termini of target proteins (1). Vertebrates contain three Mint genes, one CASK gene, and three Velis genes (2–5). Mints 1 and 2 are specifically expressed in the nervous system and bind to Munc18–1, a secl-like protein that is essential for neurotransmitter release (2, 6). Mint1 is evolutionary conserved, and the Caenorhabditis elegans lin-10 gene is a Mint1 homolog (7). Mints have been identified in different organisms and biological contexts, leading to various names (Table I). CASK was identified as a novel MAGUK (membrane-associated guanylate kinase domain-containing protein) that binds to the C-terminal cytoplasmic tail of neurexins, neuron-specific cell surface proteins (4). Neurexins were initially purified as receptors for α-latrotoxin, a neurotoxin that triggers massive exocytosis of synaptic vesicles, and function as heterophilic cell-adhesion molecules (12, 13). Independently, CASK was discovered in C. elegans as the protein encoded by the lin-2 gene, which collaborates with lin-10 and a third gene, lin-7, in vulva development (14, 15). Finally, the three widely expressed Velis genes are the vertebrate homologs of the C. elegans lin-7 gene (5). Mints, CASK, and Velis share distinct properties; all are vertebrate homologs of C. elegans genes involved in vulva development and are multidomain proteins that contain one (CASK and Velis) or two (Mints) PDZ domains. In vertebrate brain, CASK binds to Mint1, but not to Mints 2 and 3, and to all three Velis to form a tripartite complex (5). The adaptor proteins assembled into this tight complex contribute not only four PDZ domains but also SH3, phosphotyrosine-binding domain, and guanylate kinase domains (5). Velis also bind to the MAGUKs hDLG2 and hDLG3 (5).

When these multiple PDZ-domain protein complexes form, the net effect is the colocalization of free PDZ domains and other protein-protein interaction domains, ready to coordinate a large number of targets. Among such potential targets are not only the cell-surface proteins neurexins and syndecans, which interact with the PDZ domain of CASK (4, 16), but also calcium channels and amyloid precursor protein, which bind to different domains in Mints (17, 18). In the current study, we have addressed the following questions: First, do Mint1 and Mint2 share similar properties, especially because in brain both Mint1 and Mint2 bind to Munc18? Second, could Mints function to recruit Munc18 to the plasma membrane? Third, do Mints participate in the formation of distinct PDZ domain-protein complexes? We demonstrate that both Mints 1 and 2 bind directly to neurexins and that this interaction is mediated by their PDZ domains. In addition, we show that neurexin-bound CASK can constitute an alternate pathway to specifically recruit Mint1-bound Munc18. Furthermore, we provide evidence that these complex functions to localize Munc18 to neurexin. We propose that various Mint-containing complexes on neurexin could recruit Munc18 to the plasma membrane at sites of exocytosis.

EXPERIMENTAL PROCEDURES

Vectors and Antibodies—The Escherichia coli expression vectors encoding GST1-neurexin I, GST-neurexin I Δ10 (named GST-neurexin I Δ3 in the cited publication), and GST-Munc18–1 have been described previously (2, 19), as are the eukaryotic expression vectors for Mints 1 and 2 and neurexin IΔ (2, 20). Vectors encoding full-length GST-Mint1, GST-CASK, and GFP-Mint1 were a kind gift from A. Maximov (University of Texas Southwestern Medical Center, Dallas, TX). Monoclonal antibodies directed against CASK, Mint1, Mint2, and Munc18 were purchased from Transduction Laboratories (Lexington, KY). Polyclonal anti-neurexin I antibodies were raised against a peptide corresponding to the C-terminal 10 amino acids of the protein. Monoclonal antibodies against syntaxin 1 and GDP dissociation inhibitor (GDI) were a kind gift from R. Jahn (Max-Planck-Institut for Biophysical Chemistry, Göttingen, Germany).

Sample Preparation and Affinity Chromatography—GST fusion proteins were expressed in E. coli and immobilized on glutathione-agarose using standard procedures. Neurexin peptides (sequence NKKRNDDKEYY) and gp41 peptides (sequence WFSITNNLWYI)
were coupled via an added N-terminal cysteine to SulfoLink beads, whereas calcium channel peptides (sequence RHSHHHFDQDHWC) and a point-mutated variant (sequence RHSHHHFDQDWHW) were coupled via their N-terminal amino group to AminoLink beads according to the manufacturer’s instructions (Pierce). Total rat brain extracts were prepared in buffer A (25 mM Hapes, pH 7.4, 125 mM potassium acetate, 5 mM magnesium chloride, 320 mM sucrose, 1.0% Triton X-100). Recombinant full-length Mint1 was expressed as GST fusion, and the GST moiety was cleaved with thrombin (Roche Molecular Biochemicals). Affinity chromatographies were performed at 4 °C in buffer A, which was adjusted to include 1 mg/ml bovine serum albumin for binding of recombinant proteins and to 600 mM potassium acetate for chromatography on immobilized peptide. Binding steps lasted 12–16 h. For differential centrifugation, rat brain was homogenized in buffer A without detergent, and a crude membrane fraction was prepared by pelleting particles larger than 800 S. This supernatant was spun again to pellet particles larger than 30 S to prepare the cytosolic supernatant. Quantitative immunodetection on supernatant was supported by a second line of binding studies, using either membrane or cytosolic proteins prepared from rat brain. CASK was mainly recovered in the membrane pellet (Fig. 1B, lane 2) with only a very small fraction detectable in cytosol (Fig. 1B, lane 5). Mint1 was evenly distributed between membranes and cytosol (Fig. 1B, lanes 2 versus 5). Using both fractions, affinity chromatography on the immobilized cytoplasmic domain of neurexin I was performed (Fig. 1C). Surprisingly, the Mint1 from the cytosolic fraction that lacks CASK bound as efficiently to neurexin I as the membrane-bound fraction of Mint1 (Fig. 1C, lanes 4 versus 6). Likely explanations for these results were either the direct interaction of Mint1 and Mint2 with the cytosolic tail of neurexins or the existence of an unknown functional CASK analog in the cytosol.

**RESULTS AND DISCUSSION**

*Mint1 and Mint2 Interact with the Cytoplasmic Domain of Neurexin I*—Previously, the PDZ domain protein CASK has been described to bind to the extreme C terminus of neurexins (4). As CASK binds Mint1 and Velis, this tripartite complex coats the cytosolic domain of neurexins (5). To study the interaction of the tripartite complex with neurexins in vitro, we solubilized rat brain and performed affinity chromatography on the immobilized cytoplasmic domain of neurexin I (GST-NxI). CASK bound efficiently and in a salt-resistant manner to the C terminus of neurexin I and was enriched in the final eluate along with Mint1 and Velis (Fig. 1A, lane 3 and data not shown). No binding of CASK and Mint1 was observed to a truncated neurexin I lacking the C-terminal 10 amino acids (GST-NxI Δ10) (Fig. 1A, lane 5). Unexpectedly, Mint2 also interacted with the native C terminus of neurexin I (Fig. 1A, lane 3). This was surprising because Mint2, though highly similar to Mint1, does not bind CASK (5). The vesicle trafficking protein Munc18, which interacts with both Mint1 and Mint2, was recovered mainly in the salt eluate of the neurexin I affinity chromatography, albeit at lower yields, together with syntaxin 1 (Fig. 1A, lanes 2 and 3). GDI served as negative control for binding.

CASK-independent Binding of Mint1 to Neurexin I—The CASK-independent binding observed for Mint2 suggested that it might directly interact with the C terminus of neurexin I. Such a property of Mint2 could be shared by Mint1. This notion was supported by a second line of binding studies, using either membrane or cytosolic proteins prepared from rat brain. CASK was mainly recovered in the membrane pellet (Fig. 1B, lane 2) with only a very small fraction detectable in cytosol (Fig. 1B, lane 5). Mint1 was evenly distributed between membranes and cytosol (Fig. 1B, lanes 2 versus 5). Using both fractions, affinity chromatography on the immobilized cytoplasmic domain of neurexin I was performed (Fig. 1C). Surprisingly, the Mint1 from the cytosolic fraction that lacks CASK bound as efficiently to neurexin I as the membrane-bound fraction of Mint1 (Fig. 1C, lanes 4 versus 6). Likely explanations for these results were either the direct interaction of Mint1 and Mint2 with the cytosolic tail of neurexins or the existence of an unknown functional CASK analog in the cytosol.
the human immunodeficiency virus type I, which shares a hydrophobic C terminus with the neurexin peptide, was used as negative control. Mint1 and Mint2 were recovered exclusively on the immobilized neurexin I peptide (Fig. 2B, lane 2).

Our results provide evidence that besides CASK, Mint proteins can be additional direct binding partners of the extreme C termini of neurexins.

Mint1 Is Recruited from the Cytosol to Plasma Membrane-bound Neurexin in Vivo—To confirm this direct interaction with neurexin I, GFP-Mint1 was transfected alone or cotransfected with neurexin Iβ into COS cells, and its intracellular distribution was examined by confocal microscopy. In control cells, GFP-Mint1 was distributed throughout the cytosol similar to GFP (Fig. 3A and data not shown). Upon coexpression of neurexin Iβ, the majority of GFP-Mint1 was detected at the plasma membrane (Fig. 3B), where it colocalized with neurexin Iβ (Fig. 3, C and D). Expression of the plasma membrane protein syntaxin 1, which is not predicted to interact with Mint1, did not alter the cytosolic localization of GFP-Mint1 (data not shown). The almost quantitative recruitment of Mint1 from the cytosol to neurexin Iβ suggests their strong interaction in vivo.

Mint Interaction with the C Terminus of Neurexin I Is PDZ Domain-mediated—Next, we addressed whether direct binding of Mints to neurexins is mediated by their PDZ domains using recombinant proteins expressed in E. coli. We prepared both full-length Mint1 and a mutant that was truncated within the first PDZ domain (Mint1ΔPDZ; Mint1 amino acids 1–688) and therefore contains no functional PDZ domain. Binding of Mint1 and its mutant form to various peptides was quantitated (Fig. 4A). The specific binding of Mint1 to the C-terminal peptide of neurexin was abolished in the ΔPDZ mutant, demonstrating that the interaction was PDZ domain-mediated. Previously, the interaction of Mint1 with the N-type calcium channel α1B subunit has been reported (17). Binding of Mint1 to the C-terminal peptide of this calcium channel was almost as strong as to the neurexin peptide, was mediated by the Mint1 PDZ domains, and the exchange of the terminal amino acid of the peptide from Cys to Thr significantly reduced Mint1 binding (Fig. 4A).

Mixed Complexes of CASK and Mint1 Can Be Formed on Neurexins—Both CASK and Mint1 can bind directly to neurexins and to each other. Therefore, the assembly of various multimeric complexes could proceed as CASK could be indirectly recruited to neurexin-bound Mint1 and vice versa. To test these scenarios, the same molar amounts of recombinant, full-length CASK and Mint1 were bound either alone or in combination to immobilized neurexin I peptide or gp41 peptide as negative control (Fig. 4B). Equal amounts of CASK and Mint1 were specifically recovered on the neurexin peptides, indicating similar binding strengths. Upon coincubation of both CASK and Mint1, the yield approximately doubled for both proteins. A likely explanation is that in this case, each neurexin-bound CASK (or Mint1) provides for an additional binding site for Mint1 (or CASK). Additional experiments confirmed that under saturating conditions, CASK and Mint1 interact with each other in a 1:1 stoichiometry (data not shown).

Interaction of Munc18 with Neurexins—Munc18 is essential for neurotransmitter secretion (6), presumably because of its interaction with syntaxin 1 (21). It binds to the Munc18-interaction domain in the N-terminal region of Mint1, which also contains the interaction domain of Mint1 with CASK (2, 5). As shown in Fig. 1A, Munc18 can be bound to the cytoplasmic tail of neurexins, which indicates that Mints 1 and 2 recruit Munc18 to this plasma membrane protein. Notably, neurexin-bound CASK could also function as an adaptor of Mint1-bound Munc18 and thereby recruit Munc18 to neurexins in an additional pathway. However, the interaction domains of Mint1 with CASK and Munc18 are in close proximity (5), which might obstruct their simultaneous binding required for this CASK-dependent pathway. To resolve this question, two subsequent binding steps were performed on immobilized GST-Munc18. First, recombinant Mint1 was bound to the GST-Munc18-containing beads, and unbound Mint1 was washed out. Subsequently, recombinant CASK was loaded onto this Mint1/GST-Munc18 preparation (Fig. 5A, lanes 1–4). Efficient CASK binding was observed (Fig. 5A, lane 4), demonstrating that Mint1 can indeed bind simultaneously to Munc18 and CASK. In parallel, recombinant CASK was loaded onto directly immobilized GST-Mint1, i.e., in absence of Munc18. As expected, CASK bound to this preparation of uncomplexed Mint1 (Fig. 5A, lane 6) but not directly to GST-Munc18 (Fig. 5A, lane 8). Quantitative analysis showed that the recovery of CASK on Mint1/GST-Munc18 was increased 4 (± 1.4)-fold over the recovery of CASK on GST-Mint1 alone (Fig. 5A and data not shown). Soluble Mint1 bound well to GST-CASK but not to GST alone (data not shown). In summary, the interaction of Mint1 with Munc18 does not interfere with the binding of Mint1 to CASK and could even enhance this binding to CASK. There-
fore, Munc18 is likely to interact not only with Mint1-neurexin complexes but also to be incorporated via Mint1 into CASK-neurexin complexes.

**Different Neurexin Complexes Are Formed and Interact with Munc18**—We report here that Mints 1 and 2 are novel direct binding partners of neurexins and that the Mint-neurexin complexes are as likely as CASK-neurexin complexes to be formed. Distinct neurexin complexes could be assembled in different neuronal compartments or during brain development. We propose that all these neurexin complexes can interact with Munc18. Both Mint1 and Mint2 could function as direct adaptors of Munc18 to neurexins, whereas Mint1 in addition could recruit Munc18 to CASK-neurexin (Fig. 5B). Munc18 is essential for the exocytosis of synaptic vesicles, and the recruitment of Munc18 to the neuronal plasma membrane is likely to be important for its function in neurotransmission. Previously, only the interaction of Munc18 with syntaxin was known to bind Munc18 at the plasma membrane. The analysis described here provides novel evidence that in parallel to syntaxin, neurexin-bound Munc18 could help to localize Munc18 to distinct sites at the plasma membrane. As neurexins have been proposed to function in neuronal cell adhesion, it is possible that they define specific sites at the plasma membrane and that Mint complexes and Mint1-CASK complexes on neurexin are involved in the localized recruitment of Munc18 to the sites of exocytosis. In support of this hypothesis, both CASK and Mint1 have been reported to be localized at synapses (16, 22). Experiments addressing this important question of Munc18 recruitment using knockout animals are in preparation.

Acknowledgments—We thank E. Borowicz, I. Leznicki, and A. Roth for excellent technical assistance. We are grateful to Sreeganga Chandra, Ege Kavalali, and Gyo¨rgy Lionart for comments on the manuscript and to Dr. Anton Maximov for expression constructs.

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**Fig. 4.** The interaction of Mint1 with neurexins is mediated by its PDZ domains and allows the formation of mixed CASK-Mint complexes. *A,* recombinant, full-length Mint and a mutant lacking both PDZ domains (Mint1(G1484E)) were bound to immobilized peptides corresponding to the C terminus of neurexin I, a sequence from gp41, the wild type C terminus of the N-type calcium channel a1B subunit, or this calcium channel peptide mutated in the terminal Cys to Thr. Bound proteins were eluted with SDS and quantitated after Western blotting using radiolabeled secondary antibodies (n = 3). *B,* same molar amounts of recombinant full-length CASK and Mint1 were bound to immobilized neurexin I or gp41. Proteins were incubated either alone or in combination. Bound proteins were analyzed as in A (n = 3).

**Fig. 5.** Interaction with Munc18 increases Mint1 binding to CASK. *A,* a comparison of CASK binding to Munc18-bound Mint1 and to uncomplexed Mint1. Two subsequent affinity chromatography steps were performed on GST-Munc18 (see diagram). First, soluble Mint1 was incubated with bead-bound GST-Munc18 to prepare Mint1/GST-Munc18 complexes (lanes 1 and 2). Subsequently, soluble CASK was loaded onto this Mint1/GST-Munc18 preparation (lanes 3 and 4). In parallel, CASK was incubated with bead-bound GST-Mint1 (lanes 5 and 6). Bead-bound GST-Munc18 alone served as negative control for CASK binding (lanes 7 and 8). Bound proteins were eluted with SDS and quantitated after Western blotting using radiolabeled secondary antibodies. The input lanes (odd numbers) contain 5% of the recombinant proteins used for binding. *B,* model of the predicted neurexin complexes in brain. Mint1 and Mint2 can bind neurexins directly and thereby recruit Munc18 to the plasma membrane. Additionally, CASK-neurexin complexes can recruit Munc18-bound Mint1. M18, Munc18; MI, Munc18 interaction domain; C1, CASK interaction domain.