Munc13 C₂B domain is an activity-dependent Ca²⁺ regulator of synaptic exocytosis

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Munc13 is a multidomain protein present in presynaptic active zones that mediates the priming and plasticity of synaptic vesicle exocytosis, but the mechanisms involved remain unclear. Here we use biophysical, biochemical and electrophysiological approaches to show that the central C₂B domain of Munc13 functions as a Ca²⁺ regulator of short-term synaptic plasticity. The crystal structure of the C₂B domain revealed an unusual Ca²⁺-binding site with an amphipathic α-helix. This configuration confers onto the C₂B domain unique Ca²⁺-dependent phospholipid-binding properties that favor phosphatidylinositol phosphates. A mutation that inactivated Ca²⁺-dependent phospholipid binding to the C₂B domain did not alter neurotransmitter release evoked by isolated action potentials, but it did depress release evoked by action-potential trains. In contrast, a mutation that increased Ca²⁺-dependent phosphatidylinositol bisphosphate binding to the C₂B domain enhanced release evoked by isolated action potentials and by action-potential trains. Our data suggest that, during repeated action potentials, Ca²⁺ and phosphatidylinositol phosphate binding to the Munc13 C₂B domain potentiate synaptic vesicle exocytosis, thereby offsetting synaptic depression induced by vesicle depletion.

Synaptic transmission is initiated when Ca²⁺ influx during an action potential triggers neurotransmitter release. Synaptic transmission is not a constant point-to-point transfer of information from one neuron to the next but, instead, changes as a function of use, rendering synapses elementary computational units of the brain. Many different types of use-dependent synaptic plasticity have been described, among which presynaptic short-term plasticity stands out because it is universally present at synapses and can alter synaptic transmission more than tenfold. Short-term plasticity is of central importance for information processing by the brain; for example, it may underlie working memory formation in the cortex.

At first approximation, presynaptic short-term plasticity results from two opposing processes. Repeated action potentials deplete the readily releasable pool (RRP) of synaptic vesicles, thereby inducing synaptic depression. At the same time, Ca²⁺ influx during repeated action potentials causes accumulation of residual Ca²⁺, thereby inducing synaptic facilitation. As a consequence, a high release probability usually results in synaptic depression, because the RRP becomes depleted, whereas a low release probability usually results in synaptic facilitation, because vesicle depletion is delayed but accumulating residual Ca²⁺ increases the effectiveness of Ca²⁺ in triggering release.

Considerable evidence, however, indicates that presynaptic plasticity is an active, regulated and synapse-specific process that goes beyond a passive response dictated by the release probability and RRP size. For example, RIM1α and Munc13 are proteins of the active zone that interact with each other and form a heterotrimeric complex with the synaptic vesicle protein Rab3 (refs. 5–9). Mutations in each of these three proteins induce changes in short-term synaptic plasticity that cannot be accounted for by corresponding alterations in residual Ca²⁺, release probability or RRP⁷,8,10–14. These and other observations indicate not only that Ca²⁺ triggers release but that, during stimulus trains, the residual Ca²⁺ accumulating between action potentials regulates release by independent mechanisms.

At present, the major Ca²⁺ regulator in short-term synaptic plasticity is thought to be calmodulin. Calmodulin regulates neurotransmitter release by multiple mechanisms, including a direct modulation of Ca²⁺ channels, activation of protein kinases, regulation of synaptic vesicle priming via the cytoskeleton and a process involving binding to the Munc13-1 and Munc13-2 isoforms. Moreover,

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calmodulin acts in presynaptic long-term plasticity by activating adenylate cyclase during mossy-fiber long-term potentiation (LTP)\textsuperscript{20,21}. However, the large variety of types of presynaptic plasticity with distinct spatiotemporal profiles suggests that calmodulin is unlikely to account for all forms of short-term plasticity.

Munc13 and RIM proteins are essential for priming synaptic vesicles and are additionally involved in short- and long-term synaptic plasticity\textsuperscript{8,10}. Three Munc13 isoforms (Munc13-1, Munc13-2 and Munc13-3) are expressed in two principal isoforms called bMunc13-2 and ubMunc13-2(3) function in synaptic vesicle exocytosis\textsuperscript{5,10,22,23}. In addition, two ubiquitously expressed Munc13 isoforms (BAP-3 and Munc13-4) probably act in nonsynaptic forms of exocytosis\textsuperscript{24,25}. Munc13 proteins have variable N-terminal sequences, but they contain similar central and C-terminal domains: a C\textsubscript{α} domain, a large Munc13-homology region (the MUN domain\textsuperscript{133}) and contains an unusual Ca\textsuperscript{2+}-binding module. During Ca\textsuperscript{2+} binding to the Munc13-1 C\textsubscript{α} domain as monitored by \textit{H},\textsuperscript{13}N HSQC spectra. The diagrams show expansions of superpositions of selected \textit{H},\textsuperscript{13}N HSQC spectra acquired during a titration of Ca\textsuperscript{2+} from 0 mM to 0.7 mM. The contours are color coded according to the Ca\textsuperscript{2+} concentration (µM, indicated by the labels next to the contours).

**RESULTS**

The Munc13 C\textsubscript{α}B domain is a Ca\textsuperscript{2+}-binding module

The C\textsubscript{α}B domains of all Munc13 isoforms contain the canonical Ca\textsuperscript{2+}-binding sites of C\textsubscript{2} domains\textsuperscript{28} (Fig. 1a) but show only limited sequence homology to other C\textsubscript{2} domains, hindering prediction of domain boundaries. Thus, we first examined the minimum sequence necessary to obtain an autonomously folded Munc13 C\textsubscript{α}B domain; we identified a C\textsubscript{α}B domain fragment that was soluble and monomeric (residues 675–820 in Munc13-1).

To determine whether the C\textsubscript{α}B domain binds Ca\textsuperscript{2+}, we recorded fluorescence spectra of purified C\textsubscript{α}B domains from Munc13-1 and Munc13-2 in the absence or presence of Ca\textsuperscript{2+} or Mg\textsuperscript{2+} (Fig. 1b and Supplementary Fig. 1). For Munc13-2, we also examined a mutant C\textsubscript{α}B domain in which two canonical aspartates (Asp629 and Asp635) in the presumptive Ca\textsuperscript{2+}-binding sites were replaced (Fig. 1a).

We next acquired \textit{H},\textsuperscript{13}N HSQC spectra of the Munc13-1 C\textsubscript{α}B domain in the absence and presence of Ca\textsuperscript{2+} (Fig. 1c, black and red contours, respectively). Ca\textsuperscript{2+} induced extensive cross-peak changes, as expected for a Ca\textsuperscript{2+}-binding module. During Ca\textsuperscript{2+} titrations, some cross-peaks showed progressive Ca\textsuperscript{2+}-induced shifts (for example, Fig. 1d, upper left corner). Other cross-peaks disappeared during the titration, or shifted to different parts of the spectrum (Fig. 1d, e), suggesting that the exchange between the Ca\textsuperscript{2+}-free and Ca\textsuperscript{2+}-bound states is slow on the NMR timescale. The curved, progressive Ca\textsuperscript{2+}-induced shifts of some cross-peaks, and the differential shifts between cross-peaks (Fig. 1d, e), show that at least two Ca\textsuperscript{2+} ions bind to the C\textsubscript{α}B domain. At a C\textsubscript{α}B domain concentration of 120 µM, no major...
Crystal structure of Ca$^{2+}$-free and Ca$^{2+}$-bound C$_2$B domain

To study how Ca$^{2+}$ binds to the Munc13 C$_2$B domain, we crystallized the Ca$^{2+}$-free and Ca$^{2+}$-bound Munc13-1 C$_2$B domain. The Ca$^{2+}$-free and Ca$^{2+}$-bound C$_2$B domain crystals had distinct space groups (C221 and $P4_2_1_2$, respectively). Using diffraction data and the crystal structure of the synaptotagmin-1 C$_2$A domain$^{29}$ as a search model for molecular replacement, we determined the structure of the Ca$^{2+}$-free Munc13-1 C$_2$B domain to a resolution of 1.90 Å (Table 1). We then used the resulting model for molecular replacement, together with diffraction data, to determine the structure of the Ca$^{2+}$-bound C$_2$B domain to a resolution of 1.37 Å (Fig. 2).

The Ca$^{2+}$-free and Ca$^{2+}$-bound C$_2$B domains contained a typical C$_2$ domain $\beta$-sandwich fold with two four-stranded $\beta$-sheets and a type II C$_2$ domain topology (Fig. 2a,b). Ca$^{2+}$ did not cause major changes in the $\beta$-sandwich, as described for synaptotagmin-1 C$_2$ domains$^{29,31,32}$, but it did induce large changes in the top loops. In the Ca$^{2+}$-free C$_2$B domain, substantial parts of loops 1, 3 and 4 showed little electron density, indicating that the Ca$^{2+}$-free loops are disordered (Fig. 2b). In the Ca$^{2+}$-bound C$_2$B domain, however, we observed well-defined electron densities for all four top loops (Fig. 2a,b), for two bound Ca$^{2+}$ ions and for multiple water molecules (partly shown in Fig. 2d). The Ca$^{2+}$-binding region is probably stabilized by Ca$^{2+}$ binding, similar to the synaptotagmin-1 C$_2$ domains$^{31,32}$. In addition, the top loops in the Ca$^{2+}$-bound C$_2$B domain structure were involved in extensive crystal contacts that may have contributed to the stabilization of the top loops.

Ca$^{2+}$-binding mode of the Munc13 C$_2$B domain

C$_2$ domains commonly bind two or three Ca$^{2+}$ ions through five conserved aspartate side chains from loop 1 (Asp1 and Asp2) and loop 3 (Asp3–Asp5)$^{30}$. The crystal structure of the Ca$^{2+}$-bound Munc13-1 C$_2$B domain revealed two bound Ca$^{2+}$ ions (Fig. 2), consistent with the NMR data (Fig. 1d,e). The two Ca$^{2+}$ ions are coordinated by the five canonical aspartate residues, accounting for the block of Ca$^{2+}$ binding by the DN mutation (Fig. 1b). In addition, the Ca$^{2+}$ ions are coordinated by two backbone carboxyl oxygens and two water molecules (Fig. 2d,e). Loop 3 of the Munc13-1 C$_2$B domain contains additional acidic residues that do not participate in Ca$^{2+}$ binding but are oriented toward the Ca$^{2+}$-binding sites and may increase its Ca$^{2+}$ affinity (Fig. 2f). Moreover, exposed basic and hydrophobic residues confer an amphipathic character to the unique $\alpha$-helix of loop 3 in the Munc13 C$_2$B domain. This amphipathic character could be increased by extending the $\alpha$-helix toward the C terminus to include Arg769 to Arg772, and the helical structure may have been partially distorted by crystal contacts (Supplementary Fig. 2).

Hence, a longer $\alpha$-helix spanning residues 762–772 may be formed by loop 3 of the Munc13 C$_2$B domain in solution and/or upon binding of the C$_2$B domain to phospholipid bilayers (see below).

Munc13 C$_2$B domain is a Ca$^{2+}$- and phospholipid-binding module

We next examined whether Munc13 C$_2$B domains bind to phospholipids in a Ca$^{2+}$-dependent manner. First, we measured fluorescence resonance energy transfer (FRET) from the Munc13-2 C$_2$B domain to dansyl-labeled liposomes containing PIP and PIP$_2$. Ca$^{2+}$ increased FRET only when both protein and liposomes were present, and this increase was reversed by addition of EGTA (Fig. 3a and Supplementary Fig. 3). FRET may be mediated by the conserved tryptophan residue near loop 3 of the Munc13 C$_2$B domain (Fig. 1a), as this residue is present in the phospholipid-interacting sequence of C$_2$ domains.

Next, we used a centrifugation assay with liposomes containing a synaptic phospholipid composition with 0.5% (w/w) PIP and 0.1% (w/w) PIP$_2$ (ref. 34). Beside the wild-type Munc13-1 and Munc13-2 C$_2$B domains and the DN mutant Munc13-2 C$_2$B domain, we also examined an additional Munc13-2 C$_2$B domain mutant called the KW mutant, in which Lys630 in loop 1 is exchanged for Arg769 to Arg772, and the helical structure may have been partially distorted by crystal contacts (Supplementary Fig. 2).

We used tryptophan
instead of methionine at this residue to maximize the membrane penetration of the Munc13 C_B domain, as previously shown for the synaptotagmin-1 C_A domain.57,58

The Munc13-1 and Munc13-2 C_B domains bound poorly to synaptic liposomes in the absence of Ca^{2+}, but strongly in the presence of Ca^{2+} (Fig. 3b). Quantification of Coomassie blue–stained SDS gels revealed that the Munc13-1 and Munc13-2 C_B domains have similar apparent Ca^{2+} affinities (Munc13-1: EC_{50} = 5.5 ± 0.9 μM Ca^{2+} (n = 3); Munc13-2: EC_{50} = 5.3 ± 0.8 μM Ca^{2+} (n = 3); mean ± s.e.m.) that were indistinguishable from that of the synaptotagmin-1 C_A-C_B domain fragment (EC_{50} = 6.4 ± 0.5 μM Ca^{2+} (n = 4; mean ± s.e.m.)) (Supplementary Fig. 4). The Munc13-2 C_B domain bound preferentially to the PIP_3-containing liposomes, similar to the synaptotagmin-1 C_C domains (Fig. 4).

In synaptotagmin-1, C_D domains bind not only to phospholipids but also to SNARE proteins.40,43 However, pulldown experiments with solubilized brain proteins uncovered Ca^{2+}-dependent binding of the Munc13 C_B domains only to tubulin (which, as an abundant protein, binds nonspecifically to many proteins), but not to SNARE proteins, suggesting that the Munc13 C_B domain does not interact with SNARE proteins (Supplementary Fig. 6).

Role of Munc13-2 C_B domain in neurotransmitter release
To assess the functional importance of Ca^{2+} binding to the Munc13 C_B domain, we analyzed synaptic transmission in autapses formed by hippocampal neurons that were cultured on microislands of glia cells. The neurons, which were isolated from mice that lack Munc13-1 and Munc13-2, were rescued by viral expression of the ‘ubMunc13-2’ variant of Munc13-2, used because of its pronounced effects on short-term synaptic plasticity.23,44

We first analyzed synaptic transmission induced by isolated action potentials. Wild-type, DW mutant and KW mutant Munc13-2 rescued the loss of synaptic transmission induced by deletion of Munc13-1 and Munc13-2 (Fig. 5a). Rescue with wild-type and DW mutant Munc13-2 did not cause any substantial change in excitatory postsynaptic currents (EPSC) amplitudes, whereas rescue with KW mutant Munc13-2 increased the EPSC amplitudes almost two-fold (Fig. 5b). To test whether this change was due to a difference in the size of the RRP, we measured the RRP by application of hypertonic sucrose45; however, we detected no significant change (Fig. 5c). We also determined the vesicular release probability (P_{vr}) for each neuron expressing wild-type or mutant Munc13-2 by calculating the ratio of integrated EPSC and RRP charges. The KW mutation did not alter P_{vr}, whereas the DW mutation nearly doubled it (Fig. 5d).
To confirm that the KW but not the DN mutation of the C2B domain of Munc13 alters the release probability during isolated action potentials, we monitored the relative EPSC amplitudes of synapses expressing wild-type, DN or KW mutant Munc13-2 at low (1 mM) or high (12 mM) extracellular Ca\(^{2+}\) concentrations (Fig. 5e). Consistent with an unchanged basal release probability, wild-type and DN mutant Munc13-2 showed the same relative Ca\(^{2+}\)-dependent changes in ESPC amplitudes. In contrast, KW mutant Munc13-2 showed a relative increase in EPSC amplitude at the low ambient Ca\(^{2+}\) concentration, confirming the hypothesis (Fig. 5f).

In increasing release, KW mutant Munc13-2 could act either as a Ca\(^{2+}\) sensor for triggering release, analogous to synaptotagmin, or as an auxiliary Ca\(^{2+}\) regulator of Ca\(^{2+}\) triggering by synaptotagmin. To differentiate between these two possibilities, we tested whether wild-type or KW mutant Munc13-2 confer Ca\(^{2+}\)-triggered synchronous neurotransmitter release onto synapses from synaptotagmin-1 knockout mice, which lack almost all such release\(^{46}\). However, neither wild-type nor KW mutant Munc13-2 rescued the loss of Ca\(^{2+}\)-induced synchronous release in synaptotagmin-deficient synapses, suggesting that Munc13-2 functions as an auxiliary Ca\(^{2+}\) regulator in release (Supplementary Fig. 7).

The Munc13-2 C2B domain in short-term plasticity

We next monitored synaptic responses induced by 2.5-Hz, 10-Hz and 40-Hz stimulus trains in synapses expressing wild-type or mutant Munc13-2. Plots of normalized responses revealed that, as expected, synapses expressing wild-type Munc13-2 showed strong facilitation at 2.5-Hz stimulation and less facilitation at 10-Hz stimulation (Fig. 6a–d and Supplementary Fig. 8). In contrast, synapses expressing DN or KW mutant Munc13-2 both showed no facilitation, but did show transient depression, during the 2.5-Hz stimulation and showed persistent depression during the 10-Hz stimulation (Fig. 6a,c).

Notably, plots of absolute synaptic responses indicated that the analysis of normalized responses can be misleading. Specifically, synapses expressing KW mutant Munc13-2 started off with a much

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**Figure 3** Ca\(^{2+}\)-dependent binding of the Munc13 (M13) C2B domain to PIP- or PIP\(_2\)-containing liposomes. (a) FRET assays of Ca\(^{2+}\)-dependent binding of the Munc13 C2B domain to dansyl-labeled ‘synaptic’ liposomes containing 0.5% (w/w) PIP and 0.1% (w/w) PIP\(_2\) (0.03 mg ml\(^{-1}\); total volume 0.6 ml). Fluorescence spectra (excitation 282 nm) were monitored in solutions containing either the C2B domain alone, liposomes alone or both, as indicated in the right\(^{32}\). Spectra were first recorded in Ca\(^{2+}\)-free buffer (black traces covered by overlying green, red or blue traces), then after addition of 2 mM Mg\(^{2+}\) (blue traces under the overlying green or red traces), then after addition of 0.2 mM Ca\(^{2+}\) (red traces), then again after further addition of 1 mM EGTA (green trace, done only for the samples containing both liposomes and C2B domain protein). Data show a representative experiment repeated multiple times; see Supplementary Figure 3 for individual spectra. AU, arbitrary units; WT, wild-type. (b) Centrifugation assays of Ca\(^{2+}\)-dependent Munc13 C2B domain binding to ‘synaptic’ liposomes containing 0.5% (w/w) PIP and 0.1% (w/w) PIP\(_2\) (b), or 0.25% (w/w) PIP and 0.05% (w/w) PIP\(_2\) (c). GST-fused Munc13 C2B domains and the synaptotagmin-1 (Syt-1) C2A/C2B domain (C2A/B) fragment (used as an internal control) were bound to liposomes at the indicated free Ca\(^{2+}\) concentrations clamped with Ca\(^{2+}\)-EGTA buffer containing 2 mM Mg\(^{2+}\). Co-pelleted Munc13 and synaptotagmin-1 C2 domains were analyzed by SDS-PAGE and Coomassie Blue staining, and quantified by scanning (above, representative experiments; below, summary graphs (means ± s.e.m. (n = 3)); data were normalized to binding at the highest Ca\(^{2+}\) concentration; quantifications for synaptotagmin-1 for b are shown in Supplementary Fig. 4).

**Figure 4** PIP and PIP\(_2\) dependence of Ca\(^{2+}\)-induced liposome binding to Munc13 C2B domains. (a,b) Quantification of Ca\(^{2+}\)-dependent Munc13 C2B domain binding to ‘synaptic’ liposomes as a function of the PIP (a) or PIP\(_2\) concentration (b). Binding assays were carried out using the centrifugation assay (Fig. 3b,c) in the absence (open symbols) or presence of 0.1 mM Ca\(^{2+}\) (filled symbols) as a function of the concentration of PIP (a) or PIP\(_2\) (b) in the liposomes. Representative experiments are shown above and summary graphs below (means ± s.e.m. (n = 3)); data were normalized to binding at the highest free Ca\(^{2+}\) concentration). Wild-type and KW mutant C2B domains are not significantly different for the PIP titration (a), but are significantly different for the PIP\(_2\) titration (b, P = 0.0016 using a two-way ANOVA test; see Supplementary Fig. 5 for direct comparison of the binding of the wild-type (WT) C2B domain to PIP- or PIP\(_2\)-containing liposomes).
higher absolute EPSC value than did synapses expressing wild-type Munc13-2, and they showed continuously larger EPSCs (Fig. 6ac). In contrast, synapses expressing DN mutant Munc13-2 started off with an unchanged EPSC value but experienced more severe synaptic depression during the stimulus trains (Fig. 6bd). Thus, in synapses containing KW mutant Munc13-2, the initially increased release probability led to a faster depletion of the RRP, and apparent depression; in synapses expressing DN mutant Munc13-2, the initial release probability was normal, and depression developed because the accumulating Ca\(^{2+}\) that normally augments release by binding to the Munc13 C\(_B\) domain can no longer bind to the domain.

To confirm these conclusions, we analyzed a second, related form of short-term synaptic plasticity: augmentation of synaptic responses observed after a short, high-frequency stimulus train. We measured synaptic responses before and after application of a 5-s, 10-Hz stimulus train and again analyzed normalized and absolute EPSC amplitudes (Fig. 6ef). Plots of normalized responses showed that augmentation was largest in synapses expressing wild-type Munc13-2 (2.5 ± 0.1-fold; n = 77) but was impaired in synapses expressing DN or KW mutant Munc13-2 (1.3 ± 0.1 (n = 71), P < 0.001; 1.5 ± 0.1 (n = 93) P < 0.001) (Fig. 6e). Plots of absolute responses, however, showed that DN mutant–expressing synapses showed a true loss of augmentation, whereas the apparent loss of augmentation in KW mutant–expressing synapses was spurious, as the synapses start from an enhanced baseline (Fig. 6f).

Notably, the loss of augmentation in synapses expressing DN mutant Munc13-2 applied only to the very initial phase; later in the stimulus train, responses recovered, consistent with the notion that multiple Ca\(^{2+}\) regulators mediate augmentation.13,16–19.

Our data suggest that the main mechanism by which Ca\(^{2+}\) binding to the Munc13 C\(_B\) domain mediates synaptic augmentation involves a change in P\(_{\text{rev}}\), as the gain-of-function KW mutant has a two-fold higher initial release probability. To test further whether additional effects on vesicle repriming could explain the phenotype, we measured the recovery of the RRP after vesicle depletion by a high-frequency stimulus train (40 Hz for 2.5 s). Although there was a trend toward a slower EPSC recovery in synapses expressing DN or KW mutant Munc13-2, this effect was not statistically significant (Supplementary Fig. 8).

Synaptic but not nonsynaptic Munc13 isoforms contain adjacent C\(_B\) and C\(_D\) domains, similar to those in PKC, that in PKC cooperate with each other.47 At a synapse, phorbol esters increase the presynaptic P\(_{\text{rev}}\) without changing the RRP size,44 at least in part by activating Munc13 (refs. 48,49). To test whether the Munc13 C\(_B\) and C\(_D\) domains also cooperate, we analyzed the effect of phorbol esters (1 \(\mu\)M phorbol 13,14-dibutyrate (PDBu) applied for 1 min). We found that the relative potentiation by PDBu was significantly decreased in synapses expressing KW mutant Munc13-2, whereas synapses expressing wild-type or DN mutant Munc13-2 had similar degrees of potentiation (Fig. 6g). Plots of the relative potentiation of release by PDBu against the vesicular release probability for individual synapses revealed an inverse correlation (Fig. 6h), indicating that the increased P\(_{\text{rev}}\) caused by the KW mutation occludes the PDBu potentiation.

DISCUSSION

Munc13 proteins are essential components of the synaptic release machinery that prime synaptic vesicles for exocytosis, and regulate short-term plasticity of synaptic exocytosis, but their mechanisms of action remain unclear. Here we show that the central C\(_B\) domain of Munc13 shows unusual Ca\(^{2+}\)- and PIP- or PIP\(_2\)-dependent phospholipid-binding properties. These properties correlate structurally with a unique accessory \(\alpha\)-helix of the Munc13 C\(_B\) domain that is part of its Ca\(^{2+}\)-binding site. The unusual properties of the Munc13 C\(_B\) domain enable Munc13 to mediate Ca\(^{2+}\)-dependent augmentation of synaptic vesicle exocytosis during high-frequency trains of action potentials. As described below, we believe that this augmentation is probably based on the Ca\(^{2+}\)-dependent binding of the Munc13 C\(_B\) domain to the plasma membrane, which in turn is enabled by increased synthesis of PIP and PIP\(_2\) induced by accumulating Ca\(^{2+}\) during a high-frequency action-potential train.

Properties of the Munc13 C\(_B\) domain

Structurally, the Munc13 C\(_B\) domain is composed of a standard C\(_B\) domain \(\beta\)-sandwich in which aspartate residues in the ‘top’ loops
Figure 6 Ca\(^{2+}\) binding to the Munc13 C\(_2\)B domain regulates release during high-frequency action-potential trains. (a–d) Normalized (a,c) and absolute (b,d) EPSC amplitudes (ampl.) in response to a 2.5-Hz (a,b) or a 10-Hz (c,d) action-potential train in Munc13-deficient neurons expressing wild-type (WT; black), DN mutant (blue) or KW mutant Munc13-2 (red; mean ± s.e.m.). In the normalized plots (left panels), *P < 0.001 for WT compared to DN and KW mutant Munc13-2; in the absolute responses (right panels), the initial responses are significantly larger (*P < 0.01) for the KW mutant Munc13-2 compared to the WT or DN mutant protein, whereas the final responses are significantly smaller (*P < 0.001) for the DN mutant than for the WT and KW mutant Munc13-2 (2.5 Hz: WT, *n* = 18; KW, *n* = 21; DN, *n* = 16; 10 Hz: WT, *n* = 50; DN, *n* = 41; KW, *n* = 64). (e,f) Normalized (e) and absolute (f) EPSC amplitudes in response to a low-frequency stimulus train (0.2 Hz) that is interrupted by a 5-s, 10-Hz stimulus train to induce augmentation (gray area)\(^{37}\). Munc13-2-deficient neurons expressing WT (black), DN mutant (blue) or KW mutant Munc13-2 (red) were analyzed (for normalized responses, degree of augmentation is significantly higher (*P < 0.001) for WT than for DN and KW mutant Munc13-2; for absolute responses, all three Munc13 forms differ significantly from each other at the *P < 0.001 level (WT, *n* = 50; DN, *n* = 41; KW, *n* = 64)). (g) Relative potentiation by PDBu (1 μM) of EPSC amplitudes evoked at 0.2 Hz in Munc13-deficient neurons expressing WT (black), DN-mutant (blue) or KW-mutant Munc13-2 (red). The relative PDBu potentiation was significantly lower (*P < 0.001) in synapses expressing KW mutant Munc13-2 than in those expressing WT or DN mutant Munc13-2 (WT, *n* = 30; DN, *n* = 31; KW, *n* = 43). (h) Plot of the degree of PDBu potentiation as a function of the initial vesicular release probability (Pvr) in individual neurons. Each individual data point represents a Munc13-deficient neuron expressing WT (black), DN-mutant (blue) or KW-mutant Munc13-2 (red). The solid symbols represent the mean values for each group (+/− SEM).

coordinate two Ca\(^{2+}\) ions (Figs. 1 and 2). A distinctive feature of the Munc13 C\(_2\)B domain is Ca\(^{2+}\)-binding loop 3, which includes an extended sequence that folds into an amphipathic α-helix (Fig. 2). Biochemically, the Munc13 C\(_2\)B domain binds to phospholipids in a Ca\(^{2+}\)-dependent manner, similar to other C\(_2\) domains, but it differs from other known C\(_2\) domains, such as those from synaptotagmin, in that Ca\(^{2+}\)-dependent phospholipid binding requires relatively high concentrations of PIP or PIP\(_2\) (Figs. 3 and 4). This unusual biochemical property is probably mediated, at least in part, by the unique α-helix formed by Ca\(^{2+}\)-binding loop 3, which contains a highly positively charged region that may act similarly to the PIP\(_2\)-dependent amphipathic α-helix observed in epsin\(^{51}\).

Our data show that, in addition to the amphipathic α-helix, the conserved, positively charged Lys630 residue in Ca\(^{2+}\)-binding loop 1 is a major determinant of the unusual phospholipid-binding properties of the Munc13 C\(_2\)B domain. In the Ca\(^{2+}\)-binding loops of synaptotagmin-1 C\(_2\) domains, the residues analogous to Lys630 of Munc13 are hydrophobic (Met173 and Val304; Fig. 1a). During Ca\(^{2+}\)-dependent phospholipid binding of synaptotagmin-1 C\(_2\) domains, Met173 and Val304 insert into the phospholipid bilayer and contribute to the relatively nonspecific but tight Ca\(^{2+}\)-dependent phospholipid binding of these C\(_2\) domains\(^{35–38}\). Moreover, exchanging Met173 and Val304 in synaptotagmin-1 C\(_2\) domains for tryptophan further enhances their Ca\(^{2+}\)-dependent phospholipid binding, indicating that tryptophan increases Ca\(^{2+}\)-dependent phospholipid binding mediated by hydrophobic residues\(^{37,38,52}\). These observations suggested to us that Lys630 in the Munc13 C\(_2\)B domain may contribute to its unique specificity for higher PIP or PIP\(_2\) concentrations. To test this hypothesis, we substituted Lys630 of the Munc13 C\(_2\)B domain for tryptophan, resulting in the KW mutation. The KW mutation rendered the Munc13 C\(_2\)B domain responsive to PIP\(_2\) at a concentration at which wild-type Munc13 is inert but synaptotagmin-1 is active; thus, the KW mutation confers synaptotagmin-like properties onto the Munc13 C\(_2\)B domain (Fig. 4). As a result, the KW mutation constitutes a gain-of-function mutation that enables Ca\(^{2+}\)-dependent binding of the Munc13 C\(_2\)B domain to phospholipid membranes containing lower PIP or PIP\(_2\) concentrations than the Munc13 C\(_2\)B domain would normally show binding to.

The C\(_2\)B domain regulates synaptic plasticity

Notably, abolishing Ca\(^{2+}\) binding to the Munc13-2 C\(_2\)B domain with the DN mutation did not alter vesicle exocytosis triggered by isolated action potentials (Fig. 5), but it impaired facilitation of synaptic vesicle exocytosis induced by repeated action potentials (Fig. 6). Thus, the Munc13 C\(_2\)B domain acts as a Ca\(^{2+}\) regulator of short-term synaptic plasticity, consistent with the notion that synaptic facilitation during high-frequency stimulus trains is not passively caused by residual Ca\(^{2+}\) but, instead, is actively induced by Ca\(^{2+}\) binding to the Munc13 C\(_2\)B domain (and other Ca\(^{2+}\)-binding proteins). The KW mutation, in contrast, increased the amount of Ca\(^{2+}\)-triggered release during single and repeated action potentials (Figs. 5 and 6), without itself acting as a Ca\(^{2+}\) sensor for release (Supplementary Fig. 7).

Viewed together, our data suggest that, during isolated action potentials, the lower PIP or PIP\(_2\) content at rest prevents Ca\(^{2+}\)-dependent binding of the wild-type Munc13 C\(_2\)B domain to the membrane. During stimulus trains, a Ca\(^{2+}\)-dependent phosphatidylinositol kinase may increase the presynaptic PIP and PIP\(_2\) content, allowing residual Ca\(^{2+}\) to activate Munc13, and thereby boost release. Consistent with this hypothesis, Ca\(^{2+}\) stimulates PIP and PIP\(_2\) synthesis in neuroendocrine cells\(^{53}\), and dephosphorylation of neurons activates presynaptic PIP kinase Iy by dephosphorylation, thereby increasing the plasma membrane PIP and PIP\(_2\) content\(^{54–56}\). Our hypothesis explains why the DN mutation has no effect on Munc13 function during isolated action potentials but interferes with Munc13 function during repeated action potentials. The hypothesis also accounts for the gain-of-function effect of the...
KW mutation, because the PIP and PIP$_2$ content at rest is proposed to be too low to allow Ca$^{2+}$ activation of the wild-type Munc13 C$_B$ domain but may be sufficient for Ca$^{2+}$ activation of the KW mutant C$_B$ domain.

**Mechanism of the Munc13 C$_B$ domain action**

In boosting release during a stimulus train, Ca$^{2+}$ binding to the Munc13 C$_B$ domain probably increases vesicle priming by enhancing the priming function of Munc13. The KW mutation may enable the Munc13 C$_B$ domain to perform the same activity even at rest, possibly because the KW mutant C$_B$ domain binds to the plasma membrane even without the increase in PIP and PIP$_2$ concentrations that is thought to occur during repeated action potentials$^{54-56}$.

However, two of our findings with KW mutant Munc13 seem to argue against the hypothesis that the Munc13 C$_B$ domain boosts the priming function of Munc13: first, the KW mutation selectively increased the Ca$^{2+}$ sensitivity of release induced by isolated action potentials without increasing the size of the RRP (Fig. 5); second, the KW mutation did not significantly alter the rate by which evoked release recovered after depletion of the RRP, although there was a trend toward acceleration of priming (Supplementary Fig. 8).

Despite these findings, we believe that the priming hypothesis is correct for the following reasons. Priming is likely to involve a partial, if not complete assembly of SNARE complexes between vesicles and the plasma membrane$^{27}$. The number of assembled SNARE complexes per primed vesicle may determine (among others) the apparent Ca$^{2+}$ affinity of synaptic vesicle fusion$^{28}$. Thus, Ca$^{2+}$ binding to the Munc13 C$_B$ domain may increase the ability of Munc13 to catalyze SNARE complex assembly of docked vesicles during priming, which would result in the appearance of an increased Ca$^{2+}$ sensitivity of release for the KW mutation. This hypothesis is consistent with the fact that KW mutant Munc13 does not act as a Ca$^{2+}$ sensor for exocytosis itself (Supplementary Fig. 7).

Although plausible, the priming hypothesis cannot be tested directly until its underlying tenet—namely that Munc13 mediates vesicle priming by catalyzing SNARE complex assembly—has been confirmed$^{59}$.

How does increased phospholipid binding induced by Ca$^{2+}$ binding to the Munc13 C$_B$ domain potentiate Ca$^{2+}$-triggered release, be it via priming or otherwise? As a component of the biochemically insoluble active zone, Munc13 is already normally close to the plasma membrane. Thus, Ca$^{2+}$-dependent C$_B$ domain binding to the plasma membrane would not relocalize Munc13 but, instead, pull on the adjacent plasma membrane and stretch it. Such an activity may, analogous to what has been proposed for the mechanism of action of synaptotagmin$^{60}$, promote exocytosis by decreasing the energy requirement for Ca$^{2+}$-triggered fusion pore opening. Diacylglycerol binding to the C$_1$ domain of Munc13—which also induces Munc13 plasma membrane binding—may potentiate release by an analogous, but Ca$^{2+}$-independent, mechanism. An alternative hypothesis is that the Munc13 C$_1$ and C$_B$ domains are normally inhibitory, and that diacylglycerol and Ca$^{2+}$ binding reverse this inhibition$^{69}$. This second hypothesis would require that the C$_1$ and the C$_B$ domain have additional unknown biochemical interactions beyond lipid binding that are altered by the various mutations, a possibility that remains to be explored.

**Munc13 as a computational unit for synaptic transmission**

The activity of synaptic Munc13 isoforms is regulated via three distinct, adjacent signaling motifs: the previously described calmodulin-binding sequence and C$_1$ domain$^{12,13}$, and the C$_B$ domain we characterize here (Fig. 7). All three motifs are directly or indirectly activated by Ca$^{2+}$ and have profound roles in controlling neurotransmitter release during short-term plasticity, but they differ from each other in their mechanisms of activation and action. The C$_B$ domain is directly Ca$^{2+}$ activated by Ca$^{2+}$ influx during action potentials but is presumably stimulated only after the accumulating residual Ca$^{2+}$ has induced the synthesis of PIP and PIP$_2$. In contrast, the calmodulin-binding sequence is indirectly activated by binding of accumulating residual Ca$^{2+}$ to calmodulin, which probably acts on many synaptic targets simultaneously. The C$_1$ domain is activated indirectly via Ca$^{2+}$-dependent induction of phospholipase C. Thus, the Ca$^{2+}$ concentration dependence and time course of activation of the three regulatory motifs are likely to differ, leading to a common readout (synaptic potentiation) that results from the integration of multiple signals acting differentially on the three signaling motifs. Consistent with this model, the KW mutation of Munc13 occludes the effect of phorbol esters on release triggered by isolated action potentials, whereas the DN mutation (which has no effect on release triggered by isolated action potentials) has no effect on the phorbol ester potentiation under those conditions (Fig. 6).

Moreover, mutations in the calmodulin-binding motif and the C$_B$ domain at least act additively during short-term synaptic plasticity (Supplementary Fig. 9).

In summary, our data establish that the Munc13 C$_B$ domain operates as a Ca$^{2+}$ regulator of short-term synaptic plasticity. Apart from the importance of the Munc13 C$_B$ domain Ca$^{2+}$-binding properties for synaptic exocytosis, our results also suggest that this domain may act as a Ca$^{2+}$ regulator of exocytosis for nonsynaptic Munc13 isoforms, which are likely to function in other forms of exocytosis (such as Munc13-4 in lymphocyte exocytosis$^{25}$).

**METHODS**

Methods and any associated references are available in the online version of the paper at http://www.nature.com/nsmb/.

**Accession codes.** Protein Data Bank: Coordinates for the Ca$^{2+}$-free and Ca$^{2+}$-bound Munc13-1 C$_B$ domain have been deposited with accession codes 3KWT and 3KWU, respectively.
Note: Supplementary information is available on the Nature Structural & Molecular Biology website.

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AUTHOR CONTRIBUTIONS
O.-H. S. performed the protein chemistry and molecular biology experiments; J.L., D.R.T. and M.M. performed the structural biology experiments; J.-S.R., M.C.-P. and Z.P.P. performed the electrophysiology experiments; S.W. and N.B. generated the vectors for expression of mutant Munc13; J.R., C.R. and T.C.S. wrote the paper.

COMPETING INTERESTS STATEMENT
The authors declare no competing financial interests.

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ONLINE METHODS

Plasmids. We used four different types of plasmids encoding rat Munc13-1 and Munc13-3 for this study: (i) We used pGEX-KT-derived plasmids for bacterial expression. (ii) We inserted the GST-coding region into baculovirus expression plasmids derived from pFastBac (Invitrogen Life Technologies). We used the resulting plasmid to clone pFastBac-GST-Munc13-1 C\(_B\) domain (residues 675–820; numbering based on U24070), pFastBac-GST-Munc13-2 C\(_B\) domain (residues 599–744; numbering based on AF159708, pFastBac-GST-Munc13-2 C\(_B\)-DN (DE29N and D635N) and pFastBac-GST-Munc13-2 C\(_B\)-KW (K630W)). (iii) We used Semliki Forest Virus expression plasmids (pSFV ubMunc13-2 WT, pSFVbubMunc13-2 C\(_B\)-DE29N, D635N, pSFVbubMunc13-2 K630W) for neuronal cultures. (iv) We also used the lentiviral expression plasmid pFUW-Munc13-2 K630W, which encodes ub-Munc13-2 with the KW mutation.

Production of recombinant proteins. In our bacterial expression protocol, GST fusion proteins were expressed at 25 °C in Escherichia coli BL21 and isolated by affinity chromatography on glutathione-Sepharose followed by on-resin cleavage with thrombin. We further purified the cleaved proteins by ion-exchange and gel-filtration chromatography on MonoS and S75 columns (Amersham). We achieved uniform \(^{15}\)N labeling by growing the bacteria in \(^{15}\)NH\(_4\)Cl as the sole nitrogen source.

In our baculovirus expression protocol, we generated Munc13-1 C\(_B\) domain proteins using Bac-to-Bac Baculovirus Expression System (Invitrogen Life Technologies). We generated recombinant GST-Munc13-1 C\(_B\) proteins by infecting 400 ml of Sf9 cells (~2 × 10\(^6\) cells per ml) in a 2 liter flask after inoculating the bacteria in a 10% (w/v) sucrose, 20 mM MES-NaOH, pH 6.2, 0.1 M NaCl and 0.5 mM Tris (1:1 v/v) mixture. After 3 days of incubation, we harvested the liposomes by centrifugation (20,800 g for 15 min) and washed it three times with 1 ml of the corresponding buffers. We dissolved the final liposome pellets in chloroform:methanol (1:2 v/v). We recovered the lipids by precipitation at 20 °C for 15 min and then carried out manual cloning of the lipids into Munc13-1 C\(_B\) domain proteins and 30 µg liposomes containing 41% (w/w) PC, 22% (w/w) PE, 10% (w/w) daisy-PE, 12% (w/w) PS, 5% (w/w) PI, 10% (w/w) cholesterol, 0.5% (w/w) PIP and 1% (w/w) PIP\(_2\). Emission spectra (excitation 282 nm) were recorded first without metal ions, then after addition of 2 mM MgCl\(_2\) and 0.2 mM CaCl\(_2\). We then carried out manual cloning of the lipids into Munc13-1 C\(_B\) domain proteins and 30 µg liposomes containing 41% (w/w) PC, 22% (w/w) PE, 10% (w/w) daisy-PE, 12% (w/w) PS, 5% (w/w) PI, 10% (w/w) cholesterol, 0.5% (w/w) PIP and 1% (w/w) PIP\(_2\). Emission spectra (excitation 282 nm) were recorded first without metal ions, then after addition of 2 mM MgCl\(_2\), then after addition of 0.2 mM CaCl\(_2\) and then after further addition of 1 mM EGTA.

Phospholipid binding assays. For the centrifugation assay, we dissolved phospholipids and cholesterol (Avanti) in chloroform:methanol (1:1; v/v) and then carried out centrifugation assay (20,000 g for 15 min) to determine the indicated free Ca\(^{2+}\) concentration. We incubated the binding reactions for 10 min at 30 °C with 800 r.p.m. shaking, pelleted the protein by centrifugation (20,800 g for 10 min) and washed it three times with 1 ml of the corresponding buffers. We dissolved the final liposome pellets in chloroform:methanol (1:2 v/v). We recovered the precipitated proteins by centrifugation (20,800 g for 15 min), resuspended them in 30 µl of 2× SDS sample buffer and analyzed them by SDS-PAGE and Coomassie Blue staining. We quantified the bound proteins by analysis of scanned Coomassie-stained gels using the Image Quant program (version 5.2, Molecular Dynamics).

We performed FRET assays essentially as described\(^{22}\) at room temperature (23–27 °C) in 0.5 ml of 20 mM HEPES-NaOH, pH 7.2, and 0.1 M NaCl, with 1 µM of Munc13-2 C\(_B\) domain protein and 30 µg ml\(^{-1}\) liposomes containing 41% (w/w) PC, 22% (w/w) PE, 10% (w/w) daisy-PE, 12% (w/w) PS, 5% (w/w) PI, 10% (w/w) cholesterol, 0.5% (w/w) PIP and 1% (w/w) PIP\(_2\). Emission spectra (excitation 282 nm) were recorded first without metal ions, then after addition of 2 mM MgCl\(_2\), then after addition of 0.2 mM CaCl\(_2\) and then after further addition of 1 mM EGTA.

GST pulldowns. We performed GST pulldowns as described\(^{39,40}\). We homogenized one unstripped rat brain (~1.5 g per brain; Pel-Freez Biologicals) with a random subset of all data set aside for the calculation of free R-factors. After complete refinement, we added solvent molecules where chemically reasonable. The final model for the Ca\(^{2+}\)-bound Munc13-1 C\(_B\) domain contains residues 673–687 and 819–1, two Ca\(^{2+}\) ions, one glycerol molecule and 161 water molecules (final R = 17.0; Reo = 19.3; overall B-factor = 16.7). The final model for the Ca\(^{2+}\)-free Munc13-1 C\(_B\) domain contains residues 678–705, 708–763, 773–801 and 807–819, one molecule of Bis-Tris-propane, 2 Cl\(^{-}\) ions and 63 water molecules (final R = 21.7; Reo = 28.2; overall B-factor = 41.0). For data collection and refinement statistics, see Table 1.

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currents using an Axopatch 200B amplifier (Molecular Devices). Series resistance was within 10 MΩ and was electronically compensated at least 70%. We analyzed the data using AXOGRAPH software (version 4.9, Molecular Devices). For analyses of Syt-1 KO mice, we cultured primary cortical neurons from Syt-1 knockout and littermate wild-type control mice in MEM (Gibco) supplemented with B27 (Gibco), glucose, transferrin, FBS and Ara-C (Sigma). To monitor synaptic responses, we made whole-cell patch-clamp recordings with neurons at 14–16 d in vitro. Synaptic responses were triggered by a 1-ms current pulse (900 µA) through a local extracellular electrode (FHC), and recorded in whole-cell voltage-clamp mode using a Multiclamp 700B amplifier (Axon Instruments). We analyzed the data using Clampfit 9.02 (Axon Instruments) or Igor 4.0 (Wavemetrics).

**Statistical analyses.** We used a paired Student’s t-test or ANOVA.
