Molecular basis of synaptic vesicle cargo recognition by the endocytic sorting adaptor stonin 2

Nadja Jung, Martin Wienisch, Mingyu Gu, James B. Rand, Sebastian L. Müller, Gerd Krause, Erik M. Jorgensen, Jürgen Klingauf, and Volker Haucke

1Department of Membrane Biochemistry, Institute of Chemistry and Biochemistry, Freie Universität Berlin, 14195 Berlin, Germany
2Department of Membrane Biophysics, Max-Planck-Institute for Biophysical Chemistry, 37077 Göttingen, Germany
3Department of Biology, University of Utah, Salt Lake City, UT 84112
4Program in Molecular, Cell and Developmental Biology, Oklahoma Medical Research Foundation, Oklahoma City, OK 73104
5Leibniz-Institut für Molekulare Pharmakologie, 13125 Berlin, Germany

Synaptic transmission depends on clathrin-mediated recycling of synaptic vesicles (SVs). How select SV proteins are targeted for internalization has remained elusive. Stonins are evolutionarily conserved adaptors dedicated to endocytic sorting of the SV protein synaptotagmin. Our data identify the molecular determinants for recognition of synaptotagmin by stonin 2 or its Caenorhabditis elegans orthologue UNC-41B. The interaction involves the direct association of clusters of basic residues on the surface of the cytoplasmic domain of synaptotagmin 1 and a β strand within the μ-homology domain of stonin 2. Mutation of K783, Y784, and E785 to alanine within this stonin 2 β strand results in failure of the mutant stonin protein to associate with synaptotagmin, to accumulate at synapses, and to facilitate synaptotagmin internalization. Synaptotagmin-binding–defective UNC-41B is unable to rescue paralysis in C. elegans stonin mutant animals, suggesting that the mechanism of stonin-mediated SV cargo recognition is conserved from worms to mammals.

Correspondence to Volker Haucke: v.haucke@biochemie.fu-berlin.de
Abbreviations used in this paper: AP-2, assembly protein complex 2; CLASP, clathrin-associated sorting protein; HEK, human embryonic kidney; μHD, μ-homology domain; NIE, neuroimmune endocrine; SV, synaptic vesicle; WT, wild type.

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et al., 1998; Grass et al., 2004), or the endocytic adaptor stonin 2 (termed stoned B in flies and UNC-41 in C. elegans; Fergestad and Broadie, 2001; Martina et al., 2001; Stimson et al., 2001; Walther et al., 2004).

Stonin 2 represents the first, and so far only, endocytic protein specifically dedicated to SV recycling by acting as a sorting adaptor for synaptotagmin 1 (Diril et al., 2006). Expression of stonin 2 in fibroblasts is sufficient to rescue clathrin/AP-2–mediated internalization of surface-stranded synaptotagmin 1 (Jarousse and Kelly, 2001) and facilitates synaptotagmin 1 redistribution into SVs in primary neurons (Diril et al., 2006). Stonin 2 is linked to the endocytic machinery by direct interactions with AP-2 (Walther et al., 2004) and eps15 or intersectin (Martina et al., 2001), and can therefore be classified as a clathrin-associated sorting protein (CLASPs; Traub, 2005). CLASPs compose a set of adaptors, including β-arrestins (Lefkowitz and Whalen, 2004), autosomal recessive hypercholesterolemia (Edeling et al., 2006), Dab2 (Mishra et al., 2002), Huntingtin interacting protein 1/1R, and numb (Santolini et al., 2000), that target specific cargo to clathrin-coated pits or subsets thereof for regulated internalization.

In contrast to other CLASPs, we lack detailed molecular information on how stonin 2 and its orthologues recognize SV cargo during exo- and endocytic vesicle cycling. We demonstrate that an evolutionarily conserved β strand within the μ–homology domain (μHD) of stonin 2 recognizes basic patches on the surface of the cytoplasmic domain of synaptotagmin 1. Mutation of critical residues within this β strand results in failure of the mutant stonin protein to physically associate with synaptotagmin and to facilitate synaptotagmin internalization. We demonstrate that an evolutionarily conserved β strand within the μ–homology domain (μHD) of stonin 2 recognizes basic patches on the surface of the cytoplasmic domain of synaptotagmin 1. Mutation of critical residues within this β strand results in failure of the mutant stonin protein to physically associate with synaptotagmin and to facilitate synaptotagmin internalization.

Results
Stonin 2 physically and functionally interacts with the C2A domain of synaptotagmin 1 to facilitate internalization
Given the importance of functional SV recycling and the role of synaptotagmin 1 in coupling exo- and endocytosis in neuro secretory cells, we first set out to identify the domains required for its internalization. Expression of stonin 2 in human embryonic kidney (HEK) 293 cells stably transfected with FLAG–synaptotagmin 1 leads to redistribution of synaptotagmin 1 from the plasma membrane to internal compartments (Diril et al., 2006). Internalization assays based on anti-FLAG antibody uptake showed that endocytosed synaptotagmin localizes to a subset of AP-2–coated puncta and to a perinuclear endosomal compartment (Fig. 1 A; Diril et al., 2006), suggesting that stonin 2 targets synaptotagmin 1 to clathrin/AP-2–coated pits. To confine the region within synaptotagmin 1 required for stonin 2–mediated internalization deletion, constructs lacking one or both of the C2 domains were generated and analyzed by antibody uptake experiments. After antibody chase for 20 min at 37°C, surface-stranded synaptotagmin 1 was detected by Alexa Fluor 594–labeled secondary antibodies under non-permeabilizing conditions, remaining surface immunoreactivity was blocked, and internalized synaptotagmin 1 was revealed using Alexa Fluor 488–labeled secondary antibodies. Surprisingly, we found that synaptotagmin 1 lacking the C2B domain (syt1ΔC2B) was internalized, albeit with reduced efficiency, whereas mutant proteins lacking either the C2A (syt1ΔC2A) or both C2 domains (syt1ΔC2AB) were not endocytosed (Fig. 1 B). Thus, the C2A domain contributes the major internalization signal for stonin 2–dependent synaptotagmin 1 endocytosis. This interpretation is supported by membrane recruitment experiments in N1E neuroblastoma cells. Overexpression of wild-type (WT) synaptotagmin 1 or syt1ΔC2B, but not syt1ΔC2A, caused a redistribution of WT stonin 2 (stonin 2WT; Diril et al., 2006) or an AP-2 and Eps15 homology domain–binding–defective stonin 2 mutant (stonin 2WF3WF, used to exclude indirect effects mediated via AP-2 or, e.g., eps15) from the cytosol to the plasma membrane (Fig. 1 C). Stonin 2 coimmunoprecipitated with WT synaptotagmin 1 or syt1ΔC2B but not with syt1ΔC2A, which is consistent with the microscopic data (Fig. 1 D). AP-2 was found in the same complex, presumably because of its direct interaction with stonin 2. This complex was also seen in affinity chromatography experiments using extracts from transfected HEK cells incubated with GST-fused C2 domains (Figs. S1 A and S2 B, available at http://www.jcb.org/cgi/content/full/jcb.200708107/DC1). Stonin 2WT or stonin 2WF3WF preferentially associated with the C2A domain of synaptotagmin 1. AP-2 efficiently copurified with either C2A or C2B from cell extracts containing stonin 2WT but was predominantly retained by C2B if lysates from stonin 2WF3WF–expressing cells were used (Fig. S1, A and B), which is in agreement with published data (Chapman et al., 1998; Haucke et al., 2000). To further corroborate the direct association of stonin 2 with synaptotagmin 1–C2A, we performed in vitro binding experiments using GST-fused synaptotagmin 1 and His6-tagged stonin 2 purified from HEK293 fibroblasts (Fig. S2 B). Stonin 2 most efficiently bound to GST-C2A or -C2AB, whereas a much weaker interaction with the C2B domain was observed (Fig. 2 A). Stonin 2 did not bind to GST control beads. These results were confirmed by experiments using 35S-labeled stonin 2 synthesized by coupled transcription/translation in vitro (Fig. S2 A). We conclude that stonin 2 directly associates with synaptotagmin 1, mainly via determinants in the C2A domain.

Stonin 2 recognizes basic patches within the synaptotagmin 1 C2 domains
Coimmunoprecipitation experiments from transfected fibroblasts (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200708107/DC1) indicated that the major binding site for synaptotagmin 1 comprised the carboxy-terminal μHD of stonin 2. The μHD exhibits ~30% amino acid identity with the μ2 subunit of AP-2, which associates with a stretch of basic amino acid residues within the C2B domain of synaptotagmin 1 (Chapman et al., 1998; Grass et al., 2004). Considering the homology between AP-2-μ and the stonin 2 μHD and the existence of basic patches in both C2 domains, we rationalized that basic residues might play a role in stonin 2 binding. To test this,
we generated synaptotagmin 1 mutants by exchanging K or R residues within basic patches of C2A or C2B for alanines (Fig. 2 B). Additionally, we targeted for mutation residues within C2A that were not conserved between synaptotagmins 1 and 6 (Fig. 2B, C2A mut 4). Synaptotagmin 6 is an isoform that appears to lack the ability to interact with stonin 2 in living cells (Diril et al., 2006). C2A and C2B mutants were then tested for their ability to associate with AP-2 or stonin 25WFNSNNF in pull-down experiments from transfected fibroblasts. We found that the elimination of basic residues within either C2 domain severely reduced binding to AP-2 or stonin 25WFNSNNF. Exchange of six basic residues within C2B attenuated association with stonin 2 or AP-2 binding below detection limits (Fig. S1 C). AP-2 binding to the C2A domain was abolished by elimination of two or more basic residues within C2A. We still observed some residual binding of stonin 2 to a C2A mutant in which six basic
residues had been exchanged (Fig. 2 C). These data were confirmed by coimmunoprecipitation experiments using charge reversal mutants of synaptotagmin 1. A synaptotagmin 1 mutant in which six basic side chains had been exchanged for acidic ones (K189–191E, K213E, K244E; KR321, 322EE; and K324–327E) displayed strongly reduced binding to stonin 2.

Figure 2. Clusters of basic amino acids within the synaptotagmin 1 C2 domains are involved in the association with stonin 2. (A) Stonin 2 and synaptotagmin 1–C2A interact directly. Stonin 2–His6 was purified from stably transfected HEK293 cells and incubated with GST–synaptotagmin 1 fusion proteins immobilized on beads. Bound stonin 2 was detected by immunoblotting for the His6-tag. Arrows mark bound stonin 2 on the Ponceau-stained nitrocellulose membrane. 10% of the input was loaded as standard (STD). Molecular masses are indicated. (B) Alignment of basic patches within synaptotagmin 1 C2A and C2B domains and mutants analyzed. (C) GST-C2A WT or mutant fusion proteins were assayed in pulldown experiments for their ability to associate with HA–stonin 2 from HEK293 lysates. Samples were analyzed by immunoblotting for HA–stonin 2, AP-2β, and clathrin heavy chain (CHC). 7% of the input was loaded as standard. (D) Coimmunoprecipitation experiment performed from cotransfected fibroblasts. HEK293 cells were cotransfected with HA-tagged stonin 2 and FLAG-tagged synaptotagmin 1 WT and mutant (K189–191E; K213E; K244E; KR321, 322EE; and K324–327EE) constructs. Immunoprecipitation was performed using polyclonal antiserum against stonin 2. Immunoblotting was performed using antibodies against HA-stonin 2, AP-2β, anti-FLAG–synaptotagmin 1, and anti-clathrin heavy chain antibodies. 3% of the input was loaded as standard. Splices are indicated by solid lines (all taken from the same exposure). In the case of FLAG–synaptotagmin 1, the bands representing material present in 3% of the starting material were taken from a slightly longer exposure of the x-ray film from the same immunoblot to reveal the bands. The two exposures are indicated by black boxes. Black lines indicate that intervening lanes have been spliced out.
The KYE site within the stonin 2 $\mu$HD is required for synaptotagmin 1 interaction and internalization

We observed that stonin 2 associated via its $\mu$HD (Fig. S3, A and B) with basic residues within the synaptotagmin 1 C2 domains, which is similar to the interaction between AP-2$\mu$ and synaptotagmin 1 C2B. Hence, we hypothesized that these interactions might be structurally related. Previous studies had suggested that a $\beta$ strand comprising residue Y343 within subdomain B of AP-2$\mu$ is part of the synaptotagmin 1 binding site (Haucke et al., 2000). Structural data are available for AP-2 (Owen and Evans, 1998; Collins et al., 2002) including its $\mu_2$ subunit, which displays high sequence homology ($\sim$52%) to the carboxy-terminal region of stonin 2. We used $\mu_2$ as a template to generate a molecular homology model of the stonin 2 $\mu$HD (Fig. S3 D). The stonin 2 $\mu$HD model displays an overall $\beta$-fold structure very similar to that of AP-2$\mu$, except for a few loops that differ from the template. Conserved features include the $\beta$ strand suggested to harbor the synaptotagmin binding site within AP-2$\mu$, which contains the aforementioned tyrosine residue (Y343 in AP-2$\mu$). This tyrosine (corresponding to Y784 in human stonin 2), as well as several flanking residues, is also evolutionarily conserved within stonin family members from humans to nematodes (Fig. 3 A), which is suggestive of its functional importance.

We hypothesized that Y784 and its neighbors form part of the binding site for synaptotagmin 1. To test this, we generated a stonin 2 mutant in which residues K783, Y784, and E785 (Fig. 3 A, red; and Fig. S3 D, right, red) had been exchanged by alanines (stonin 2$\delta$KYE). We transfected fibroblasts with stonin 2$\delta$WF$\delta$NPF, the KYE mutant stonin 2 (stonin 2$\delta$KYE), or a mutant of stonin 2 combining these mutations (stonin 2$\delta$WF$\delta$NPF$\delta$KYE) and performed affinity chromatography experiments using immobilized GST-fused synaptotagmin 1 C2 domains. Although stonin 2$\delta$WF$\delta$NPF readily copurified with synaptotagmin 1 C2A or AB, the double mutant of stonin 2 (stonin 2$\delta$WF$\delta$NPF$\delta$KYE) had completely abolished its ability to bind to synaptotagmin 1. 

Figure 3. Evolutionarily conserved residues within a $\beta$ strand of its $\mu$HD are required for association of stonin 2 with synaptotagmin 1.
(A) The KYE site is evolutionarily conserved. Multiple protein sequence alignment of stonin 2 and orthologues from rat, mouse, human, cattle, zebrafish, D. melanogaster, and C. elegans. Numbers refer to the last amino acid residue within the predicted $\beta$ strand. Conserved residues K783, Y784, and E785 are colored in red. (B) Mutation of conserved residues K783 and Y784 abolishes its ability to bind to synaptotagmin 1. GST–synaptotagmin 1 fusion proteins immobilized on beads were incubated with cell extracts derived from HEK293 cells transfected with AP-2$\delta$WF$\delta$NPF, HA–stonin 2$\delta$KYE, or HA–stonin 2$\delta$WF$\delta$NPF$\delta$KYE, respectively. Bound proteins were detected by immunoblotting for clathrin heavy chain (CHC), HA–stonin 2, and AP-2$\mu$. 8% of the input was loaded as standard (STD). (C) In vitro binding experiment using purified recombinant proteins. GST-C2AB immobilized on beads was incubated with purified stonin 2–His$_6$ WT or $\delta$KYE. Bound stonin 2–His$_6$ was detected by immunoblotting for the His-tag. The arrow marks the stonin 2–His$_6$ band on the Ponceau S–stained nitrocellulose membrane. 10% of the input was loaded as standard.
Figure 4. Stonin 2–mediated synaptotagmin 1 internalization requires an intact KYE site within stonin 2. [A] Synaptotagmin 1 does not associate with mutant stonin 2\( ^{\delta{\text{KYE}}} \) in coimmunoprecipitation experiments. HEK293 cells were cotransfected with FLAG–synaptotagmin 1 and HA–stonin 2\( ^{\text{WT}} \), AP-2–binding–deficient HA–stonin 2\( ^{\delta{\text{WF}}} \), or HA–stonin 2\( ^{\delta{\text{NPF}}} \), respectively. Proteins immunoprecipitated with antibodies against stonin 2 were detected by immunoblotting for HA–stonin 2, AP-2β, FLAG–synaptotagmin 1, and clathrin heavy chain (CHC). 2% of the input was loaded as standard. [B] The Y784R point mutant of stonin 2 is unable to associate with synaptotagmin 1. Experiments were done as described in A. 2.5% of the input was loaded as standard. [C] Stonin 2\( ^{\delta{\text{KYE}}} \) and stonin 2\( ^{YR} \) mutants lack the ability to facilitate synaptotagmin 1 internalization. HEK-syt1 cells were transfected with HA-tagged stonin 2\( ^{\text{WT}} \), stonin 2\( ^{\delta{\text{KYE}}} \), or...
lost its ability to associate with synaptotagmin (Fig. 3 B). Trace amounts of stonin 2\(^{\text{KYE}}\) were retained on GST-C2B– or -C2AB–containing beads. This is owed to the fact that this mutant retains the ability to bind to AP-2 (Fig. 4 A) and, thus, indirectly associates with the C2B domain of synaptotagmin 1 via AP-2. Similar results were obtained in direct binding experiments using His6-tagged stonin 2\(^{\text{KYE}}\) purified from HEK cells (Fig. 3 C) or synthesized by coupled transcription/translation in vitro (Fig. S3 C).

To further corroborate these findings, we performed coimmunoprecipitation experiments. Fibroblasts were cotransfected with synaptotagmin 1 and stonin 2\(^{\text{WT}}\), stonin 2\(^{\text{SWP}}\), or the \(\delta\text{KYE}\) mutant of stonin 2. As expected, synaptotagmin 1 was found in immunoprecipitates containing stonin 2\(^{\text{WT}}\) or stonin 2\(^{\text{SWP}}\) but not stonin 2\(^{\text{KYE}}\). Conversely, stonin 2\(^{\text{KYE}}\), but not stonin 2\(^{\text{SWP}}\), retained its ability to associate with AP-2 (Fig. 4 A). Similar results were seen for a stonin 2 mutant in which Y784 had been exchanged for R (stonin 2\(^{\text{R}}\); Fig. 4 B). This was corroborated in direct binding assays using \(^{35}\text{S}\)-labeled stonin 2\(^{\text{R}}\) (Fig. S3 C). To exclude the possibility that the introduced mutations negatively impact protein folding and, therefore, nonspecifically affect synaptotagmin binding, we probed the structure of the generated stonin 2 mutants by limited proteolysis. The digestion patterns of stonin 2\(^{\text{WT}}\), stonin 2\(^{\text{KYE}}\), and stonin 2\(^{\text{R}}\) were not found to be significantly different (Fig. S4, available at http://www.jcb.org/cgi/content/full/jcb.200708107/DC1), excluding gross structural changes induced by the mutations.

**Synaptotagmin-binding-defective stonin 2 is unable to facilitate synaptotagmin 1 internalization in fibroblasts or neurons and fails to accumulate at synapses**

To study the functional importance of the interaction between stonin 2 and synaptotagmin 1, we performed endocytosis experiments in HEK–synaptotagmin 1 cells coexpressing either stonin 2\(^{\text{WT}}\) or synaptotagmin 1–binding–deficient mutants thereof. Neither stonin 2\(^{\text{KYE}}\) nor stonin 2\(^{\text{R}}\) mutants were able to facilitate AP-2–dependent synaptotagmin 1 internalization (Fig. 4, C and D). Stonin 2\(^{\text{KYE}}\) and stonin 2\(^{\text{R}}\) also failed to become recruited to the plasmalemma by overexpressed synaptotagmin 1 in N1E neuroblastoma cells (Fig. 5). Quantitative fluorescence-based analysis of synaptotagmin 1 internalization indicated that mutations of individual residues within the KYE site to alanines reduced the efficiency of endocytosis and that these effects were additive (Fig. 4 D). Based on these findings, we conclude that the direct association between stonin 2 and synaptotagmin 1 is necessary for the physiological function of stonin 2 as a synaptotagmin-specific endocytic sorting adaptor.

In primary neurons, stonin 2 localizes to pre-SV clusters (Diril et al., 2006). We hypothesized that this localization might be caused by its interaction with synaptotagmin 1, at least in part. We transfected primary rat hippocampal neurons with HA-tagged stonin 2\(^{\text{WT}}\), stonin 2\(^{\text{KYE}}\), or stonin 2\(^{\text{R}}\), respectively, and studied their localization by indirect immunofluorescence microscopy. As expected, stonin 2\(^{\text{WT}}\) colocalized with synaptotagmin 1 in pre-SV clusters. In contrast, we were unable to detect such colocalization in the case of the synaptotagmin-binding–defective stonin 2 mutants, stonin 2\(^{\text{KYE}}\), or stonin 2\(^{\text{R}}\) (Fig. 6 A). Thus, stonin 2 represents the first example of an endocytic protein known to be targeted to synapses by interaction with a SV protein, further emphasizing its important role as a specialized adaptor dedicated to SV recycling.

We then quantitatively analyzed the effect of stonin 2\(^{\text{WT}}\) or the synaptotagmin-binding–defective KYE mutant on the partitioning of synaptotagmin 1 between the presynaptic plasmalemma or an internal SV-localized pool in primary hippocampal neurons in culture. To this aim, we used a previously described approach using eclipitic pHluorin-tagged synaptotagmin 1 (syt-pHluorin). SytPHluorin is properly targeted to synapses where it undergoes activity-dependent exo-endocytic cycling (Diril et al., 2006). The pH dependence of the pHluorin fluorescence allows quantitative monitoring of the distribution of the synaptotagmin chimera (Wienisch and Klingauf, 2006). Fluorescence analysis after acid quenching and ammonium dequenching (Fig. 6, B and C) revealed that coexpression of stonin 2\(^{\text{WT}}\) significantly decreased the relative steady-state plasmalemmal fraction of sytPHluorin at presynaptic boutons, resulting in a strong increase of the vesicular/surface stranded pool ratio, as reported previously (Diril et al., 2006). In contrast, stonin 2\(^{\text{KYE}}\) had lost the ability to facilitate targeting of sytPHluorin to the recycling vesicle pool but instead displayed a modest, albeit statistically insignificant, dominant-negative effect, i.e., led to a small decrease in the vesicular/surface stranded pool ratio of sytPHluorin (Fig. 6 D). Thus, the ability of stonin 2 to associate with synaptotagmin 1 is essential for its role as a synaptotagmin-specific sorting adaptor in neurons.

**Synaptotagmin-binding-defective UNC-41B is unable to rescue paralysis in *C. elegans* mutant animals**

As mentioned in the preceding paragraph, the \(\mu\text{HD}\) of stonins, and in particular the KYE site responsible for its association with synaptotagmin 1, is highly conserved between different stonin family members from mice to worms (Fig. 3 A). The *C. elegans* genome contains a single member of the stonin family encoded by *unc-41*, expressed as two alternative transcripts A and B (unpublished data). UNC-41B expressed in fibroblasts, like its mammalian counterpart stonin 2, was able to associate with the C2 domains of synaptotagmin 1, which is consistent with its putative role in SV recycling. Mutation of the KYE site within UNC-41B strongly impaired its synaptotagmin 1–binding ability (Fig. 7 A). To investigate whether UNC-41 plays a
role in neurotransmission, we monitored animal movement. As expected, unc-41(e268) mutant worms carrying a nonsense mutation that abrogates UNC-41 protein expression displayed severe locomotion defects, presumably because of impaired neurotransmission. This phenotype was rescued by microinjection of a construct expressing WT UNC-41B cDNA but not the synaptotagmin interaction–defective δKYE mutant (Fig. 7, B and C), although both proteins were expressed at roughly similar levels (Fig. 7 D). Moreover, a 25-fold increase in injection concentration of the δKYE mutant protein-encoding plasmid also did not result in rescue of the locomotory phenotype (unpublished data). Similar results were seen in rescue experiments using GFP-tagged UNC-41B variants (unpublished data).

When analyzed by confocal microscopy, WT GFP–UNC-41B exhibited a punctate synaptic distribution within the C. elegans nervous system, whereas the δKYE mutant protein failed to accumulate at synapses (Fig. 7 E). These experiments suggest that the KYE site is important for UNC-41B association with synaptotagmin and its targeting to synapses, although at this stage we cannot rule out the possibility that other factors contribute to this phenotype in vivo.

In summary, our data reveal the molecular basis for the recognition of synaptotagmin 1 by stonin 2 and suggest an evolutionarily conserved mechanism of cargo recognition by stonin family members during exo-endocytic SV cycling.

Discussion
This study provides the first detailed molecular characterization of the interaction interface between the SV protein–specific CLASP stonin 2 and its cargo, synaptotagmin 1. Previous studies have implicated the synaptotagmin 1 C2B domain in clathrin/AP-2–mediated endocytosis and SV recycling (Haucke et al., 2000; Littleton et al., 2001; Poskanzer et al., 2006). We demonstrate a role for the synaptotagmin 1 C2A domain as the main recognition site for stonin 2 and as a major determinant for synaptotagmin 1 internalization. The following lines of evidence support this. First, we find that synaptotagmin 1, lacking the C2B domain, is readily internalized in a stonin 2–dependent manner in transfected fibroblasts. Second, stonin 2 is recruited to the plasma membrane of transfected neuroimmune endocrine (NIE) cells by synaptotagmin 1 ΔC2B but not by a ΔC2A domain mutant.
Figure 6. The stonin 2 KYE site is required for synaptic localization of stonin 2 and facilitates targeting of synaptotagmin 1 to recycling vesicles in primary neurons. (A) Primary hippocampal neurons at DIV9 were transfected with HA–stonin 2WT, –stonin 2YR, or –stonin 2δKYE, respectively. Monoclonal antibodies against synaptotagmin 1 are used to decorate presynaptic sites (red). Low-power views (top) exemplify the overall distribution of HA-tagged stonin 2 or mutants (green; insets, threefold magnified images of boxed area). High-power views (bottom) represent the localization of HA–stonin 2 or mutants in selected neurites. Bars, 10 μm. (B–D) Stonin 2WT but not stonin 2δKYE mutant enhances targeting of sytpHfluorin to recycling vesicles in primary hippocampal neurons. (B) To assess the surface and vesicular pools of sytpHfluorin, the relative fluorescence (F) values were measured after acid quenching and ammonium dequenching. Bar, 10 μm. (C) Quantitative analysis of n = 7 experiments, each comprising >50 boutons as in B. Mean time course of sytpHfluorin at synaptic boutons normalized to initial F values is shown. (D) Ratios of vesicular/surface stranded pools of sytpHfluorin (n = 7 experiments; error bars represent SEM; control, 3.05 ± 0.3; stonin 2WT, 4.28 ± 0.19; stonin 2δKYE, 2.6 ± 0.15; **, P < 0.01). Expression of WT stonin 2 results in a significant increase in the vesicular/surface pool ratio, whereas stonin 2δKYE has lost this ability.
The KYE site is required for UNC-41 function in locomotory behavior in *C. elegans.* (A) Mutation of the KYE site within *C. elegans* UNC-41B abolishes its ability to bind to synaptotagmin 1. GST or GST fused to the C2 domains of rat or *C. elegans* (Ce) synaptotagmin 1 immobilized on 30-μg protein beads were incubated with cell extracts derived from Cos7 cells transfected with HA–UNC-41B or HA–UNC-41B δKYE. Bound proteins were detected by immunoblotting for HA–UNC-41B, actin, and AP-2μ. 4% of the input was loaded as standard. (B and C) Worm tracking assay for unc-41 mutant (unc-41) and rescued animals. (B) Representative worm moving traces over 10 min. The number of worms on each plate is indicated. (C) The mean speed for each 2 s was used and the percentage of the speed below a certain threshold was plotted for each genotype. Data are given as mean ± SEM. (D) Confocal images of immobilized worms expressing endogenous UNC-41 or plasmid-borne UNC-41B WT or the δKYE mutant analyzed by indirect immunofluorescence microscopy using UNC-41–specific antibodies. Bar, 10 μm. (E) Confocal images of immobilized worms expressing GFP–UNC-41B WT or the δKYE mutant. A magnified view of the boxed area from the dorsal cord is shown to the right of the image. Bar, 10 μm.
Third, direct binding, as well as coimmunoprecipitation experiments, reveals that conserved basic residues within C2A form the main interaction site for stonin 2. In contrast, AP-2 predominantly associates with the C2B domain, which is consistent with earlier data (Chapman et al., 1998; Haucke et al., 2000). We hypothesize that the identified basic patch within the C2A domain is part of an endocytosis signal. However, synaptotagmin 1 mutants displaying reduced affinity for stonin 2 (Fig. 2D) appeared to retain the ability to undergo stonin 2-dependent internalization (not depicted), suggesting that additional determinants and/or cooperative effects are likely to be involved. It is worthwhile to note that both C2 domains contain additional positively charged surface-exposed side chains that could well serve as additional interaction sites for stonin 2.

Site-directed mutagenesis paired with structure-based homology modeling has allowed us to unravel the cognate recognition site for synaptotagmin 1 within the μHD of stonin 2. Mutational analysis reveals the functional importance of a β strand, including residues K783–E785, that is evolutionarily conserved between different members of the stonin/stoned B family of adaptors from nematodes to mammals (Fig. 3A). We propose that the function of stonin 2 as a synaptotagmin-specific endocytic sorting adaptor dedicated to SV recycling is based on the ability of residues outlined by the KYE site to interact with patches of basic side chains within the synaptotagmin C2 domains. This proposal is based on the observations that stonin 2KYE is unable to interact with synaptotagmin directly in vitro or in living cells, to facilitate synaptotagmin 1 endocytosis in fibroblasts, to become enriched at presynaptic sites in primary neurons, or to target synaptotagmin 1 to the recycling vesicle pool at synapses. With all mutant proteins, a valid concern is that the mutations may affect protein structure and/or stability.

We feel that this is unlikely for several reasons. When expressed in fibroblasts or primary neurons, stonin 2KYE appeared to be expressed at levels comparable to those of its WT counterpart. Moreover, it retained its ability to associate with AP-2 in vitro and in living cells or to become targeted to clathrin-coated pits in primary astrocytes (unpublished data). When probed for structural integrity by limited proteolysis using different proteases, two independent mutants of stonin 2 (6KYE and Y784R) gave rise to fragmentation patterns virtually identical to those seen for the WT. A possible, yet speculative model for the recognition of synaptotagmin 1–C2A by the μHD of stonin 2 based on our collective mapping data is shown in Fig. S5 (available at http://www.jcb.org/cgi/content/full/jcb.200708107/DC1). Considering the high degree of sequence conservation with regard to synaptotagmin 1–C2 domains and stonin/stoned B family members, we consider it likely that other stonins, such as stoned B, for D. melanogaster, where stonin 2/stoned B is encoded by an essential gene (Ferguson and Brodie, 2001; Stimson et al., 2001). One possibility to explain the pivotal roles of stoned proteins could be the need for sorting during SV cycling. SV recycling requires constitutive cargo to be excluded from the forming vesicle and, thus, might benefit from the presence of a specific stonin family sorting adaptor. The presence of stonin 2 allows concentration of SV proteins, including synaptotagmin 1 and its partners, independently of the requirements of AP-2 for recognition of constitutive cargo (i.e., activation by kinases such as adaptor-associated kinase 1 and cyclin G–associated kinase), such as transferrin or EGF receptors. In addition, the use of C2A as an interaction interface for stonin 2 may alleviate constraints on SV cargo recognition imposed by the multiplicity of binding partners targeting the C2B domain of synaptotagmin 1. Multiple mechanisms of regulation of SV endocytosis by synaptotagmin 1 have recently been observed in D. melanogaster (Poskanzer et al., 2006). Mechanistically, the action of stonin 2 and its orthologues stoned B (D. melanogaster) and UNC-41B (C. elegans) may therefore resemble that of other CLASPs, including β-arrestins (Traub, 2005) or dishevelled-2 (Yu et al., 2007), in targeting cargo to subsets of clathrin-coated vesicles (Puthenveedu and von Zastrow, 2006). In the case of the presynaptic compartment, a precise fine tuning of the endocytic process is required to maintain the exact composition of SV proteins and lipids (Takamori et al., 2006) and to ensure release competence. The present study could form a first basis for the mechanistic understanding of this process.

Materials and methods

Cell culture and transfections

HEK293 and NIE-115 cells were cultured in DMEM (Invitrogen) containing 4.5 g/liter glucose and NIE cells in DMEM containing 1 g/liter glucose, supplemented with 10% FCS, penicillin, and streptomycin. Culturing of primary hippocampal neurons has been previously described (Mueller et al., 2004). Cell lines as well as primary neurons were transfected using Lipofectamine 2000 (Invitrogen). Calcium phosphate transfection was used for spfHLucin recycling assays. Doxycycline-regulable HEK293 cell lines were generated using the T-Rex system (HEK-Kstr2WT, HEK-Kstr2KYE, Invitrogen). HEK293 cells stably expressing luminaly FLAG-tagged synaptotagmin 1 (HEK-syt1) have been previously described (Diril et al., 2006). For morphological experiments, cells were grown on Matrigel-coated glass coverslips.

Plasmids and DNA constructs

The following amino terminally HA-tagged human stonin 2 constructs were made by inserting PCR products into EcoRV-Xbal restriction sites in pCH2A: HA–stonin 2 (aa 421–899), HA–stonin 2 (aa 1–553), and HA–stonin 2KYE (W15A, W102A, and W232A). We generated plasmid construct, allowing for the expression of luminaly FLAG-tagged rat synaptotagmin 1 by introducing the cDNA into the BamHI–XhoI restriction sites into the pFLAG vector. By application of PCR site-directed mutagenesis, we prepared several synaptotagmin 1 mutation constructs: ΔC2B (1–265), ΔC2A (Δ140–270), ΔC2AB [1–139], and syt1mut (K189–192E; K213E; K244E; and KR321,322EE, K324–327E). The following rat synaptotagmin 1 GST fusion constructs were generated using pGEX4T-1 (BamHI–XhoI): C2A (Δ140–265), C2B (Δ271–421), C2AB (Δ140–421), C2Amut (KK189, 190AA), C2Amut (KK191, 192AA), C2Amut (KK189, 192AA), C2Amut (KK191H, K213E, and K244s), C2Amut (KK189–192A, K213E, and K244s), C2Amut (KK326, 327AA), C2Bmut (KK324–327A), and C2Amut (KK321, 322AA; and K324–327A). Constructs allowing for the expression of HA-tagged human stonin 2 were generated as previously described (Walker et al., 2004; Diril et al., 2006). The following mutants were prepared by PCR.
site-directed mutagenesis: HA-stonin 2 was generated as described previously (Wolther et al., 2004). Monoclonal antibodies against the α subunit of AP-2 (clone AP6) and clathrin heavy chain (clone CD1) were a gift from P. De Camilli (Yale University, New Haven, CT). Mono- (clone M2) or poly- (clone 2H3) antibodies to α-actin were obtained from Babco, Santa Cruz. E. coli strain DH5α was transformed with pET-28a-α-actin, pET-28a-α-actin::HA-stonin 2 (E785A), and HA-stonin 2YR (Y784A). Cell extracts were prepared and proteins were affinity purified using GST-bind resin (EMD). 

Expression and purification of GST–synaptotagmin 1 fusion proteins in Escherichia coli (ER2566) at 25°C for 3 h after induction with 0.5 mM IPTG. Bacterial pellets obtained from 1-liter cultures were suspended in 100 ml PBS. Cells were lysed using lysozyme, 1% Triton X-100, benzamidine (to remove possible nucleic acid contaminants), and sonication. The bacterial extract was cleared by centrifugation at 39,000 g for 15 min and GST fusion proteins were affinity purified using GST-bind resin (EMD). 

Stonin 2 WT and ΔkYΕ mutant proteins were affinity purified from HEK12-sr2mβ and HEK12-sr2mα cells using Ni-NTA agarose (Qiagen). Protein expression was induced by addition of 1 μg/ml doxycycline to the growth medium for at least 16 h before cells were lysed in homogenization buffer (20 mM Hepes, pH 7.4, 150 mM NaCl, 2 mM MgCl₂, 1 mM PMSF, 0.1% mammalian protease inhibitor cocktail [Sigma-Aldrich]) using a ball-bearing cell cracker with a clearance of 12 μm. The cell extract was cleared by centrifugation at 20,000 g for 15 min and 100,000 g for 15 min. The supernatant was supplemented with 500 mM NaCl, 1% CHAPS, 1 mM DTT, 10 mM imidazole, and 1 mM PMSF before application on Ni-NTA Agarose for 2 h at 4°C on a rotating wheel. The beads were washed twice in washing buffer (20 mM Hepes, pH 7.4, 500/150 mM NaCl, 2 mM MgCl₂, 320 mM sucrose, 1% CHAPS, 1 mM DTT, and 10 mM imidazole) for 10 min. 

Affinity chromatography, in vitro binding, and immunoprecipitation experiments 24–48 h after transfection, transiently transfected HEK293 cells were lysed in 20 mM Hepes, 100 mM KCl, 2 mM MgCl₂, 1% Triton X-100, 1 mM PMSF, 0.3% mammalian protease inhibitor cocktail for 10 min on ice. Cell extracts were incubated with 1 μg/ml of GST fusion proteins on a shaker at 56°C for 15 min. The supernatant was removed and sub-duced to SDS-PAGE and immunoblotting with the His-tag. 

Samples were analyzed by SDS-PAGE and immunoblotting. For in vitro binding experiments, 2–3 μg of immobilized GST–synaptotagmin 1 fusion proteins were incubated with 600 ng to 1 μg stonin 2–His, in 100 μl of binding buffer (20 mM Hepes, pH 7.4, 150 mM NaCl, 2 mM MgCl₂, 320 mM sucrose, 1% CHAPS, 1 mM DTT, and 10 mM imidazole) for 2 h at 4°C. After three washes in binding buffer, the beads were eluted in 50 μl of sample buffer. Samples were analyzed by SDS-PAGE and immunoblotting. 

For some experiments, radioactively labeled 35S-labeled stonin 2 was synthesized by the coupled TN2 in vitro transcription/translation kit (Promega), incubated with 2 μg GST–synaptotagmin 1 fusion proteins in 100 μl of binding buffer for 2 h at 4°C on a rotating wheel. After three washes in binding buffer, the beads were eluted in 50 μl SDS-PAGE sample buffer and the entire sample was analyzed by SDS-PAGE and autoradiography. 

Tryptic in gel digest and mass spectrometry The SDS polyacrylamide gel, containing the proteins of interest, was stained using the freshly prepared colloidal Coomassie and destained according to the manufacturer’s instructions (Roth). Gel bands were excised under clean conditions with new razor blades and cut into 1-mm³ pieces. Gel fragments were transferred to a 500-μl reaction tube and incubated in a shaker in 20 μl of a 1:1 solution of acetonitrile/100 mM NH₄HCO₃ for 15 min. Samples were centrifuged for a short time and supernatant was exchanged for 100% acetonitrile and incubated for 5 min or until the gel pieces turned white. Acetonitrile was removed and gel pieces lyophilized for 10 min. For reduction of disulfide bridges, the lyophilized gel pieces were incubated in 20 μl of 100 μM DTT in 100 mM NH₄HCO₃ for 30 min at 56°C. After incubation, samples were centrifuged for a short time, supernatant was removed, and volume was measured. Gel fragments were again dehydrated twice by the addition of 20 μl of 100% acetonitrile. Cysteine residues were covalently modified by carbodiimide/methylenediamine by addition of 20 μl of 55 mM iodoacetamide in 100 mM NH₄HCO₃ and incubation for 20 min at room temperature in the dark. Supernatant was removed and exchanged for 100 mM NH₄HCO₃ and incubated for 15 min at room temperature. Gel pieces were incubated in 20 μl of 100% acetonitrile until they turned white and lyophilized for 10 min. 12.5 μg/ml trypsin in 25 mM NH₄HCO₃ was prepared and added to the lyophilized gel pieces (volume = 20 μl – volume of supernatant, measured after reduction in DTT + 3 μl). Samples were placed for 30 min on ice before incubation at 37°C overnight. Samples were centrifuged and again incubated in DTT for 30 min before –3 μl of the supernatant were removed and subjected to mass spectrometric analyses.

Limited proteolysis We performed limited proteolysis to assess the folding properties of stonin 2 mutants by digestion of stonin 2–His, purified from stable HEK cells in vitro-translated stonin 2, 0–20 μg/ml Tryptsin and 0–80 ng/ml Proteinase K were chosen for this purpose. Proteolysis reactions were performed in 20 mM Hepes, pH 7.4, 100 mM KCl, and 2 mM MgCl₂ for in vitro–translated stonin 2 and in 20 mM Hepes, pH 7.4, 150 mM NaCl, 2 mM MgCl₂, 320 mM sucrose, 1% CHAPS, 1 mM DTT, and 10 mM imidazole for purified stonin 2–His, for 10 min at 37°C. In vitro–translated digested protein was analyzed by SDS-PAGE and autoradiography and stonin 2–His, by immunoblotting for the His-tag.

Antibody internalization and membrane recruitment assays For indirect immunofluorescence microscopy, HEK293 cells stably expressing FLAG–synaptotagmin 1 were incubated with indicated field of stonin 2 mutants by digestion of stonin 2–His, purified from stable HEK cells or in vitro–translated stonin 2, 0–20 μg/ml Tryptsin and 0–80 ng/ml Proteinase K were chosen for this purpose. Proteolysis reactions were performed in 20 mM Hepes, pH 7.4, 100 mM KCl, and 2 mM MgCl₂ for in vitro–translated stonin 2 and in 20 mM Hepes, pH 7.4, 150 mM NaCl, 2 mM MgCl₂, 320 mM sucrose, 1% CHAPS, 1 mM DTT, and 10 mM imidazole for purified stonin 2–His, for 10 min at 37°C. In vitro–translated digested protein was analyzed by SDS-PAGE and autoradiography and stonin 2–His, by immunoblotting for the His-tag.

Membrane recruitment experiments in NIE-115 cells were performed as in Diril et al. (2006). Membrane recruitment experiments in NIE-115 cells were performed as in Diril et al. (2006).

Images were taken at room temperature by a charge-coupled device camera (AxioCam; Carl Zeiss, Inc.) mounted on an inverted microscope (Axiovert 200M; Carl Zeiss, Inc.) with an oil-immersion objective (1.4 NA; Carl Zeiss, Inc.) illuminated and controlled by the Stentor Kinetic Imaging system (Intelligent Imaging Innovations, Inc.). Imaging data were digitized, analyzed, and processed by nearest neighbor deconvolution with Slidebook 4.210 software (Intelligent Imaging Innovations, Inc.).
Syntrophilin recycling assays in living neurons

Published procedures were used to assay syntrophilin recycling in neurons (Diril et al., 2006). In brief, hippocampal neurons from 1–3-d-old Wistar rats were transfected by calciumphosphate DNA coprecipitation and used for SYTOX Green staining. Neurons were fixed with 4% paraformaldehyde for 20 min, followed by permeabilization with 0.1% Triton X-100 for 5 min and blocked with 1% BSA. Primary antibodies were diluted in blocking buffer and incubated overnight at 4°C. Secondary antibodies were diluted in blocking buffer and incubated for 1 h at room temperature. After incubation, the coverslips were mounted on slides using Prolong Gold antifade reagent (Invitrogen). The images were obtained with a confocal microscope (LSM510, Carl Zeiss) using an oil immersion objective (Plan-Apochromat 100×/1.4 NA; Carl Zeiss, Inc.) and analyzed by confocal software (Image J).

C. elegans strains, microinjection, and confocal imaging

The following strains were used for rescue experiments: WT C. elegans [Bristol N2]; C836B: unc-41[e268]; EG499: unc-41[e268]; oxEx920[Punc-41::unc-41 B and unc-41 cDNA], Pcc::GFP, Pcc::GFP; and oxEx922[Punc-41::unc-41 B KYE→AAA cDNA], Pcc::GFP, Pcc::GFP. Strains used for confocal imaging were: EG4741: oxEx1050[Punc-41::unc-41 B and unc-41 cDNA WT], Pcc::GFP; EG4746: oxEx1055[Punc-41::unc-41 B and unc-41 cDNA KYE→AAA WT], Pcc::GFP; and EG4751: oxEx1059[Punc-41::unc-41 B and unc-41 cDNA KYE→AAA mutant], Pcc::GFP. Strains used for rescue experiments: 50 ng/μl Punc-122: GFP were used as an injection marker was mixed with 1 kb DNA ladder (Fermentas) as carrier DNA to give a final DNA concentration of 100 ng/μl. RM#536p[Punc-41::unc-41 B and unc-41 cDNA WT], or KYE→AAA mutant constructs or analogous GFP fusion proteins (pMG313/14: Punc-41::unc-41 B and WT) were injected at 1 ng/μl into unc-41 WT embryos. Worms were immobilized with 2% phenoxyl propanol, and GFP fluorescence was imaged at room temperature on a confocal laser-scanning microscope (LSM510 Pascal) using an oil-immersion objective (plan-Neofluar 100×/1.3 NA; Carl Zeiss, Inc.) and analyzed by confocal software (Carl Zeiss, Inc.).

Worm-tracking assay

1% unseeded 50-mm agar plates containing 0.004% Bromphenol blue were prepared. Right before the assay, 200 ml 2% OP50 E. coli were spread onto the assay plates to analyze worm feeding behavior. Four to five worms were put on a single plate. Worm positions were recorded every 2 s for 10 min. Images were analyzed by worm tracker 06 (An Image) plug-in developed by J. White, University of Utah, Salt Lake City, UT. 300 mean speed values were collected for each worm. 6–10 worms were analyzed per genotype.

Molecular modeling and multiple sequence alignments

Multiple protein sequence alignments were performed using the ClustalW program [available at http://www.ebi.ac.uk/ClustalW/]. The x-ray structure of μ2 was used as a structural template for the model of stonin 2–μHD (PDB:563 EB75; 1GWS: chain M, G165-C435). Additional fragments with sequence homology to other known Protein Database structures (1BWU and 2FEA) were used to complete the model for residues 622–638 and 736–772 of stonin 2 for which corresponding loop regions in μ2 were not resolved or no homologous sequence was available in the μ2 template. The x-ray structure of syntaptotagmin 1–C2A (PDB:1BYN: E140K267) was used to create the interaction model shown in Fig. S5. Interaction models considering complementary shape, electrostatic potentials, and data from rotational analysis were generated by manual docking using the bio-polymer module of SYBYL 7.2 and minimized with an AMBER 7.0 force field within SYBYL (Tripos, Inc.).

Online supplemental material

Fig. S1 shows association of stonin 2 with syntaptotagmin 1. Fig. S2 shows that stonin 2 directly binds to syntaptotagmin 1 in vitro. Fig. S3 shows that the interaction between stonin 2 and syntaptotagmin 1 depends on residues within the carboxy-terminal μHD of stonin 2. Fig. S4 shows that limited proteolytic digests of stonin 2 WT, ΔKYE, and ΔY mutants indicate structural integrity of the mutated proteins. Fig. S5 shows a hypothetical model of the interaction between stonin 2 (gray) and the C2A domain of syntaptotagmin 1 (yellow) via two parallel β strands. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200708017/DC1.

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