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Stonin 2 Is an AP-2-Dependent Endocytic Sorting Adaptor for Synaptotagmin Internalization and Recycling

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Summary

Clathrin-mediated endocytosis is involved in the internalization, recycling, and degradation of cycling membrane receptors as well as in the biogenesis of synaptic vesicle proteins. While many constitutively internalized cargo proteins are recognized directly by the clathrin adaptor complex AP-2, stimulation-dependent endocytosis of membrane proteins is often facilitated by specialized sorting adaptors. Although clathrin-mediated endocytosis appears to be a major pathway for presynaptic vesicle cycling, no sorting adaptor dedicated to synaptic vesicle membrane protein endocytosis has been indentified in mammals. Here, we show that stonin 2, a mammalian ortholog of *Drosophila* stoned B, facilitates clathrin/AP-2-dependent internalization of synaptotagmin and targets it to a recycling vesicle pool in living neurons. The ability of stonin 2 to facilitate endocytosis of synaptotagmin is dependent on its association with AP-2, an intact μ-homology domain, and functional AP-2 heterotetramers. Our data identify stonin 2 as an AP-2-dependent endocytic sorting adaptor for synaptotagmin internalization and recycling.

Introduction

Clathrin-mediated endocytosis represents the major route by which hormones, receptors, and signaling factors are internalized. While most constitutively internalized cargo proteins may be recognized directly by the heterotetrameric adaptor complex AP-2 via conventional tyrosine- (Yxxø, with ø representing a large hydrophobic residue) or dileucine-based signals (Bonifacino and Traub, 2003), stimulation- or ligand-induced endocytosis of membrane proteins is often facilitated by specialized sorting adaptors, including β-arrestins (Lefkowitz and Whalen, 2004), Dab2, ARH, HIP1 (Traub, 2003), Numb (Santolini et al., 2000), or eps15 (Bonifacino and Traub, 2003). The phosphotyrosine binding domain within Dab2 or ARH simultaneously interacts with an FxNPxY internalization motif within the cytoplasmic tail of the low-density lipoprotein receptor (LDLR) and with phosphatidylinositol (4,5)-bisphosphate, thereby targeting LDLRs for clathrin-mediated endocytosis. ARH- or Dab2-mediated LDLR internalization also requires an AP-2 appendage domain and clathrin binding sequences, suggesting that both proteins function as sorting adaptors specifically dedicated to endocytosis of LDLR family members. Consistent with this, mutations within ARH and Dab2 have been linked to familial hypercholesterolemia (Traub, 2003). Similar roles are fulfilled by β-arrestins, which undergo signal-induced phosphorylation-dependent interactions with G protein-coupled receptors, clathrin, and the β appendage of heterotetrameric AP-2 adaptor complexes (Lefkowitz and Whalen, 2004).

Although multiple lines of evidence suggest that clathrin/AP-2-mediated endocytosis is a major pathway for the activity-dependent recycling of synaptic vesicles (SVs) (Brodin et al., 2000; Murthy and De Camilli, 2003), the precise molecular mechanisms of SV cargo protein recognition have remained elusive. Synaptotagmin 1, an important modulator of calcium-dependent neurosecretion within the SV membrane (Tucker and Chapman, 2002), has been proposed to regulate the exo- and endocytic limbs of the vesicle cycle. Deletion of synaptotagmin 1 in mice (Geppert et al., 1994; Nicholson-Tomishima and Ryan, 2004), worms (Jorgensen et al., 1995), and flies results in pleiotropic defects in neuroexocytosis and impaired retrieval of SV membranes. Interfering with synaptotagmin function by mutation (Littleton et al., 2001), acute chemical inactivation (Poskanzer et al., 2003), or injection of antibodies (Llinas et al., 2004) leads to a partial depletion of the SV pool. How precisely synaptotagmin regulates SV endocytosis is unclear, but direct interactions with the μ2 subunit of the heterotetrameric clathrin adaptor complex AP-2 (Grass et al., 2004; Haucke et al., 2000), the μ-homology domain of stonin 2 (Martina et al., 2001; Walther et al., 2001) (see below), and/or phosphoinositides (Tucker and Chapman, 2002) may contribute to this.

Surprisingly, synaptotagmin 1 itself undergoes efficient endocytic internalization in neurons and neuroendocrine cells, but remains at the plasmalemma in transfected fibroblasts (Jarousse and Kelly, 2001). The molecular mechanisms for synaptotagmin 1 internalization have thus remained obscure. In *Drosophila*, synaptotagmin 1 function is tightly linked to the expression of the stoned B gene product, a putative endocytic accessory protein expressed in the nervous system and enriched at presynaptic nerve terminals (Fergestad and Broadie, 2001; Stimson et al., 2001). Stonins 1 and 2 are the only orthologs of stoned B in mammals (Martina et al., 2001). Stonin 2 has been linked to clathrin-mediated endocytosis by its interactions with AP-2 (Walther et al., 2004) and the accessory proteins eps15 and intersectin (Martina et al., 2001). Binding of stonin 2 to AP-2 is mediated by interactions between its WVxF motifs and the β sandwich domain of the α-adaptin appendage as well as indirect associations bridged by

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eps15 and perhaps other eps15-homology (EH) domain-containing factors.

However, the relationship between stonin 2 function in clathrin-dependent endocytosis and synaptotagmin internalization has not been explored. Here, we show that stonin 2 is an AP-2-dependent sorting adaptor for endocytic internalization and recycling of synaptotagmin.

Results

Interaction of Stonin 2 with Neuronal Synaptotagmin Family Members In Situ and in Living Cells

In order to gain insights into the function of stonin 2 in brain, we first analyzed the distribution of stonin 2 and its binding partners. Antibodies against stonin 2 coimmunoprecipitated the adaptor complex AP-2 and synaptotagmin 1 (Figure 1A). Stonin 2 and synaptotagmin 1 were also both present in material affinity purified with a GST-tagged α-adaptin ear domain fusion protein (Figure S1A; see the Supplemental Data available with this article online), suggesting that stonin 2 may link synaptotagmin 1 to the endocytic adaptor protein complex AP-2. Indeed, direct binding of the GST-tagged μ-homology domain of stonin 2 to the recombinantly expressed purified cytoplasmic C2AB domain of synaptotagmin 1 was observed (Figures S1B and S1C). Consistent with their direct physical association, stonin 2 and synaptotagmin 1 were colocalized at synapses in cultured rat cortical neurons (Figure 1B). The presynaptic distribution of stonin 2 at synaptotagmin 1-immunopositive sites was even more apparent in primary neurons transfected with EGFP-stonin 2. EGFP-stonin 1, by contrast, was largely soluble. (Figure 1C). Transfected stonin 2 also colocalized with other presynaptic vesicle markers such as synaptobrevin 2 and SV2a (Figure S2).

To further explore the putative physical and functional interaction between stonin 2 and different synaptotagmin family members, we made use of N1E-115 neuroblastoma cells. Confocal imaging revealed that overexpressed EGFP-stonin 2 displays a largely cytoplasmic distribution in these cells. Upon cotransfection of synaptotagmin 1, which is targeted to the plasma membrane in N1E cells, stonin 2 became colocalized at the plasmalemma, consistent with our previous data (Walther et al., 2004). Likewise, stonin 2 colocalized with overexpressed synaptotagmins 2 and 9 at the plasma membrane (Figure 1D). In the case of synaptotagmin 9, both proteins were also present on internal, presumably endosomal structures. A similar, albeit much weaker, membrane recruitment phenotype was seen upon cotransfection of EGFP-stonin 2 with synaptotagmins 8 and 11 (Figure S3A). Synaptotagmins 3, 4, 5, 6, 7, and 10 were unable to recruit stonin 2 to the plasmalemma or other internal membranes (Figure 1D and Figure S3A). By contrast, EGFP-stonin 1 retained its cytoplasmic distribution irrespective of synaptotagmin expression (Figure S3B). These data indicate that stonin 2 can functionally interact with synaptotagmins 1, 2, and 9 in living cells. Upon closer inspection of N1E cells coexpressing synaptotagmin 1 and stonin 2 by three-dimensional deconvolution microscopy, we noticed that stonin 2 targeted to the plasmalemma attained a punctate distribution reminiscent of clathrin/AP-2-coated pits. Stonin 2 indeed colocalized with the clathrin adaptor complex AP-2 at the membrane. Synaptotagmin 1 thus targets stonin 2 to plasmalemmal clathrin/AP-2 coated pits in neuroblastoma cells (Figure 1E). Synaptotagmin 1-mediated recruitment of stonin 2 to the plasma membrane could also be observed in transfected Cos7 or HEK293 fibroblasts and in PC12 neuroendocrine cells (data not shown; see below).

Stonin 2 Specifically Facilitates Synaptotagmin 1 Internalization in Fibroblasts by Providing a Physical Link with AP-2

Since AP-2 is involved in clathrin-mediated endocytosis (Robinson, 2004), we analyzed the internalization of synaptotagmin 1 in stonin 2-expressing HEK293 fibroblasts. FLAG-tagged synaptotagmin 1 was targeted to the plasma membrane, from where it was endocytosed very ineffectively. Coexpression of wild-type stonin 2 dramatically increased synaptotagmin 1 endocytosis (Figure 2 and Figure S4). Internalized synaptotagmin 1 and a fraction of stonin 2 were targeted to a perinuclear compartment, resembling early recycling endosomes (Movie S1; see the Supplemental Data available with this article online). The ability of stonin 2 to target synaptotagmin 1 for internalization was strictly dependent on its ability to associate with AP-2. A mutant in which its three α-adaptin appendage domain binding WVxF motifs (Mishra et al., 2004; Praefcke et al., 2004) had been mutationally inactivated (stonin $2^{\delta WWW}$) (Walther et al., 2004) weakly stimulated synaptotagmin 1 endocytosis (Figure 2A), measured either by fluorescence-based quantification of internalized antibodies in stably transfected HEK293 cells (Figure 2B) or by using a biochemical assay (Figure S4). This effect was completely lost upon mutation of its two eps15 binding NPF motifs (Figure S4; compare also Figure 6). However, the stonin $2^{\delta WWW}$ mutant retained the ability to become recruited to the plasma membrane in synaptotagmin 1-transfected HEK293 cells (Figure 2A). Expression of stonin 2 could also facilitate endocytosis of synaptotagmin 2, but not synaptotagmin 6, a family member that was unable to recruit stonin 2 to membranes (Figures S5A and S5B). The ability of stonin 2 to facilitate synaptotagmin 1 internalization correlated with the efficiency with which stonin 2 could be coimmunoprecipitated with both AP-2 and synaptotagmins 1 or 2, but not 6 (Figure S5C), from HEK293 cell extracts. Stonin 2 and synaptotagmin 1 were both found in AP-2 immunoprecipitates obtained from lysates of cells coexpressing wild-type stonin 2 and synaptotagmin 1. By contrast, neither stonin 2 nor synaptotagmin 1 were found to be associated with AP-2 in cells transfected with the stonin $2^{\delta WWW}$ mutant. Clathrin was absent from all of these samples (Figure 2C). These data suggest that synaptotagmin 1 is physically linked to AP-2 via stonin 2. In agreement with this, both AP-2 and synaptotagmin 1 could be coimmunoprecipitated with antibodies against stonin 2 from lysates of transfected fibroblasts (Figure S5C) or rat brain extracts (see Figure 1A).

In order to further explore the specificity of stonin 2 functions, we analyzed the endocytic internalization of other clathrin-dependent cargo membrane proteins. A HEK293 cell line stably expressing stonin 2 after induction with doxycycline endocytosed fluorescently

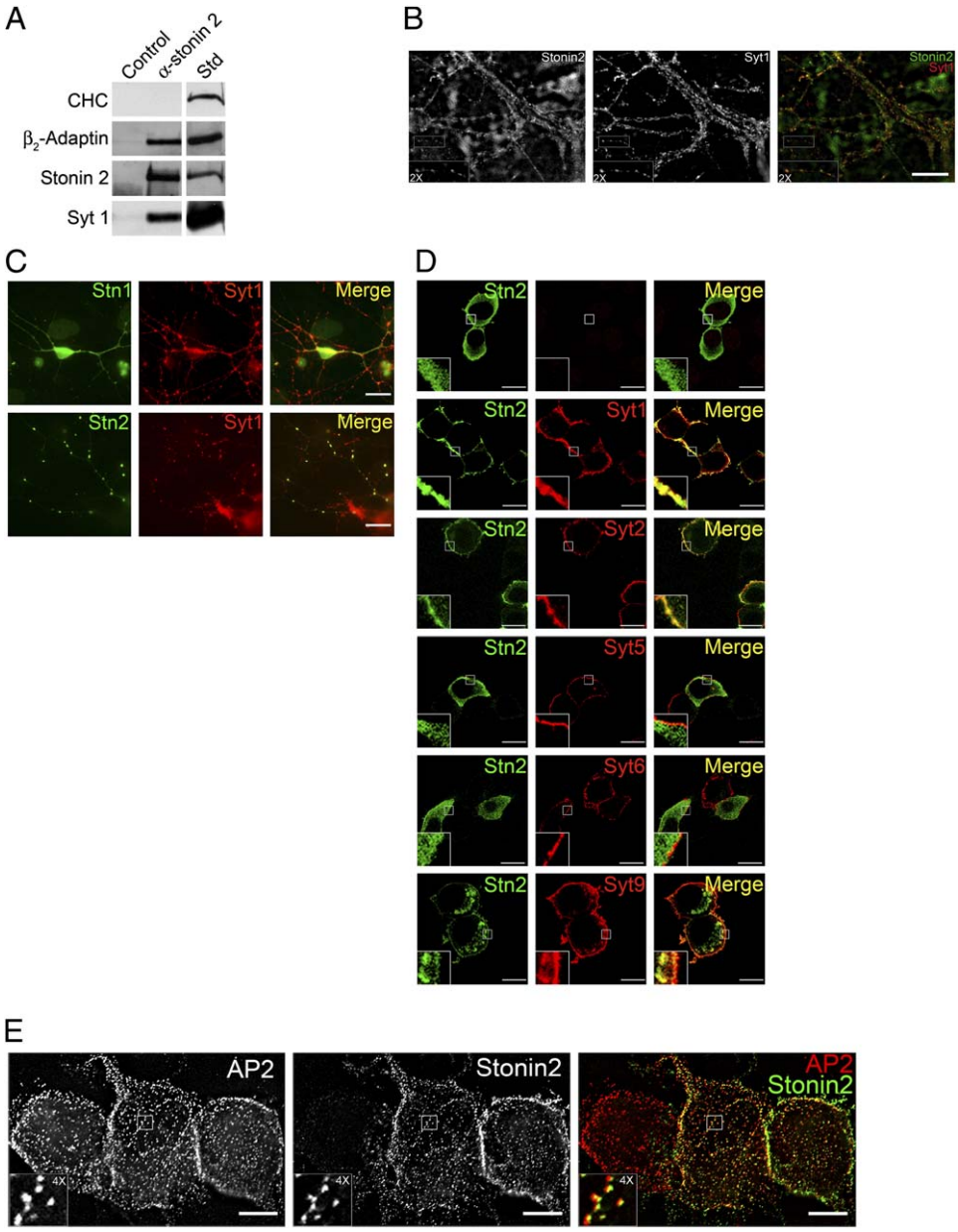

Figure 1. Colocalization and Functional Interaction of Stonin 2, but Not Stonin 1, with Synaptotagmin Family Members

(A) Stonin 2 coprecipitates with AP-2 and synaptotagmin 1 in brain. 1 mg rat brain Triton X-100 extracts were treated with either control beads or stonin 2 antibody-coupled protein A/G sepharose beads. AP-2 and synaptotagmin 1, but not clathrin, are coimmunoprecipitated together with stonin 2. Std, 20 μg total protein extract.

(B) Rat brain cortical neurons cultured for 3 weeks in vitro were fixed and immunostained with antibodies against stonin 2 (green) and synaptotagmin 1 (red) and were analyzed by deconvolution fluorescence microscopy. Inset magnification: 2×. The scale bar is 20 μm.

(C) Rat brain hippocampal neurons were transfected with EGFP-stonin 1 or EGFP-stonin 2 and were analyzed 1 week posttransfection. The scale bar is 20 μm.

(D) Confocal images of N1E-115 neuroblastoma cells cotransfected with EGFP-stonin 2 and synaptotagmins 1, 2, 5, 6, and 9. Inset magnification: 4×. The scale bar is 16 μm.

(E) N1E-115 cells were cotransfected with stonin 2 and synaptotagmin 1. Pictures were obtained by constrained iterative deconvolution of serial images taken along the z axis. Inset magnification: 4×. The scale bar is 10 μm.

labeled transferrin at approximately the same rate as noninduced controls (Figures 3A and 3B), whereas FLAG-synaptotagmin 1 internalization was greatly facilitated (Figure 3D). Consistent with our previous results, we observed an inhibition of transferrin internalization in cells transiently overexpressing stonin 2 to very high levels, resulting in sequestration of AP-2 (data not shown; Walther et al., 2004). Tac receptor chimeras harboring either a tyrosine (Yxxø)- or a dileucine (D/ExxxLL)-based endocytosis signal (Letourneur and Klausner, 1992), both of which have been shown to bind directly to AP-2 (Robinson, 2004), were also endocytosed in

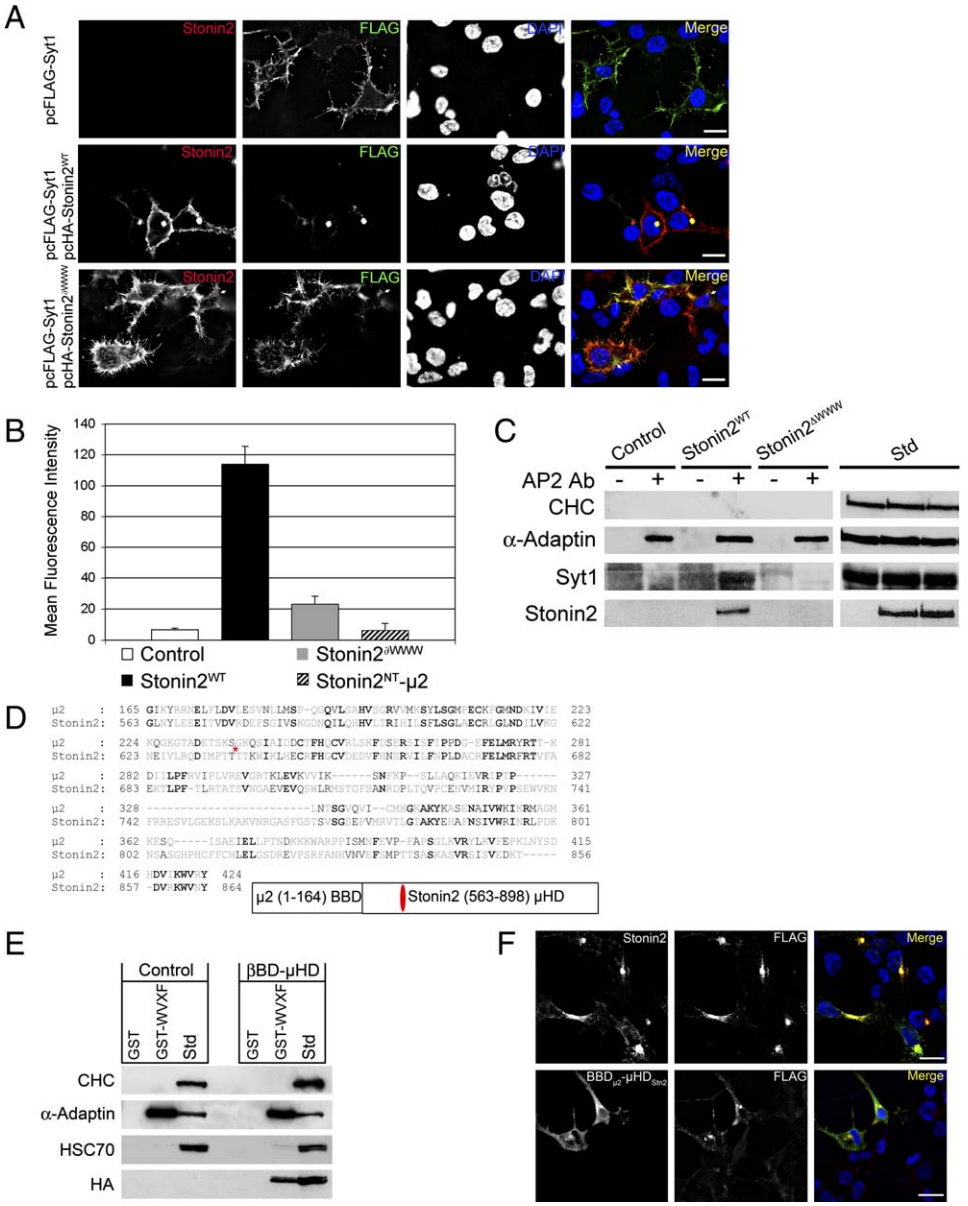

Figure 2. Stonin 2 Facilitates Endocytosis of Plasma Membrane-Stranded Synaptotagmin 1 in HEK293 Cells

(A) HEK293 cells expressing lumenally FLAG-tagged synaptotagmin 1 were labeled with anti-FLAG antibodies for 30 min on ice, followed by incubation for 2 hr at 37ºC. Coexpression of stonin 2^{WT}, but not its AP-2 binding mutant (stonin $2^{\partial WWW}$), facilitates endocytosis of synaptotagmin 1. Stonin $2^{\partial WWW}$, however, colocalizes with synaptotagmin 1 at the plasma membrane. White arrows in the bottom panel show cells that internalize small amounts of FLAG-synaptotagmin 1 due to stonin2$^{\partial WWW}$ expression. The scale bar is 16 μm.

(B) Quantification of stonin 2-induced internalization of synaptotagmin 1. HEK293 cells stably expressing FLAG-synaptotagmin 1 were transfected with (1) mock, (2) stonin 2^{WT}, (3) stonin $2^{\partial WWW}$, or (4) stonin 2^{NT}-μ2 chimera-expressing plasmids. Antibody uptake assays were performed as described in (A). Cell surface bound antibodies were blocked, and internalization was quantified as described in Experimental Procedures. Error bars represent mean arbitrary fluorescence units (AU) ± standard deviation (SD).

(C) AP-2 antibodies coimmunoprecipitate synaptotagmin 1 only in the presence of overexpressed stonin 2. HEK293-FLAG-Syt 1 cells were transfected with (1) mock, (2) stonin 2^{WT}, and (3) stonin $2^{\partial WWW}$ constructs. Cellular protein extracts were incubated with control beads or AP-2 antibody-coupled beads. Synaptotagmin 1 was coprecipitated with AP-2 only when the cells had been transfected with stonin 2^{WT}, but not with stonin $2^{\partial WWW}$. Std, 4% of total extract used.

(D) Alignment of human stonin 2 and rat μ2-adaptin amino acid sequences. For incorporation of stonin 2-μHD into the AP-2 complex, a chimeric construct comprising the μ2-β binding domain (BBD, residues 1–164) fused to the stonin 2 μ-homology domain (μHD, residues 563–898) was generated. A hemagglutinin (HA) epitope tag was introduced into the stonin 2-μHD between residues 635 and 636 (i.e., at a site analogous to that previously used for labeling of transfected μ2-adaptin) for detection of cells transfected with the $BBD_{\mu2}$-μHD_{stn2} chimera.

(E) Incorporation of the $BBD_{\mu2}$-μHD_{stn2} chimera into the AP-2 complex. AP-2 was affinity purified from the lysates of control or $BBD_{\mu2}$-μHD_{stn2}-transfected cells by using a GST fusion protein harboring the stonin 1 AP-2 binding motif (GST-WVxF [Walther et al., 2004]). The incorporated chimeric subunit was detected by anti-HA tag antibodies. Std, 5% of total extract used.

(F) HEK293-FLAG-Syt 1 cells were transfected with stonin 2^{WT} or $BBD_{\mu2}$-μHD_{stn2} chimeric constructs. Uptake experiments were performed as described in (B). Overexpression of the $BBD_{\mu2}$-μHD_{stn2} construct stimulates synaptotagmin 1 endocytosis, albeit slightly less efficiently than stonin 2^{WT}. The scale bar is 20 μm.

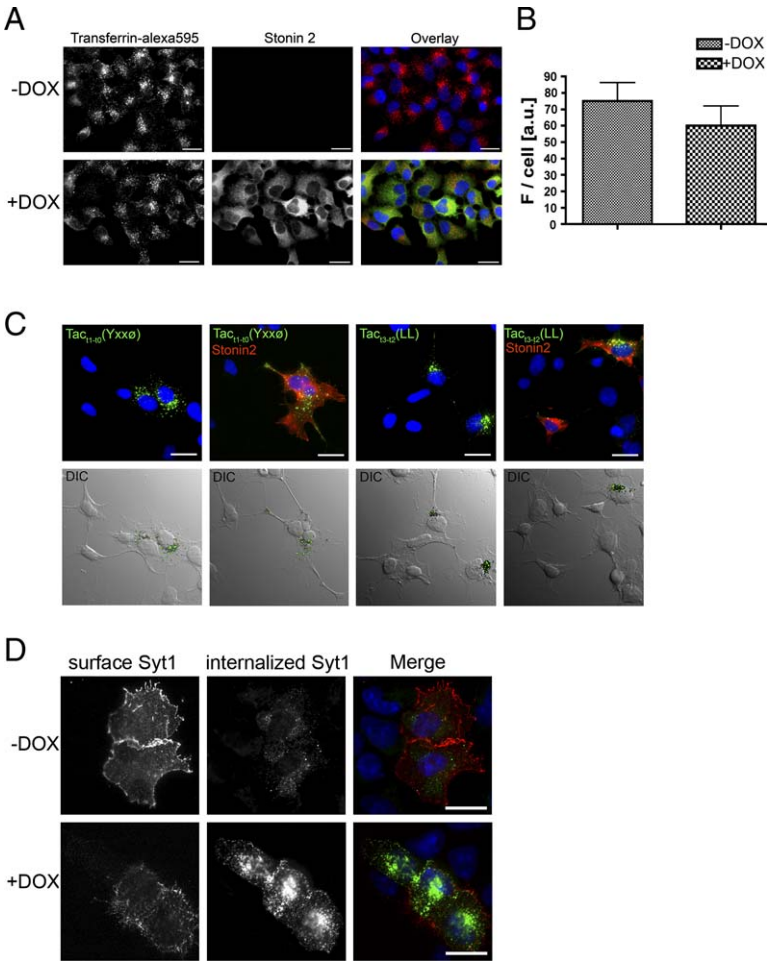

Figure 3. Overexpression of Stonin 2 Does Not Facilitate Endocytosis of Other Plasma Membrane Proteins

(A) Transferrin internalization assays were performed by using a HEK293 cell line stably expressing stonin 2 under the control of an inducible promoter. Prior to a transferrin uptake assay, both induced (1 μg/ml doxycycline overnight) and uninduced cells were starved for 4 hr in serum-free medium. After incubation with 20 μg/ml Alexa^{594}-labeled transferrin for 15 min at 37ºC, surface bound ligand was removed with 0.1 M Na-Acetate, 0.5 M NaCl (pH 5.2). Cells were fixed, stained, and analyzed. The scale bar is 20 μm.

(B) Internalized transferrin was quantified by using the Mask function of the Slidebook 4.0.10 software (Intelligent Imaging Innovations) applied on the Texas red channel. Nine data sets were acquired from 2 independent experiments (277 uninduced and 241 induced cells). The bars represent the mean fluorescence value from nine data sets in arbitrary units (±SD).

(C) HEK293 cells were transfected with Tac chimeras composed of the extracellular and transmembrane domains fused to cytosolic tails bearing either dileucine (t_3-t_2)- or tyrosine (t_1-t_0)-based endocytosis motifs. Uptake (15 min at 37ºC) was assayed essentially as described in Figure 2 by using anti-Tac antibodies. Overexpression of stonin 2 (red) does not affect internalization of Tac chimeras (green). Equal exposure times and identical intensity normalization were used during the acquisition of the images. The scale bar is 20 μm.

(D) Internalization of FLAG-synaptotagmin 1 transiently transfected into a HEK293 cell line stably expressing stonin 2 under the control of an inducible promoter. Where indicated, cells were induced by treatment with 1 μg/ml doxycycline overnight. Uptake experiments (1 hr at 37ºC) were essentially performed as described in Figure 2. The scale bar is 20 μm.

stonin 2-expressing HEK cells with an efficiency that was indistinguishable from nontransfected controls (Figure 3C). This suggests that stonin 2 specifically facilitates endocytosis of synaptotagmin, but not of other clathrin/AP-2-dependent cargos, consistent with its inability to interact with either tyrosine- or dileucine-based internalization signals (Martina et al., 2001; Walther et al., 2001).

In order to understand the molecular requirements for the ability of stonin 2 to target synaptotagmin for internalization, we constructed chimeric proteins between stonin 2, which harbors a putative synaptotagmin binding μ-homology domain (μHD; residues 563–898) (Martina et al., 2001; Walther et al., 2001), and AP-2μ. Replacement of its μ-homolgy domain (μHD; residues 563–898) (Martina et al., 2001; Walther et al., 2001) with the corresponding cargo binding domain of μ2-adaptin (C-μ2) rendered stonin 2 unable to facilitate endocytosis of synaptotagmin 1 (Figure 2B). Conversely, if the μHD of stonin 2 was fused to the β binding domain of μ2-adaptin (Collins et al., 2002), thereby replacing its endogenous cargo binding domain (C-μ2), the resulting chimeric $BBD_{\mu 2}$-μHD_{stn2} fusion protein (Figure 2D) was incorporated into α-adaptin-containing AP-2 complexes (Figure 2E) and facilitated synaptotagmin 1 internalization (Figure 2F), though with slightly reduced efficiency. These data indicate that the stonin 2 μHD is needed to physically connect synaptotagmin 1 with the endocytic machinery and AP-2.

Stonin 2-Mediated Synaptotagmin Internalization Is AP-2 Dependent

Next, we analyzed directly whether AP-2 was required for stonin 2-mediated synaptotagmin 1 internalization. To this aim, we created HEK293 cell lines stably expressing synaptotagmin 1 (see also Figure 2B and Figure S4). We then used siRNAs against μ2-adaptin (Motley et al., 2003) to specifically knock down AP-2 in these cells. HEK293 cells transfected with anti-μ2 siRNA displayed strongly reduced levels of μ2- and α-adaptins (Figure 4A) and lacked detectable AP-2α-coated pit staining in more than 80% of the cells (Figure 4B, left). The expression levels of clathrin and stably transfected synaptotagmin 1 were unaffected by the siRNA. As expected (Motley et al., 2003), AP-2 knockdown cells showed a severely reduced ability to internalize fluorescently labeled transferrin (Figure 4B, right), indicating

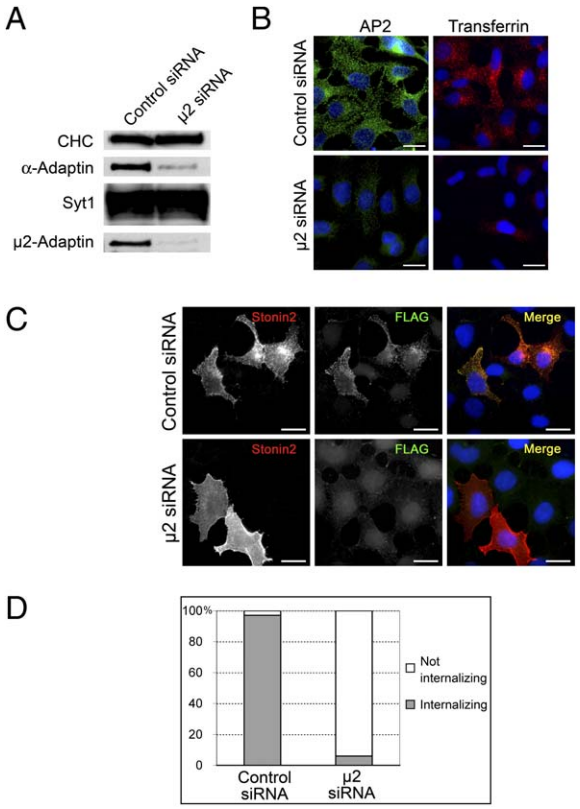

Figure 4. RNAi-Mediated Knockdown of μ2-Adaptin Expression Abolishes Synaptotagmin 1 Endocytosis in HEK293-FLAG-Syt 1 Cells

(A) HEK293-FLAG-Syt 1 cells were transfected with control or μ2 siRNAs. Consistent with previous results (Motley et al., 2003), μ2 knockdown causes degradation of other AP-2 subunits, including α-adaptin, but it has no effect on clathrin or synaptotagmin 1 levels.

(B) RNAi-mediated knockdown causes a loss of AP-2-coated pits from the plasma membrane and an inhibition of transferrin uptake. HEK293-FLAG-Syt 1 cells were transfected with control or μ2 siRNAs as described in (A). For transferrin uptake assays, cells were incubated in OPTIMEM containing 20 μg/ml Alexa^{594}-labeled human serum transferrin on ice for 30 min and were warmed up to 37ºC for 15 min. The scale bar is 20 μm.

(C) HEK293-FLAG-Syt 1 cells were transfected with stonin 2^{WT} in the presence of control or μ2 siRNAs. Antibody uptake assays were performed as described in Figure 2. Cells were incubated at 37ºC for 30 min for internalization. Those cells treated with μ2 siRNAs were unable to internalize FLAG-synaptotagmin 1. AP-2 knockdown abolishes stonin 2-mediated synaptotagmin 1 endocytosis. The scale bar is 20 μm.

(D) Quantification of the data shown in (C). Control or μ2 siRNA-treated HEK cells expressing moderate levels of stonin 2 were scored for synaptotagmin 1 internalization. Synaptotagmin 1 was efficiently internalized in 97% of the control cells, but in only 4% of the AP-2 knockdown cells (n = 100 for both conditions).

that clathrin/AP-2-dependent endocytosis is impaired. When cells treated with siRNA against μ2, but not those treated with a scrambled control siRNA, were cotransfected with stonin 2, little, if any, synaptotagmin 1 internalization was detectable (Figures 4C and 4D). Together with the data regarding the stonin 2$^{\delta WWW}$ mutant defective for binding to AP-2 (compare Figures 2A and 2B; Figure S4), our analyses suggest that stonin 2-mediated endocytosis of synaptotagmin 1 is an AP-2-dependent process. As discussed above, HEK cells lacking exogenous stonin 2 expression displayed a very low, but still detectable, rate of synaptotagmin 1 internalization, and this could be completely inhibited by siRNAs against AP-2μ (Figure S6), whereas scrambled control siRNA was without such effect. Synaptotagmin 1 internalization thus is an AP-2-dependent process, at least in transfected HEK cells.

Internalized Synaptotagmin 1 Is Targeted to Early Recycling Endosomal Vesicles

Since stonin 2 seemed to specifically affect the ability of cells to internalize synaptotagmin, but not transferrin or other Yxxø- or LL-motif-dependent cargos, we wanted to analyze the endosomal sorting of endocytosed synaptotagmin 1. We first visualized the distribution of transferrin and synaptotagmin 1 cointernalized for 20 min under conditions of reduced rates of exit from early endosomes (i.e., reduced temperature of 20ºC). Endocytosed transferrin and synaptotagmin 1 displayed a high degree of colocalization in punctate, presumably early endosomal compartments (Figure S7). By contrast, synaptotagmin 1 and transferrin became segregated into distinct vesicular structures if endocytosis was allowed to proceed for 1 hr at 37ºC (Figure S7B). The compartments to which synaptotagmin 1, internalized for 20 min at 20ºC, is sorted were indeed identified as early and recycling endosomes based on the large degree of colocalization between endocytosed synaptotagmin 1 and the early endosomal marker protein EEA1 (Bonifacino and Traub, 2003) (Figure S7D) as well as the transferrin receptor (Figure S7C). Colocalization between endocytosed synaptotagmin 1 and EEA1 was seen within 5 min of internalization at 20ºC and significantly decreased if cells were incubated for 1 hr at 37ºC (data not shown). As indicated before, a fraction of stonin 2 (see also Figure 2A) and AP-2 (data not shown) were cosorted together with synaptotagmin 1 to endosomal compartments, providing further support for the intimate functional and physical connection between stonin 2, AP-2, and synaptotagmin 1 endocytosis.

Stonin 2 Targets Synaptotagmin to a Recycling Vesicle Pool in PC12 Cells and in Primary Neurons

PC12 cells endogenously express synaptotagmin 1, which gets sorted to both large dense core vesicles (DCVs) and SLMVs, the neuroendocrine counterpart of small synaptic vesicles (SVs) in neurons. We thus wanted to know whether stonin 2 expression affects endocytic sorting of synaptotagmin 1 to synaptic-like microvesicles (SLMVs) in these cells (Tucker and Chapman, 2002). FLAG-tagged synaptotagmin 1 coexpressed with synaptopHluorin (a pH-sensitive variant of GFP fused to synaptobrevin 2 [Miesenbock et al., 1998]), a major component of SLMVs (but also present in DCVs), displayed a low level of internalization. The majority of endocytosed synaptotagmin 1 localized to cytoplasmic organelles, distinct from the synaptopHluorin-containing puncta. If, however, stonin 2 was coexpressed with synaptotagmin 1, a dramatic increase in synaptotagmin 1 endocytosis was observed (at least 10-fold), and the majority of internalized protein colocalized with synaptopHluorin in cytoplasmic vesicles,

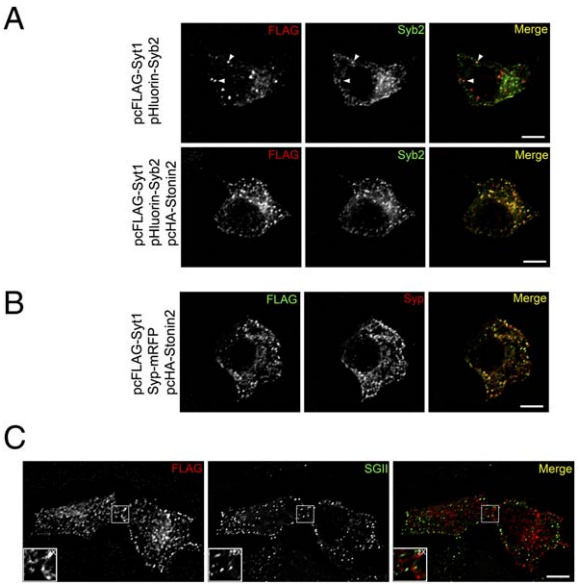

Figure 5. Stonin 2 Facilitates Endocytic Internalization of Synaptotagmin 1 and Its Sorting to SLMVs in Neuroendocrine Cells

(A) PC12 cells were cotransfected with synaptotagmin 1 and pHluorin-synaptobrevin 2 constructs (synaptopHlourin) in the presence or absence of stonin 2. Antibody uptake assays were performed as described in Figure 2. Stonin 2 expression facilitates synaptotagmin 1 endocytosis and sorting to synaptopHluorin (pHluorin-synaptobrevin 2)-containing SLMVs. In the absence of transfected stonin 2, endocytosed synaptotagmin 1 displays only limited colocalization with synaptopHluorin (white arrowheads). The scale bar is 5 μm.

(B) As in (A), except that PC12 cells were transfected with a synaptophysin-mRFP construct that partially localizes to SLMVs and to the plasma membrane in the absence of transfected stonin 2. Endocytosed synaptotagmin 1 colocalizes with synaptophysin to SLMVs only in the presence of stonin 2. The scale bar is 5 μm.

(C) Internalized synaptotagmin 1 does not colocalize with the DCV marker secretogranin (SG) II in neuroendocrine cells. PC12 cells were cotransfected with synaptotagmin 1 and stonin 2 constructs. Cells were stained postfixation with polyclonal antisera against the DCV marker secretogranin II and were analyzed by deconvolution fluorescence microscopy. The scale bar is 5 μm.

presumably SLMVs (Figure 5A). We did not detect any colocalization of endocytosed synaptotagmin 1 with the DCV marker secretogranin II (Figure 5C), suggesting that stonin 2 specifically facilitates endocytic sorting to SLMVs. Similar results were seen if the distribution of internalized synaptotagmin 1 was compared to that of synaptophysin-mRFP, another marker for SLMVs (Figure 5B). These data identify stonin 2 as an endocytic sorting adaptor dedicated to synaptotagmin 1 internalization and sorting to neurosecretory vesicles in PC12 cells.

In cultured primary hippocampal neurons, SV proteins, including synaptotagmin 1, undergo constitutive cycling between SVs and the presynaptic plasma membrane. We were interested in quantitatively assessing the partitioning of synaptotagmin 1 between vesicular and plasmalemmal pools, and, therefore, we constructed a chimeric reporter protein consisting of a signal-sequence-tagged, pH-sensitive "pHluorin" mutant of GFP fused to the lumenal domain of synaptotagmin 1 (sytpHluorin; Figure 6A). The sytpHluorin chimera is properly targeted to presynaptic vesicles, as electrical stimulation with 200 action potentials (APs) at 20 Hz gave rise to a stimulation-dependent sytpHluorin fluorescence increase at synaptic boutons (Figures 6B and 6C) with similar characteristics as described before for pHluorin-tagged synaptobrevin (Miesenbock et al., 1998; Sankaranarayanan and Ryan, 2001). The pH dependence of the fluorescence enabled us to monitor the effect of stonin 2 on sytpHluorin distribution within the presynaptic compartment (Figure 6A) of living neurons by using a simple acid quenching-dequenching protocol. Acid quenching resulted in a near complete loss of fluorescence (Figure 6D), whereas alkalinization of the vesicular lumen with NH_4Cl produced a maximum intensity signal at synaptic boutons (Figure 6E), indicating that the transient fluorescence increase after stimulation was due to exocytotic externalization of sytpHluorin. Together with the colocalization of sytpHluorin with other presynaptic vesicle proteins (Figure S8C) and the kinetics of sytpHluorin exo-endocytosis (see Figures 6G–6I), these data indicate that sytpHluorin can serve as a faithful reporter of synaptotagmin 1 recycling at presynaptic nerve terminals. This system then allowed us to assess the effect of stonin 2 on synaptotagmin 1 recycling in living neurons both at steady state and under stimulating conditions.

Fluorescence analysis after acid quenching-dequenching revealed that, under resting conditions, coexpression of wild-type stonin 2 significantly decreased the relative steady-state plasmalemmal fraction of sytpHluorin at presynaptic boutons, resulting in a nearly 2-fold increase of the vesicular-to-surface-stranded pool ratio (Figure 6F). Conversely, a stonin two-point mutant lacking the ability to bind to either AP-2 or eps15 and intersectin (stonin $2^{\delta WF\delta NPF}$) exhibited a dominant-negative phenotype, leading to increased surface levels of our reporter protein (Figure 6F). This mutant had no effect on internalization of transferrin or AP-2 distribution when expressed in fibroblasts (Figures S8A and S8B). Stimulation of neurons by 200 APs at 20 Hz produced a transient fluorescence increase followed by a decline reflecting sytpHluorin endocytosis (Figure 6G). As expected, the amplitudes of these fluorescence changes significantly differed depending on the presence of stonin 2 or the dominant-negative mutant. The largest increase was seen for wild-type stonin 2, whereas stonin $2^{\delta WF\delta NPF}$ displayed a decrease in amplitude compared to controls (Figure 6G). The different steady-state levels observed should be a consequence of small differences in retrieval efficiency of exocytosed synaptotagmins after synaptic stimulation, accumulated during several days (6–9 days posttransfection) of ongoing synaptic activity.

In an attempt to resolve such expectedly minute differences in retrieval after 200 APs, we reanalyzed the sytpHluorin responses, restricting analysis, however, to those boutons that gave signals with good signal-to-noise ratios (>50, measured as peak signals over standard deviation). Normalization of these responses revealed that the overall time course of individual endocytic events remained largely unchanged (Figure 6H). However, small differences in retrieval efficiency (i.e., relative percentage of reinternalized sytpHluorin molecules) could be observed: boutons overexpressing wild-type stonin 2 displayed a slightly increased retrieval

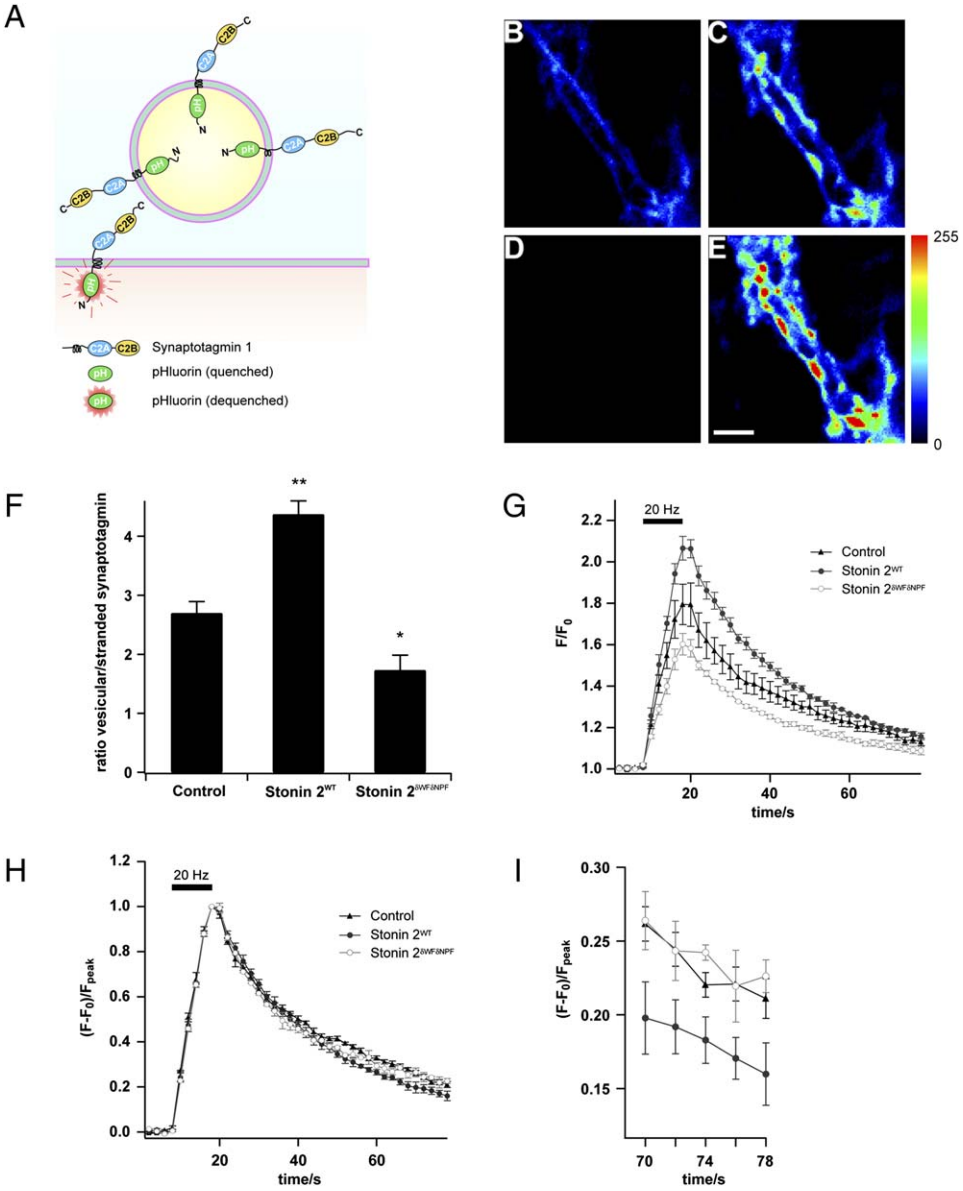

Figure 6. Stonin 2 Targets Synaptotagmin 1-pHluorin to Recycling Vesicles in Primary Hippocampal Neurons

(A) The schematic illustrates how 1-pHluorin (sytpHluorin) can be used to probe synaptotagmin 1 localization: its fluorescence is quenched in the acidic vesicular lumen, but not when residing at the plasmalemma.

(B–E) SytpHluorin fluorescence (B) before and (C) immediately after stimulation with 200 APs at 20 Hz. Challenge with acidic solution results in (D) fluorescence quenching, whereas alkalinization with NH_4Cl produces (E) maximum fluorescence. The scale bar is 5 μm. Fluorescence values are color coded according to the depicted scale.

(F) Based on the relative fluorescence values measured before and after acid quenching-dequenching, the vesicular-to-surface-stranded pool ratios of sytpHluorin were calculated and plotted ± SEM (control: 2.69 ± 0.20; stonin 2^{WT}: 4.37 ± 0.23; stonin $2^{\delta WF\delta NPF}$: 1.72 ± 0.26; *$p < 0.01$; **$p < 0.05$). Expression of wild-type stonin 2 results in a significant increase, whereas stonin $2^{\delta WF\delta NPF}$ produces a decrease in the vesicular-to-surface pool ratio.

(G) Time course of sytpHluorin fluorescence ratios (F/F_0 ± SEM) after stimulation with 200 APs at 20 Hz. The bar indicates the stimulation period (10 s). Peak values ± SEM are for control: 1.80 ± 0.10; stonin 2^{WT}: 2.07 ± 0.06 ($p < 0.03$ versus control); and stonin $2^{\delta WF\delta NPF}$: 1.60 ± 0.05; ($p < 0.01$ versus stonin 2^{WT}).

(H) Time course of sytpHluorin fluorescence ratios (F/F_0 ± SEM) as in (G), but normalized with respect to peak amplitudes (F_{peak}), reveals no differences in kinetics, but small differences in retrieval efficiency. Data are a subset of those in (G); only recordings with a signal-to-noise ratio >50 (measured as peak amplitude over the standard deviation of the baseline) have been included to better resolve the decay characteristics of the responses.

(I) Detail of (H), documenting differences in the retrieval efficiencies at t = 78 s (% ± SEM) for control: 78.9 ± 0.01; stonin 2^{WT}: 84.0 ± 0.02 ($p < 0.06$ versus control); and stonin $2^{\delta WF\delta NPF}$: 77.4 ± 0.01 ($p < 0.05$ versus stonin 2^{WT}). Retrieval efficiency is defined as $(1 - [(F - F_0)/F_{peak}]) \times 100$.

efficiency compared to control neurons or those expressing the dominant-negative mutant (Figures 6H and 6I).

Our combined data thus suggest that stonin 2 acts as an endocytic sorting adaptor that targets synaptotagmin to presynaptic recycling vesicles in neurons.

Discussion

Our data described here suggest that stonin 2, a brain-enriched endocytic protein, serves as an AP-2-dependent sorting adaptor that targets synaptotagmin for clathrin/AP-2-mediated internalization. This proposal is based on the following lines of evidence. First, stonin 2 colocalizes with synaptotagmin 1 at presynaptic sites and is found complexed with both AP-2 and synaptotagmin 1 in the brain, to which it directly binds. Second, expression of synaptotagmins 1, 2, and 9 recruits stonin 2 to plasmalemmal AP-2-coated pits, and this, in turn, facilitates synaptotagmin internalization and sorting to early endosomal compartments. The stimulatory effect of stonin 2 is specific to synaptotagmin internalization, but not to other AP-2-dependent endocytic pathways, including uptake of transferrin receptors. RNA interference in combination with site-directed mutagenesis reveals a strict AP-2 dependence for synaptotagmin 1 internalization. Third, stonin 2-mediated synaptotagmin 1 internalization facilitates sorting of synaptotagmin 1 to SLMVs and to a recyling vesicle pool in primary hippocampal neurons in culture. The latter observation strongly suggests that stonin 2-mediated endocytic recycling of synaptotagmin is a physiologically relevant pathway.

To our knowledge, stonin 2 is the first endocytic adaptor protein dedicated to the recycling of a SV protein, i.e., synaptotagmin. Its spatial distribution and concentration at presynaptic sites allows for a specific function in facilitating SV protein recycling without affecting other clathrin-dependent endocytic pathways. Stonin 2 may thus contribute to the clathrin-mediated recycling of SVs, which is in agreement with the observation that SV proteins, including synaptotagmin 1, represent the main cargo of clathrin-coated vesicles isolated from presynaptic nerve terminals (Maycox et al., 1992). Our observations are also consistent with and further support a role for synaptotagmin 1 in regulating the endocytic limb of the vesicle cycle (Jorgensen et al., 1995; Littleton et al., 2001; Llinas et al., 2004; Nicholson-Tomishima and Ryan, 2004; Poskanzer et al., 2003). Interestingly, different synaptotagmin isoforms have been implicated in regulating the choice between a kiss-and-run mode of vesicle cycling and a slower, presumably clathrin/AP-2-dependent pathway (Virmani et al., 2003; Wang et al., 2003). Consistent with its function in a slow, clathrin/AP-2-dependent pathway, we find that stonin 2 selectively interacts with synaptotagmin 1, but not synaptotagmin 4, which has been postulated to promote kiss-and-run exocytosis (Wang et al., 2003).

How stonin 2 physically interacts with synaptotagmin 1 at the structural level remains an open question, but our experiments with chimeric proteins as well as direct binding studies (Figures S1B and S1C) indicate that its μHD is required and sufficient for this. Surprisingly, at least to us, the cargo binding domain of μ2-adaptin (C-μ2) was unable to substitute, suggesting that perhaps the association of synaptotagmin 1 with stonin 2 is of higher affinity than its binding to AP-2μ (Grass et al., 2004). While the basic AP-2μ binding motif derived from synaptotagmin's C2B domain when transplanted onto an oligomeric reporter protein is sufficient to trigger endocytosis in fibroblasts (Grass et al., 2004), interaction of C2B with other binding partners, including phosphoinositides, calcium channels, or SNAREs (Tucker and Chapman, 2002), may impose the need for a specialized sorting adaptor to target native synaptotagmin 1 for clathrin/AP-2-mediated internalization. The dual requirement for stonin 2 and AP-2 in synaptotagmin internalization can thus be explained by a hypothetical model. According to this model, both adaptors cooperate by interacting with the C2 domains of plasma membrane-stranded synaptotagmin 1 oligomers either by forming a multimeric complex (Figure 7, I) or via a sequential mode in which stonin 2 delivers synaptotagmin 1 to AP-2μ (Figure 7, II).

Alternatively, it is also possible that stonin 2 executes a more specific role in regulating the amount of synaptotagmin targeted to the recycling vesicle pool. Our kinetic data (see Figures 6G–6I) in transfected hippocampal neurons expressing wild-type or dominant-negative stonin 2 mutant proteins argue in favor of this possibility. Although stonin 2 appears to affect the ratio of synaptotagmin 1 present at the presynaptic plasmalemma or within SVs, we were unable to detect any differences in the kinetics of individual endocytic events. How could targeting of synaptotagmin 1 to sites of endocytosis not be rate limiting for endocytosis of synaptic vesicles, i.e., not alter the kinetics of synaptotagmin 1 retrieval? One attractive hypothesis is that cargo molecules stranded at the plasmalemma are targeted to and concentrated at sites of endocytosis at rest, i.e., prior to stimulation, thereby constituting a partially preassembled pool of "early retrievable vesicles" (Mueller et al., 2004) that is endocytosed first. Our observations therefore may suggest that the decreased ratio of vesicular-to-surface-stranded pools of synaptotagmin seen in stonin $2^{\delta WF\delta NPF}$-expressing cells could arise by progressive accumulation of synaptotagmin 1 molecules at the plasma membrane, perhaps owed to competition between endogenous stonin 2 and the dominant-interfering stonin $2^{\delta WF\delta NPF}$ mutant protein. Conversely, overexpression of wild-type stonin 2 is sufficient to rescue synaptotagmin 1 stranded at the plasmalemma and for targeting synaptotagmin 1 to the recycling vesicle pool. Whatever the exact molecular mechanism may be, our findings suggest that stonin 2 acts at an early cargo selection step of clathrin/AP-2-mediated SV endocytosis. This proposal is in line with our observation that synaptotagmin 1 overexpression targets stonin 2 to plasmalemmal AP-2-coated pits (see Figure 1E), and that stonin 2 is enriched in synaptotagmin-containing clathrin/AP-2-coated vesicles isolated from presynaptic nerve terminals (Walther et al., 2001). It is also consistent with observations made in stoned mutant animals in *Drosophila*, in which loss of stoned B/stonin function is correlated with synaptotagmin mislocalization and degradation (Fergestad et al., 1999).

Last, we cannot exclude that stonin 2 executes additional functions in recycling vesicle endocytosis, as indicated by its persistent association with synaptotagmin 1 cargo molecules throughout the endosomal itinerary, at least in transfected fibroblasts.

Taken together, our data demonstrate that stonin 2 exhibits specific functions with regard to targeting synaptotagmin for endocytic recycling and sorting to

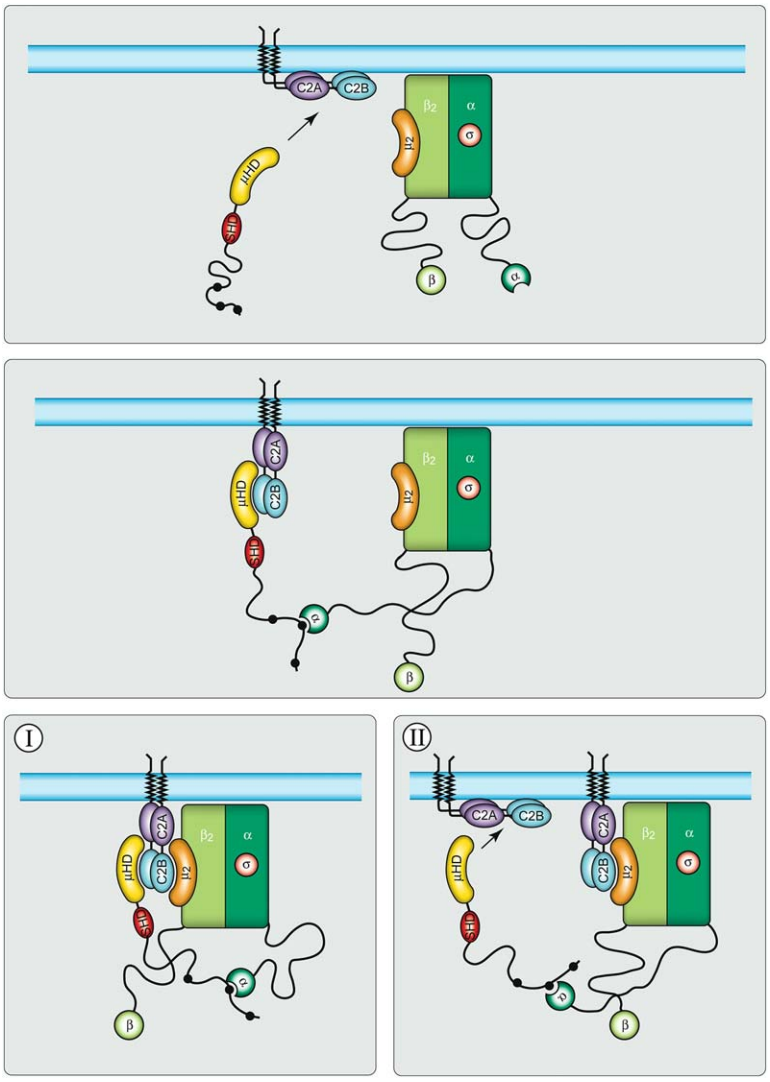

Figure 7. Hypothetical Model for How Stonin 2 and AP-2 May Cooperate in Synaptotagmin Internalization

Stonin 2 via its μ-homology domain may bind to plasma membrane-stranded synaptotagmin 1 oligomers (top). Interactions with the ear domain of AP-2α aid in recruitment of the synaptotagmin-stonin 2 complex to membrane bound AP-2 (middle). Clathrin/AP-2-mediated synaptotagmin internalization might be driven either by a multimeric synaptotagmin-stonin 2-AP-2 complex (bottom, I) or via a sequential mode involving stonin 2-mediated delivery of synaptotagmin to AP-2μ (bottom, II).

neurosecretory vesicles. To our knowledge, it is the first endocytic protein identified that facilitates recycling of a SV protein. Stonin 2 thereby also joins a small group of proteins, including Dab2, ARH, HIP1, and β-arrestins, that modulate endocytosis of select cargo proteins (Traub, 2003). It will be interesting to see whether additional endocytic adaptors exist that facilitate endocytic recycling of other SV proteins.

Experimental Procedures

Cell Culture, Transfections, Plasmids, and Antibodies

HEK293, N1E-115, and PC12 cells were cultured in DMEM (GIBCO-BRL) with appropriate supplements and antibiotics. Culturing of primary cortical or hippocampal neurons has been described before (Mueller et al., 2004). Plasmid and siRNA transfections were done with Lipofectamine 2000 or Oligofectamine (GIBCO-BRL). Additional details regarding plasmids, antibodies, transfection procedures, as well as the generation of stable cell lines can be found in the Supplemental Data.

Antibody Internalization Assays and Quantification

HEK293 cells were incubated (30 min) on ice with α-FLAG antibody (10 μg/ml) before incubation at 20°C or 37°C for the indicated times. Cells were fixed with 4% PFA, and primary antibodies bound to non-internalized synaptotagmin 1 were blocked with goat anti-mouse IgG (1:5) for 2 hr at room temperature. Cells were permeabilized and stained with primary and secondary antibodies (diluted in GSDB). Slidebook 4.0.10 software (Intelligent Imaging Innovations, Inc.) was utilized for quantifying results from three independent experiments. Using equal exposure times, 3 fluorescence images were acquired from each experiment, yielding 9 data sets (each containing 10–30 cells). Regions of interest (ROI) were defined as the total surface area of all cells or stonin 2-expressing cells in each data set. ROIs were selected by using the fluorescence intensity derived from transfected stonin 2 or the total synaptotagmin 1 fluorescence obtained by a polyclonal antibody after permeabilization. Fluorescence intensity of internalized antibodies was calculated by subtracting the background fluorescence from the ROI fluorescence. For experiments with different stonin 2 constructs, the fluorescence intensity was measured only from the transfected cells. The fluorescence intensity of any particular data set represents the average intensity of the cells in that data set. Data are given as arbitrary units of mean fluorescence intensity (±SD) obtained from nine data sets.

SytpHLuorin Recycling Assays in Living Neurons

Hippocampal neurons (1- to 3-day-old Wistar rats) prepared in sparse culture were transfected by calciumphosphate-DNA coprecipitation and were used after 11–14 days in vitro. A modified Tyrode solution (150 mM NaCl, 4 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM glucose, 10 mM HEPES [pH 7.4]) was used for all experiments. Synaptic boutons were stimulated by electric field stimulation (platinum

electrodes, 10 mm spacing, 200 pulses of 50 mA and alternating polarity, 10 μM CNQX, and 50 μM AP-5) at room temperature. Fast solution exchanges were achieved by a piezo-controlled stepper device (SF77B/Warner Instruments). Ammonium chloride solution (pH 7.4) was prepared by substituting 50 mM NaCl in normal saline with NH_4Cl; all other components remained unchanged. Acidic solution with a final pH of 5.5 was prepared by replacing HEPES with 2-[N-morpholino]ethane sulphonic acid (pK = 6.1). Images were obtained by using a cooled slow-scan CCD camera (PCO SensiCam-QE, Kelheim, Germany) and a Zeiss inverted microscope (Axiovert S100TV) with a Zeiss ×63, 1.2 NA water-immersion objective and an eGFP filter set. Fluorophores were excited at 470 nm every 2 s for 500 ms. Imaging data were digitized and preanalyzed with Till Vision Software (Till Photonics, Germany) by using regions of interest to delimit puncta. For further analysis, data were collected, normalized, and averaged by using self-written macros in Igor Pro (Wavemetrics, Oregon, USA).

Immunofluorescence Microscopy

Confocal images of N1E cells were obtained by a Carl Zeiss laser scanning microscope II. For acquisition of all other images, a motorized Carl Zeiss Axiovert 200M inverted microscope equipped with a Stallion system (Intelligent Imaging) was used. Images were processed by deconvolution of serial image sections taken along the z axis, in order to minimize out-of-focus fluorescence.

Biochemical Procedures

Biochemical experiments were essentially done as described before (Walther et al., 2004). See the Supplemental Data for additional information.

Supplemental Data

Supplemental Data including Supplemental Experimental Procedures, eight supplemental figures, and a supplemental movie are available at http://www.developmentalcell.com/cgi/content/full/10/2/233/DC1/.

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