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Human stoned B interacts with AP-2 and synaptotagmin and facilitates clathrin-coated vesicle uncoating

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Synaptic vesicle biogenesis involves the recycling of synaptic vesicle components by clathrin-mediated endocytosis from the presynaptic membrane. stoned B, a protein encoded by the *stoned* locus in *Drosophila melanogaster* has been shown to regulate vesicle recycling by interacting with synaptotagmin. We report here the identification and characterization of a human homolog of stoned B (hStnB). Human stoned B is a brain-specific protein which co-enriches with other endocytic proteins such as AP-2 in a crude synaptic vesicle fraction and at nerve terminals. A domain with homology to the medium chain of adaptor complexes binds directly to both AP-2 and synaptotagmin and competes with AP-2 for the same binding site within synaptotagmin. Finally we show that the μ2 homology domain of hStnB stimulates the uncoating of both clathrin and AP-2 adaptors from clathrin-coated vesicles. We hypothesize that hStnB regulates synaptic vesicle recycling by facilitating vesicle uncoating.

INTRODUCTION

Synaptic vesicle biogenesis involves the rapid recycling of synaptic vesicle membrane proteins and lipids to allow for sustained neurotransmission (Hannah *et al.*, 1999; De Camilli *et al.*, 2001). Clathrin-mediated endocytosis represents a major mechanism for local regeneration of synaptic vesicles (Heuser and Reese, 1973; Brodin *et al.*, 2000). Presynaptic clathrin coats comprise clathrin, the adaptor complex AP-2 (a heterotetrameric complex composed of α, β2, μ2 and σ2 subunits) and AP180 (Hirst and Robinson, 1998; Kirchhausen, 2000). Coat assembly may involve interactions of the AP-2 adaptor complex with the synaptic vesicle protein synaptotagmin (Zhang *et al.*, 1994; Haucke and De Camilli, 1999; Haucke *et al.*, 2000; Littleton *et al.*, 2001) and lipids (Takei *et al.*, 1998; Arneson *et al.*, 1999; Cremona *et al.*, 1999; Gaidarov and Keen, 1999). Completion of assembly is followed by vesicle fission which requires dynamin and a number of accessory factors (reviewed in Hannah *et al.*, 1999; Brodin *et al.*, 2000; De Camilli *et al.*, 2001) including endophilin (Ringstad *et al.*, 1999; Schmidt *et al.*, 1999). Finally, clathrin and adaptors are removed from clathrin-coated vesicles in an ATP-dependent reaction mediated by the molecular chaperone hsc70 (Jiang *et al.*, 2000), auxilin (Ungewickell *et al.*, 1995), and a yet unidentified co-factor (Hannan *et al.*, 1998).

Recent studies in *Drosophila* have identified stoned proteins as a novel class of regulators of synaptic vesicle endocytosis (Stimson *et al.*, 1998; Fergestad *et al.*, 1999; Phillips *et al.*, 2000; Fergestad and Broadie, 2001). The *stoned* locus encodes two essential proteins, stoned A and stoned B, which share a number of structural features with accessory proteins of endocytosis (Stimson *et al.*, 1998) and localize to the presynaptic compartment (Stimson *et al.*, 1998; Fergestad *et al.*, 1999; Fergestad and Broadie, 2001). *stoned* mutants display defects in neurotransmission, endocytosis and synaptic vesicle protein localization (Fergestad *et al.*, 1999; Fergestad and Broadie, 2001). Stoned B is a synaptotagmin-binding protein (Phillips *et al.*, 2000) containing seven copies of the tripeptide NPF implicated in binding to Eps15 family members (Stimson *et al.*, 1998) and a region with homology to μ2 adaptin. These data have suggested that *Drosophila* stoned proteins may regulate synaptic vesicle endocytosis by interacting with synaptotagmin.

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We report here the identification and characterization of a human homolog of *Drosophila* stoned B (hStnB) and provide evidence for its involvement in the uncoating of clathrin-coated vesicles.

RESULTS

Identification and localization of human stoned B

Upon searching the human genome database we identified an incomplete sequence with homology to *Drosophila* stoned B. This sequence overlapped at its 5′ region with a commercially available Stratagene cDNA clone (#14981) containing the putative start ATG codon, which was preceded by an in-frame stop codon. We used the genomic information to assemble a full-length clone of hStnB by PCR from human brain cDNA and verified its sequence by DNA-sequencing. The identity of its 5′ end was further confirmed by 5′ RACE analysis. The human stoned B (hStnB) open reading frame (ORF) (Figure 1) encodes a novel protein of 886 amino acids with a predicted molecular weight of 100 kDa. Like *Drosophila* stoned B, it comprises a domain with high homology to μ2-adaptin (amino acids 547–790; 34.8% identity between *Drosophila* and human stoned B) as well as two NPF motifs. Affinity-purified antibodies to hStnB recognized a single band of ~120 kDa in total brain extracts (Figure 2A). This immunoreactive band disappeared if the antibodies were preincubated with the antigenic peptide (Figure 2A, + peptide) indicating that our antisera specifically recognize mammalian StnB. Western-blotting of extracts from various rat tissues revealed that mammalian StnB, like synaptotagmin I, appears to be a brain-specific protein, although it may be present at low levels in other tissues. In contrast, the molecular chaperone hsc70 was expressed ubiquitously in all tissues examined (Figure 2B). In subcellular fractions of rat brain mammalian StnB was co-enriched with AP-2 and synaptotagmin I in crude synaptic vesicles (LP2) and its distribution roughly paralleled that of AP-2 in other fractions (Figure 2C). Mammalian StnB, was moderately enriched in clathrin-coated vesicles which contained large amounts of clathrin, AP-2, and the uncoating factor auxilin. In contrast, μ3, a subunit of the AP-3 adaptor complex was de-enriched (Figure 2D).

Immunostaining of rat brain sections with affinity-purified antisera to StnB revealed a punctate nerve terminal pattern which co-localized with synaptotagmin I and AP-2 immunoreactivity (Figure 2E and F).

hStnB binds to both AP-2 and synaptotagmin

In order to identify potential binding partners of hStnB, Triton X-100-extracted rat brain homogenate was affinity-purified on either glutathione *S*-transferase (GST) or a fusion protein comprising the μ2 homology domain of hStnB fused to GST (Figure 3A). The hStnB fusion protein specifically retained synaptotagmin I and AP-2, but not clathrin or the abundant synaptic vesicle protein synaptophysin (Figure 3B). None of these proteins bound to GST. Since synaptotagmin is known to bind AP-2 with high affinity (Zhang *et al.*, 1994) we sought to determine whether hStnB was capable of binding directly to AP-2. To this aim we immunoaffinity-purified AP-2 from clathrin coat proteins (Figure 3C, bottom) and analyzed whether *in vitro* translated ^{35}S-labeled full-length hStnB or its μ2 homology domain were capable of binding to immobilized AP-2. Indeed, hStnB efficiently bound to AP-2-containing immunobeads, but not to beads lacking AP-2. Radiolabeled luciferase did not bind irrespective of the presence of AP-2 on the beads (Figure 3C, top). Moreover, either full-length *in vitro* synthesized ^{35}S-labeled hStnB or the μ2-homology domain-containing fragment were capable of binding directly to a fusion protein between GST and the C2B domain of synaptotagmin, but not to GST alone (Figure 3D). These data indicate that hStnB is capable of associating directly with both AP-2 and synaptotagmin. One of the major functions of the μ2 subunit of AP-2 is to recognize plasma membrane cargo proteins by binding to tyrosine-based endocytosis signals (Bonifacino and Dell'Angelica, 1999). To test whether hStnB was also able to recognize these signals we immobilized 14-mer peptides containing the tyrosine-based endocytic motif YQRL or an inactive mutant (AQRL; Haucke and De Camilli, 1999) on beads. Upon incubation with purified μ2 (amino acids 157–435; Owen and Evans, 1998) or the purified μ2 homology domain of hStnB (amino acids 536–791), we found that only μ2, but not hStnB (536–791), could bind efficiently to immobilized YQRL peptide (Figure 3E). Similar results were obtained with *in vitro* translated full-length hStnB (not shown). We conclude that hStnB unlike μ2 may not function as an endocytic sorting receptor for tyrosine-based endocytic motifs.

AP-2 and hStnB compete for binding to synaptotagmin

μ2 harbors the major binding site for synaptotagmin within AP-2 (Haucke *et al.*, 2000), and hStnB appears to associate with the C2B domain of synaptotagmin primarily via its μ2 homology domain (see Figure 3D). This poses the question as to whether AP-2 and hStnB compete for binding to synaptotagmin. To directly address this possibility we determined the binding of ^{35}S-labeled *in vitro* translated hStnB to synaptotagmin in the presence of clathrin and adaptors. Addition of increasing concentrations of a clathrin/adaptor pool competitively inhibited binding of hStnB to GST–synaptotagmin C2B (Figure 4A). Conversely, addition of the purified hStnB μ2 homology domain competed the binding of native AP-2 to synaptotagmin (Figure 4B). Moreover, a point mutant of synaptotagmin in which two lysines within the AP-2 binding site had been replaced by alanines displayed a decreased affinity not only for AP-2 (Chapman *et al.*, 1998) but also for hStnB (Figure 4C). A double point mutant of hStnB in which W719 and K721 had been changed to alanines, a region implicated in binding of μ2 to synaptotagmin (Haucke *et al.*, 2000), showed a reduced ability to bind to the C2B domain of synaptotagmin (Figure 4D).

hStnB can facilitate uncoating of clathrin-coated vesicles

Human stoned B appears to compete with AP-2 for binding to synaptotagmin (see Figure 4) suggesting that it might be a negative regulator of coat formation such as in vesicle uncoating. We decided to test directly the possibility that hStnB might affect clathrin-coated vesicle uncoating. When the purified μ2 homology domain of hStnB (Figure 5A) was incubated with clathrin-coated vesicles in the presence of ATP

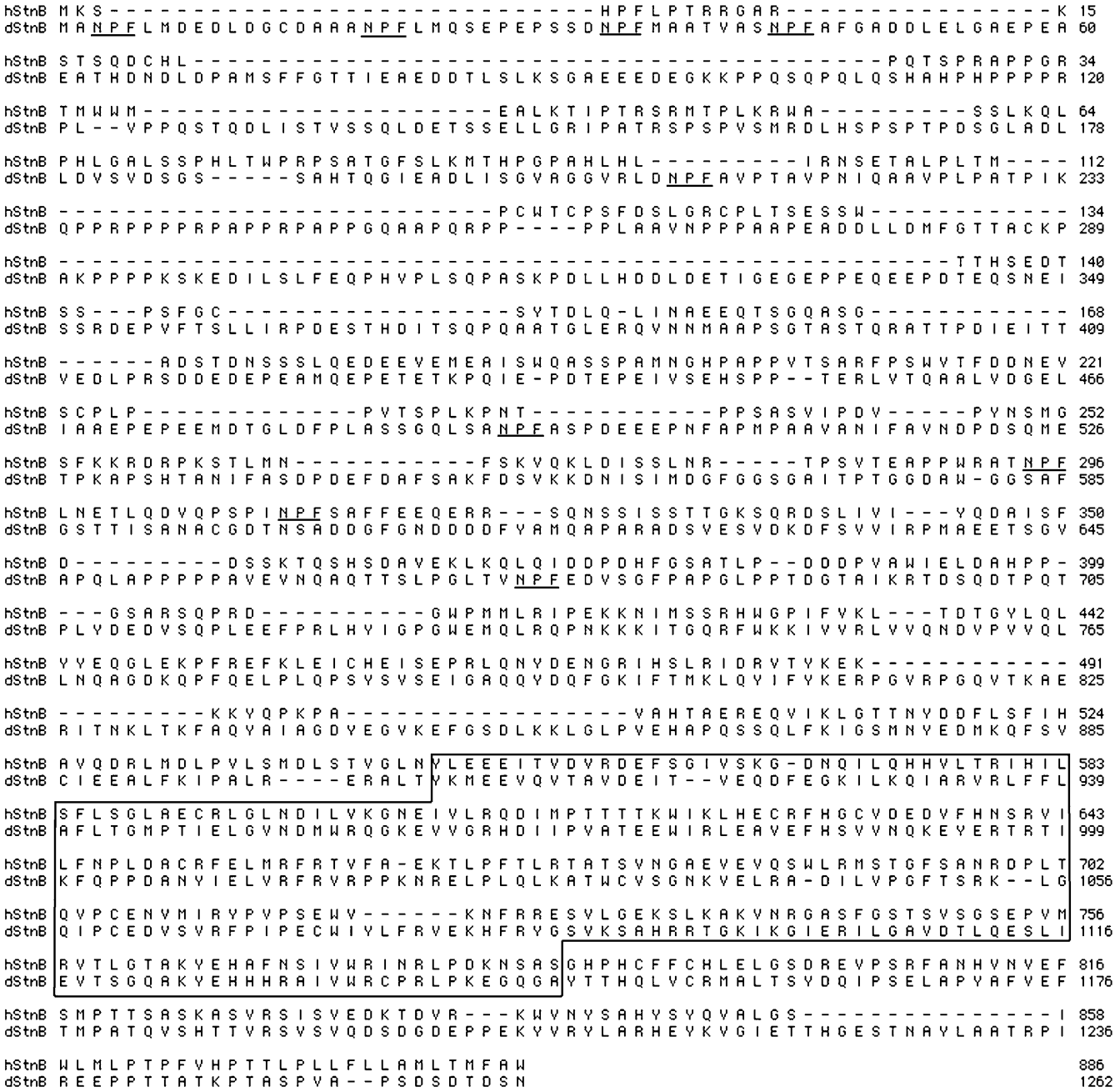

Fig. 1. Alignment of the deduced amino acid sequences of *Drosophila* (dStnB) and human stoned B (hStnB). The μ2 homology domain is boxed. NPF motifs are underlined. Sequence conservation is highest within the μ2 homology domain (34.8% identity; 66.4% similarity), the overall identity is 22.4% (44% similarity).

and cytosol a significant stimulation of clathrin and AP-2 uncoating was observed (Figure 5B). hStnB (amino acids 536–791) stimulated both the rate and extent of uncoating (Figure 5C). During the uncoating reaction the hStnB fragment mostly associated with the vesicular pellet fraction while clathrin and adaptors were released into the supernatant (Figure 5D). Bovine serum albumin (BSA) remained in the supernatant fraction (Figure 5D) and did not affect uncoating (not shown). In contrast, addition of purified soluble AP-2 had, if any, a slightly inhibitory effect on clathrin release (see Supplementary data). hStnB specifically catalyzed uncoating of clathrin/AP-2-coated vesicles but not release of AP-1 (see Supplementary data).

We conclude that hStnB facilitates the uncoating of clathrin/AP-2 coated vesicles.

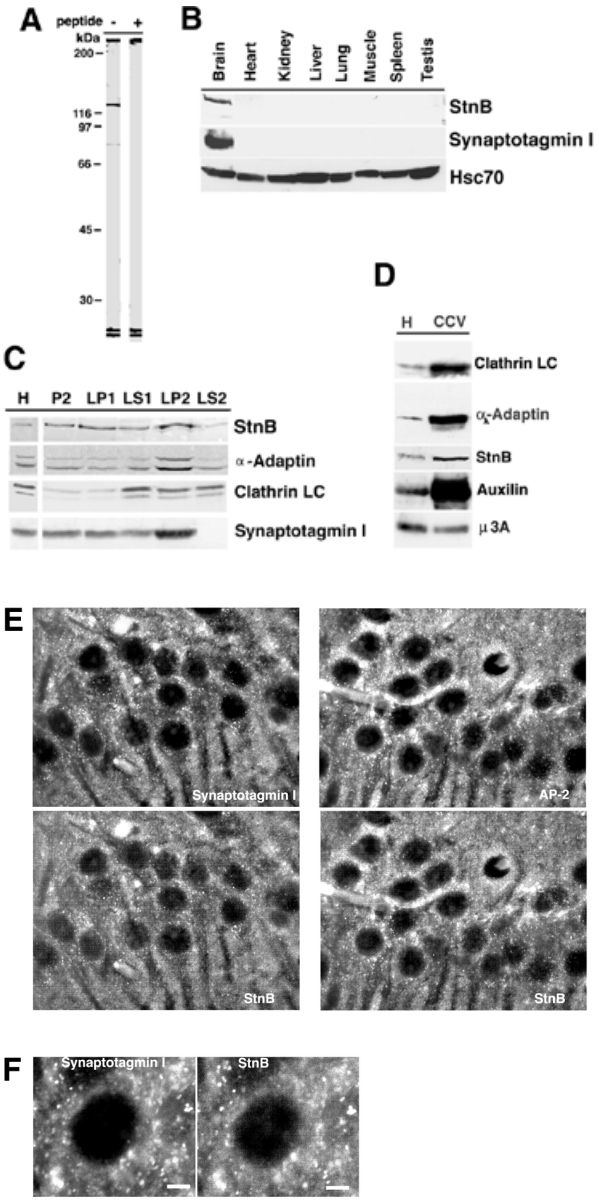

Fig. 2. Tissue distribution and localization of StnB. (**A**) Electrophoretic mobility of mammalian StnB in total brain extracts. Western blot of rat brain homogenates (100 μg total protein) using affinity-purified anti-hStnB antibodies in the absence (–) or presence (+) of the antigenic peptide (400 μM). (**B**) Western-blot analysis of the pattern of expression of mammalian StnB in various rat tissues (50 μg total protein per lane). Synaptotagmin I and hsc70 imunoreactivities are shown for comparison. (**C**) Subcellular fractions (50 μg of total protein per lane) of rat brain during the preparation of crude synaptic vesicles (LP2) were immunodecorated with antibodies against hStnB, α-adaptin, synaptotagmin I, and the light chains (LC) of clathrin. (**D**) Clathrin-coated vesicles and total brain homogenate (40 μg total protein per lane) were immunodecorated with antibodies against hStnB, α_A-adaptin (a subunit of AP-2), the light chains (LC) of clathrin, auxilin and μ3 (a subunit of AP-3). (**E**) Colocalization of StnB immunoreactivity with synaptotagmin I and AP-2 in sections of rat hindbrain by indirect immunofluorescence microscopy. In all cases immunoreactivity is represented by a punctate nerve terminal pattern. (**F**) Higher magnification illustrating the precise co-localization of StnB with synaptotagmin at nerve terminals by indirect immunofluorescence microscopy. Scale bar, 10 μM.

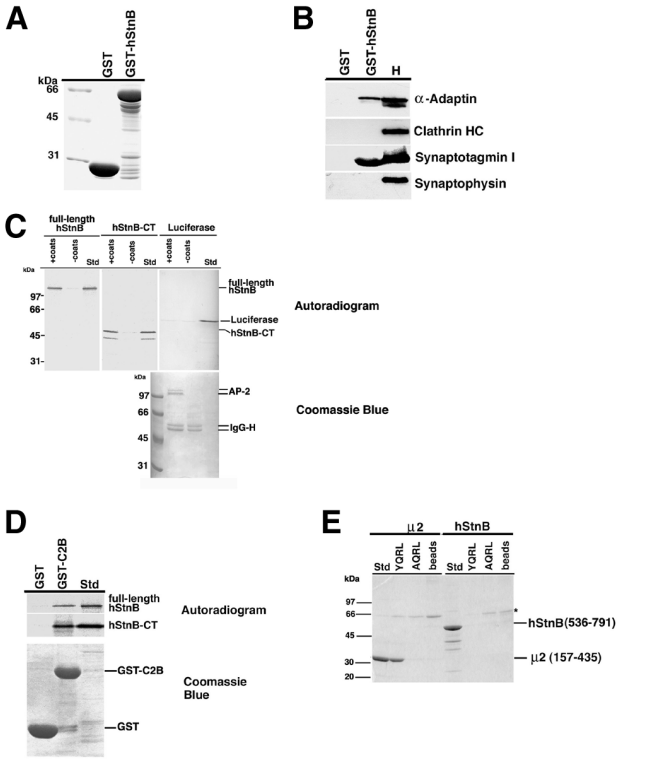

Fig. 3. hStnB directly interacts with AP-2 and synaptotagmin. (**A**) Coomassie Blue stained gel of GST and GST–hStnB (amino acids 445–791) fusion proteins (20 μg). (**B**) A Triton X-100 extract of rat brain was affinity-purified on GST or a GST fusion protein comprising amino acids 445–791 of hStnB (20 μg each). The bound material was eluted with sample buffer and analyzed by western-blotting for α-adaptin, the heavy chain of clathrin, synaptotagmin I and synaptophysin. Std, 100 μg rat brain homogenate. (**C**) Direct binding of ^{35}S-labeled *in vitro* translated hStnB or a μ2 homology domain fragment (hStnB-CT; amino acids 536–791) to immunoaffinity-purified AP-2 (1 μg). Samples incubated with beads lacking AP-2 (-coats) or ^{35}S-labeled luciferase were taken as controls. After extensive washing, eluted proteins were analyzed by SDS–PAGE and autoradiography (top) or staining with Coomassie Blue (bottom). Std, 10% of the radiolabeled protein added to the assay. (**D**) ^{35}S-labeled *in vitro* translated full-length hStnB or a μ2 homology domain fragment (hStnB-CT) were incubated with GST or a GST fusion protein (20 μg) encoding the C2B domain of synaptotagmin for 2 h at 4°C, washed, eluted with sample buffer and analyzed by autoradiography. Std, 10 % (full-length hStnB) or 20 % (hStnB-CT) of the radiolabeled protein added to the assay. (**E**) The μ2 homolgy domain of hStnB is unable to recognize tyrosine-based endocytic motifs. Recombinant hStnB (amino acids 536–791) or μ2 (amino acids 157–435) (10 μg each) were incubated with peptide-coupled beads containing the tyrosine-based endocytic motif YQRL, an inactive mutant (AQRL) or no peptide. After extensive washing the eluted protein samples were analyzed by SDS–PAGE and staining with Coomassie Blue. Std, 20% of the protein added to the assay. The asterisk denotes traces of BSA which was present in the binding buffer.

DISCUSSION

In the present study we have identified a human homolog of *Drosophila* stoned B, an important regulator of vesicle recycling at the synapse. We have shown that hStnB interacts directly with both AP-2 and synaptotagmin via its μ2 homology domain, suggesting that hStnB may play a role in endocytosis. Moreover,

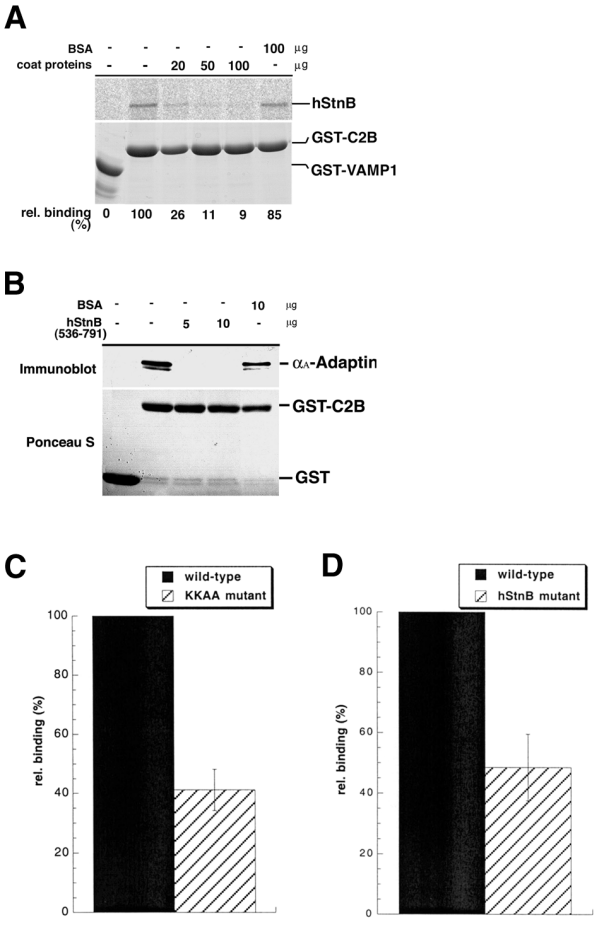

Fig. 4. AP-2 and hStnB compete for the same binding site within synaptotagmin. (**A**) Binding of ^{35}S-labeled *in vitro* translated full-length hStnB to GST–synaptobrevin or GST–synaptotagmin C2B domain fusion proteins (10 µg) in the presence of the indicated amounts of BSA or clathrin/AP coat proteins. Samples were analyzed by SDS–PAGE and staining with Coomassie Blue or quantitative analysis of the radioactive bands on a Fuji phosphoimager. (**B**) Binding of AP-2 from a coat protein fraction (corresponding to ~1 µg AP-2) in the presence of the indicated amounts of purified hStnB (amino acids 536–791) or BSA. Samples were analyzed by SDS–PAGE and immunoblotting for α-adaptin or staining with Ponceau S. (**C**) Binding of wild-type GST–synaptotagmin C2B or its KKAA mutant (10 µg) to ^{35}S-labeled *in vitro* translated hStnB-µ2 homology domain. Binding to the wild-type versus mutant versions of synaptotagmin was quantified from three different independent experiments and plotted as mean ± SD. (**D**) Binding of ^{35}S-labeled *in vitro* translated wild-type or mutant hStnB-µ2 homology domain to GST–synaptotagmin C2B domain fusion protein (10 µg) for 1 h at 4°C. Samples were washed and analyzed as described in (A). Binding of the wild-type versus mutant versions of hStnB was normalized to the respective standard of *in vitro* translated protein. Data were acquired from three different independent experiments and plotted as mean ± SD.

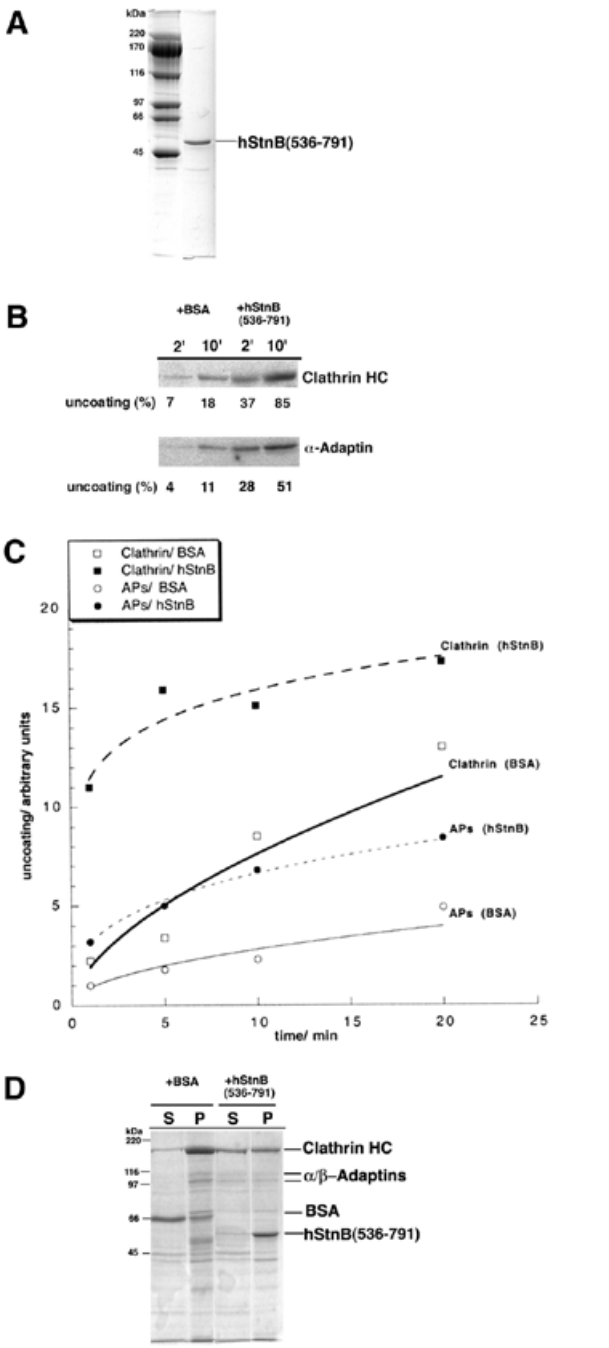

Fig. 5. hStnB facilitates uncoating of clathrin-coated vesicles. (**A**) Purified hStnB (amino acids 536–791; 2 µg) analyzed by SDS–PAGE and staining with Coomassie Blue. (**B**) Uncoating of clathrin-coated vesicles (1 µM final clathrin concentration) in the presence of 5 µg of BSA or hStnB (amino acids 536–791). Supernatant fractions were analyzed by SDS–PAGE and immunoblotting for clathrin heavy chain (top) and α-adaptin (bottom). Immunoreactive bands were detected with ^{125}I-labeled protein A, autoradiography and quantitative analysis on a Fuji phosphoimager. Clathrin and adaptor uncoating is given as a percentage of the total amount of each protein present in the assay. (**C**) Time course of uncoating in the presence or absence of hStnB (amino acids 536–791). Experiments were done as described in (B). The total amounts of clathrin and AP-2 released into the supernatant fraction during uncoating are given as a function of time. (**D**) Clathrin-coated vesicle uncoating after 5 min incubation as described in (B), analyzed by SDS–PAGE and stained with Coomassie Blue.

AP-2 and hStnB compete for binding to synaptotagmin and presumably use a common mechanism of association via µ2 homology domains. Finally, we have presented functional evidence for the involvement of hStnB in the uncoating of clathrin-coated vesicles.

Our data support a role for hStnB in synaptic vesicle endocytosis in mammals and lend further support to the idea that

stoned B facilitates synaptic vesicle recycling by interacting with synaptotagmin (Fergestad and Broadie, 2001). The finding that hStnB competes with AP-2 for binding to synaptotagmin and that it may be able to disrupt the synaptotagmin–AP-2 complex suggests that hStnB might act as a negative regulator of coat assembly. Disassembly of clathrin/AP-2 coats occurs during the uncoating reaction, which presumably requires the dissociation of AP-2 from synaptotagmin at the membrane. Impairment of clathrin-coated vesicle uncoating has been shown to inhibit endocytosis and synaptic vesicle formation in a number of organisms (Cremona *et al.*, 1999; Harris *et al.*, 2000; Pishvaee *et al.*, 2000; Greener *et al.*, 2001; Zhao *et al.*, 2001). The data presented here support a putative role for hStnB in the uncoating of clathrin-coated vesicles: first, hStnB can bind directly to both AP-2 and synaptotagmin via its μ2 homology domain, properties that would allow the protein to disrupt the AP-2-synaptotagmin complex. The observation that hStnB is unable to recognize tyrosine-based endocytic motifs also supports the notion that it may act at a different, presumably later step of the vesicle cycle. Secondly, hStnB and AP-2 compete for the same binding site within synaptotagmin, suggesting that they might act at different steps within the vesicle cycle. Thirdly, the μ2 homology domain of hStnB facilitates uncoating of clathrin and AP-2 from clathrin-coated vesicles *in vitro*.

In summary, we hypothesize that hStnB regulates synaptic vesicle recycling by interacting with AP-2 and synaptotagmin and by promoting clathrin-coated vesicle uncoating. How exactly hStnB triggers uncoating and whether this requires its binding to AP-2 needs further investigation. One could imagine that efficient AP-2 release in addition to disrupting its binding to synaptotagmin might require some 'chaperone-like' activity of hStnB that would help to guide AP-2 away from the vesicle membrane. We also do not yet understand the function of the NPF motifs and whether they play a role in uncoating or whether they serve as targeting determinants that guide hStnB to sites of endocytosis. Putative NPF-binding partners include the endocytic factors intersectin and Eps15 (Brodin *et al.*, 2000; De Camilli *et al.*, 2001). Future studies will be aimed at addressing these issues as well as at dissecting the order of events during hStnB-mediated vesicle uncoating.

METHODS

Antibodies. Polyclonal antibodies against human stoned B (hStnB; residues 464–477 or 536–791) were raised in rabbits. Antiserum was affinity-purified either on cyanogen bromide activated Sepharose covalently linked to the antigenic peptide or an immobilized fusion protein between GST and the hStnB μ2 homology domain (amino acids 445–791). Mouse monoclonal antibodies against clathrin light chains, synaptophysin and synaptotagmin I were a kind gift of Dr Reinhard Jahn (MPI, Göttingen), antibodies against clathrin heavy chain were kindly provided by Dr Pietro De Camilli (Yale University), anti-α-adaptin antibodies were from Affinity Bioreagents (clone AC1-M11) and Transduction Labs. Antisera against μ3 were from Transduction Labs.

Cloning of hStnB cDNA. NCBI data base searches using *Drosophila* stoned B as a bait identified partial human sequences corresponding to the C-terminal half of the hStnB ORF (amino acids 408–886) including an in-frame stop codon. This incomplete cDNA was cloned by PCR using human cDNA as a template. It was found to overlap with a Stratagene clone (Cat. No.14981) containing the 5′ end of the hStnB ORF including a putative start ATG preceded by an in-frame stop codon. A complete cDNA clone was assembled by PCR, cloned into pcDNA3 and verified by DNA sequencing. The complete gene including its 5′ end could also be amplified from human brain cDNA (Invitrogen Inc.) by 5′-RACE suggesting that a full-length clone had been identified.

Site-directed mutagenesis. A double point mutant of the μ2 homology domain of hStnB (amino acids 559–791) in which W719 and K721 were changed to alanines was created by PCR using mutagenic primers and cloned along with a corresponding wild-type version of the gene into a hemagglutinin epitope-tagged derivative of pcDNA3. The presence of the mutation was verified by DNA sequencing.

Production of recombinant proteins. GST- (amino acids 445–791) and His_6-tagged (amino acids 536–791) fusion-proteins comprising the μ2 homology domain of hStnB were cloned into pGEX5T or pET28a, respectively, by PCR and verified by restriction analysis and DNA sequencing. Fusion proteins were expressed in *E. coli* and purified according to standard procedures (see Supplementary data).

Affinity chromatography. Affinity chromatography was essentially performed as described previously (Haucke and De Camilli, 1999) (see Supplementary data for details).

Uncoating assays. Clathrin coated vesicles from pig brain (1 μM final clathrin concentration) were incubated with 2 mM ATP and rat brain cytosol (1 mg/ml) in the presence or absence of BSA or recombinant hStnB (amino acids 536–791) in buffer C (20 mM imidazole pH 7.0, 25 mM KCl, 10 mM ammonium sulfate, 2 mM $MgCl_2$, 1 mM DTT) at 20°C. After the indicated times the samples were centrifuged in a TL100.1 rotor at 100 000 r.p.m. for 5 min at 4°C. The top 80% of the supernatants or the pellet fractions was analyzed by SDS–PAGE and Coomassie Blue staining or immunoblotting for clathrin heavy chain and α-adaptin using ^{125}I-labeled protein A for detection.

Miscellaneous. Standard procedures were used for SDS–PAGE, immunoblotting, *in vitro* transcription/translation (Promega Inc.) and indirect immunofluorescence microscopy. Subcellular fractions of rat brain, clathrin-coated vesicles and clathrin/adaptor coat proteins were prepared as previously described (Haucke and De Camilli, 1999). Rat brain cytosol was prepared according to Takei *et al.* (1998). Rat brain thin sections were obtained from Novagen Inc.

Accession numbers. The nucleotide sequence reported in this paper has been deposited at DDBJ/EMBL/GenBank (accession No. AF380833).

Supplementary data. Supplementary data are available at *EMBO reports* Online.

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NOTE ADDED IN PROOF

A recent study has reported on a ubiquitously expressed isoform of hStnB, which is involved in endocytosis. [Martina, J.A., Bonangelino, C.J., Aguilar, R.C. and Bonifacino, J.S. (2001) Stonin 2: an adaptor-like protein that interacts with components of the endocytic machinery. *J. Cell Biol.*, **153**, 1111–1120.]

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