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

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Synaptic vesicle biogenesis involves the rapid 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 Strategene 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 auxin. In contrast, μ1, 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 immunoperoxidase-stained AP-2 from clathrin coat proteins (Figure 3C, bottom) and analyzed whether in vitro translated 35S-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 35S-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.

We determined the binding of 35S-labeled hStnB to immobilized synaptotagmin (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, human stoned B in vesicle recycling

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, hStnB binds to both AP-2 and synaptotagmin

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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 35S-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

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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. 1. Alignment of the deduced amino acid sequences of Drosophila (dStnB) and human stoned B (hStnB). The $\mu_2$ homology domain is boxed. NPF motifs are underlined. Sequence conservation is highest within the $\mu_2$ homology domain (34.8% identity; 66.4% similarity), the overall identity is 22.4% (44% similarity).
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,

**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,
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 Broadsie, 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 μ2 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 His6-tagged (amino acids 536–791) fusion-proteins comprising the μ2 homology domain of hStnB were cloned into pGEXGST 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 MgCl2, 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 125I-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 hSNB, which is involved in endocytosis. [Martina, J.A., Bonangelino, C.J., Aguilar, R.C. and Bonifacio, J.S. (2001)


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