

# Stonin 2: An Adaptor-like Protein that Interacts with Components of the Endocytic Machinery

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**Abstract.** Endocytosis of cell surface proteins is mediated by a complex molecular machinery that assembles on the inner surface of the plasma membrane. Here, we report the identification of two ubiquitously expressed human proteins, stonin 1 and stonin 2, related to components of the endocytic machinery. The human stonins are homologous to the *Drosophila melanogaster* stoned B protein and exhibit a modular structure consisting of an NH<sub>2</sub>-terminal proline-rich domain, a central region of homology specific to the stonins, and a COOH-terminal region homologous to the  $\mu$  subunits of adaptor protein (AP) complexes. Stonin 2, but not stonin 1, interacts with the endocytic machinery proteins Eps15, Eps15R, and intersectin 1. These interactions occur via

two NPF motifs in the proline-rich domain of stonin 2 and Eps15 homology domains of Eps15, Eps15R, and intersectin 1. Stonin 2 also interacts indirectly with the adaptor protein complex, AP-2. In addition, stonin 2 binds to the C2B domains of synaptotagmins I and II. Overexpression of GFP-stonin 2 interferes with recruitment of AP-2 to the plasma membrane and impairs internalization of the transferrin, epidermal growth factor, and low density lipoprotein receptors. These observations suggest that stonin 2 is a novel component of the general endocytic machinery.

**Key words:** receptor-mediated endocytosis • clathrin • AP-2 • Eps15 • intersectin

## Introduction

The internalization of plasma membrane proteins is critical to many important physiological processes such as synaptic vesicle recycling and receptor-mediated endocytosis. These processes are mediated by a complex molecular machinery that is recruited from the cytosol to the inner surface of the plasma membrane, where it assembles into a supramolecular structure known as a protein coat (for reviews see Owen and Luzio, 2000; Kirchhausen, 2000; Slepnev and De Camilli, 2000). The coat selects integral membrane proteins destined for internalization and participates in the formation of endocytic vesicles. The main structural constituent of plasma membrane coats is the protein clathrin. Assembly of clathrin coats onto the plasma membrane is initiated by binding of the heterotetrameric adaptor protein (AP)<sup>1</sup> complex, AP-2, to a member of the synaptotagmin family of integral membrane proteins (Zhang et al., 1994; Haucke et al., 2000). Clathrin triskelions then poly-

merize onto the membrane-bound AP-2 to form a lattice that serves as a scaffold for the protein coat. Integral membrane proteins containing either tyrosine-based or dileucine-based endocytic signals are concentrated within the assembling coats by interaction of these signals with AP-2 (for review see Bonifacino and Dell'Angelica, 1999; Heilker et al., 1999). Various accessory proteins (e.g., amphiphysins, Eps15, intersectins, dynamins, etc.) become associated with the coat by either direct or indirect interaction with clathrin and/or AP-2. These accessory proteins are thought to regulate coat assembly and vesicle budding, as well as interactions of the endocytic machinery with signal transduction pathways and the actin cytoskeleton (Kirchhausen, 2000; Owen and Luzio, 2000; Slepnev and De Camilli, 2000).

Novel components of the machinery are still being identified through biochemical and genetic approaches. A particularly useful approach to identify physiologically critical components of the endocytic machinery has been the study of temperature-sensitive paralytic mutants of *D. melanogaster* pioneered by David Suzuki and colleagues (Grigliatti et al., 1973). Some of these mutants bear defects in genes involved in synaptic vesicle recycling from the plasma membrane. Inactivation of these genes upon shift to the nonpermissive temperature results in an inability to reform synaptic vesicles after exocytosis. One of these mu-

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<sup>1</sup>Abbreviations used in this paper: AP, adaptor protein; EH, Eps15 homology; GAL4ad, GAL4 transcription activation domain; GFP, green fluorescent protein; GST, glutathione S-transferase.

tant genes, *shibire*, was found to encode a *D. melanogaster* homologue of mammalian dynamins (Chen *et al.*, 1991; van der Blik and Meyerowitz, 1991). Another paralytic mutant, *stoned*, was found to bear mutations in a dicistronic transcript encoding two structurally unrelated proteins, stoned A and stoned B (Andrews *et al.*, 1996). Although stoned A has no homology to any other known protein, stoned B comprises a COOH-terminal domain with homology to the  $\mu$ 2 subunit of AP-2 (Andrews *et al.*, 1996). Combination of hypomorphic *shibire* and *stoned* mutations produces synthetic lethality, suggesting that the products of these genes participate in either the same or parallel pathways of synaptic vesicle biogenesis (Petrovich *et al.*, 1993). It remains to be established, however, whether the *stoned* gene products play a role in endocytosis itself, and whether this role is limited to neurons or general to all cells.

To investigate the possible involvement of *stoned* gene products in endocytosis in a system that is more amenable to biochemical and cellular analyses, we sought to identify human homologues of these proteins. Here, we report the identification and characterization of two ubiquitously expressed human homologues of the *D. melanogaster* stoned B protein named stonin 1 and stonin 2. The results of our study suggest that stonin 2 is a component of the general endocytic machinery in higher eukaryotes.

## Materials and Methods

### Cloning of Stonin 1 and Stonin 2 cDNAs

A search of DNA databases for human homologues of *D. melanogaster* stoned B identified the SALF protein (Upadhyaya *et al.*, 1999) and a related fetal lung protein encoded by a novel cDNA sequence (GenBank/EMBL/DBJ accession no. AI734225). Primers corresponding to SALF were used in 5'- and 3'-RACE PCR on a Marathon-Ready™ human heart cDNA library (CLONTECH Laboratories, Inc.) to isolate a novel transcript, herein termed Stonin 1 (GenBank/EMBL/DBJ accession no. AF255310), encoding the NH<sub>2</sub>-terminal 711 of the 1,182-amino acid SALF. The complete ORF of the second human cDNA, herein termed Stonin 2 (GenBank/EMBL/DBJ accession no. AF255309) was similarly obtained from a Marathon-Ready™ human spleen cDNA library (CLONTECH Laboratories, Inc.), using primers designed on the basis of partial genomic DNA sequences (GenBank/EMBL/DBJ accession nos. AC010582 and AL136040). The identical stonin 1 and stonin 2 full-length sequences were amplified by RT-PCR from mRNA of multiple human cell lines (HeLa, Jurkat, 293T, and Caco-2A). The above PCR products were cloned into the pCR2.1 vector (Invitrogen) and sequenced.

### Northern Blotting

Northern blot analysis was performed on multiple human tissue blots (CLONTECH Laboratories, Inc.) as described previously (Dell'Angelica *et al.*, 1997). Stonin 1 and stonin 2 cDNA probes were obtained by PCR. For stonin 1, two different probes were used in separate experiments: the proline-rich domain probe comprised nucleotides 1–615 and the  $\mu$ -homology domain probe comprised nucleotides 1187–2509 of the full-length stonin 1 cDNA. The probe for stonin 2 corresponded to the complete coding sequence.

### Antibodies

Polyclonal antibodies to human stonin 1 and stonin 2 were generated by immunizing rabbits with the corresponding proline-rich domains fused to a deca-histidine tag. The fusion proteins were recovered from inclusion bodies by extraction in 6 M urea. A monoclonal antibody to human Eps15 and a polyclonal antibody to *Xenopus* intersectin were gifts from Dr. Pier Paolo Di Fiore (European Institute of Oncology, Milan, Italy) and Dr. Peter McPherson (McGill University, Montreal, Canada), respectively. The following monoclonal antibodies were also used: HA.11; to the hemagglutinin epitope and 9E10; to the Myc epitope (Covance), 100/3; to  $\gamma$ 1-adap-

tin and AP.6; to  $\alpha$ -adaptin (Sigma-Aldrich), clones 7.1 and 13.1; to the green fluorescent protein (GFP; Roche Molecular Biochemicals) and H4A3; to lamp-1 (Developmental Studies Hybridoma Bank). The following polyclonal antibodies were also used: anti-GFP antiserum (Medical and Biological Laboratories Co.) and serum anti-caveolin antiserum (Transduction Laboratories).

### Recombinant DNA Constructs

GFP fusion constructs, comprising the proline-rich domain of human stonin 2, namely GFP-stonin 2<sub>PRD</sub> (residues 1–223) and the full-length GFP-stonin 2, were cloned into the pEGFP-C1 (HindIII-SmaI) vector (CLONTECH Laboratories, Inc.). The complete ORF of human stonin 1 was fused to GFP by cloning into pEGFP-C2 (GFP-stonin 1, KpnI-BamHI). Epitope-tagging at the NH<sub>2</sub> terminus of stonin 2 was performed through PCR amplification of the full-length stonin 2 cDNA using 5'-primer containing the nucleotide sequence for the Myc epitope. This product was cloned into the XhoI-XbaI sites of the mammalian expression vector pCI-neo (Promega). The PRD + SHD domains of stonin 2 were subcloned into the XhoI-XmaI sites of the pCI-neo vector (Myc-stonin 2<sub>PRD+SHD</sub>; residues 1–359) using the same approach. Two-hybrid constructs in the pGBKT7 (TRP1) vector (CLONTECH Laboratories, Inc.) were prepared by cloning of nucleotide sequences coding for the following amino acid residues: 1–296 of human Eps15 (Eps15-Eps15 homology (EH); NdeI-BamHI); 1–395 of human Eps15R (Eps15R-EH; EcoRI-BamHI); and 1–333 of human intersectin 1 (intersectin 1-EH; NdeI-XmaI). Similarly, the stonin 1 (full-length; XhoI-XmaI), stonin 2 (full-length; NdeI-XmaI), stonin 2<sub>PRD+SHD</sub> (NdeI-XmaI), and human epsin 2 (full-length; NdeI-EcoRI) cDNAs were cloned into the pGADT7 (LEU2) vector (CLONTECH Laboratories, Inc.) to obtain the corresponding GAL4 transcription activation domain (GAL4ad) fusion constructs. The constructs (protein names and amino acid numbers indicated in parentheses) glutathione *S*-transferase (GST)-intersectin 1-EH (intersectin 1, 1–333), GST-synaptotagmin I-C2AB (murine synaptotagmin I, 96–421), GST-synaptotagmin I-C2A (synaptotagmin I, 96–265), GST-synaptotagmin I-C2B (synaptotagmin I, 248–421), GST-synaptotagmin II-C2AB (murine synaptotagmin II, 124–422), GST-synaptotagmin II-C2A (synaptotagmin II, 124–272), and GST-synaptotagmin II-C2B (synaptotagmin II, 249–422) were cloned into the EcoRI-XhoI sites of the pGEX-5X-1 vector (Amersham Pharmacia Biotech). GST-Eps15-EH was generated by cloning of the cDNA sequence corresponding to residues 1–296 of Eps15 into the BamHI-XhoI sites of the pGEX-4T-1 vector (Amersham Pharmacia Biotech). For protein expression and purification, competent *E. coli* strains DH5 $\alpha$  (PGC Scientific) or BL21 (DE3) (Novagen Inc.) cells were transformed with the above constructs. Recombinant GST fusion proteins were purified by using glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech), and amino acid substitutions in the proline-rich domain of stonin 2 (N110A, P111A, F112A, P127A, and F128A) were made using the QuickChange mutagenesis kit (Stratagene), according to the manufacturer's instructions.

### Transfection, Immunofluorescence, and Internalization Assays

The sources and culture conditions for all the human cell lines used in this study are described elsewhere (Dell'Angelica *et al.*, 1997). Human HeLa or M1 cells, grown on glass coverslips, were transfected by using FuGENE-6 (Roche Molecular Biochemicals). Cells were incubated with 7 mM Na-butyrate for 12 h before fixation. At 24 h after transfection, cells were either fixed and processed for immunofluorescence (Dell'Angelica *et al.*, 1997) or used directly for analysis of rhodamine-EGF (500 ng/ml), Dil-LDL (10 ng/ $\mu$ l), or Cy3-transferrin (530 ng/ml) internalization (Bemerah *et al.*, 2000; Cy3-transferrin was a gift from Timothy McGraw, Weill Medical College, New York, NY). The internalization of rhodamine-EGF was performed using the same conditions as for Cy3-transferrin, with the exception that the serum starvation was much longer (12 h) and was done in the presence of 0.5% (vol/vol) serum. Images were acquired on an LSM 410 confocal microscope (Carl Zeiss, Inc.).

### General Biochemical Procedures

Metabolic labeling of cells in culture with [<sup>35</sup>S]methionine, preparation of Triton X-100, detergent-free extracts of post-nuclear membranes, ultracentrifugation on sucrose gradients, and immunoprecipitation–recapture were carried out as described (Dell'Angelica *et al.*, 1997). For cell fractionation, [<sup>35</sup>S]methionine-labeled cells were disrupted in lysis buffer (10 mM Tris-HCl, pH 7.4, 250 mM sucrose with a cocktail of protease inhibi-

tors) (Dell'Angelica et al., 1997) by 20 passages through a 25-gauge needle on ice. Samples were centrifuged at 500 *g* for 10 min at 4°C, and the resulting supernatants (postnuclear supernatant) were subjected to ultracentrifugation at 105,000 *g* for 1 h at 4°C to obtain cytosolic (supernatant) and membrane (pellet) fractions. For extraction experiments, the membrane fraction was resuspended in lysis buffer and divided into six equal aliquots. Aliquots were then incubated for 45 min at 4°C in lysis buffer containing either 1 M Tris-HCl (pH 7.4), 1 M NaCl, 0.2 M Na<sub>2</sub>CO<sub>3</sub> (pH 11.3), 1.6 M urea, 2% (wt/vol) Triton X-100, or left untreated. After the incubation period, samples were centrifuged at 105,000 *g* for 1 h, and both pellets and supernatants were collected for further analysis. To obtain a fraction enriched in detergent-resistant membranes, cellular extracts were subjected to a discontinuous sucrose gradient as described (Ilangumaran and Hoessli, 1998), with the exception that 0.9-ml fractions were collected from the top of the gradient. Then, 0.5 ml of each fraction was diluted 1:1 in immunoprecipitation buffer and used for immunoprecipitation–recapture with the antibodies described in the corresponding figure legend. For *in vitro* translation of Myc-stonin 2<sub>PRD+SHD</sub>, [<sup>35</sup>S]methionine-labeled proteins were synthesized *in vitro* using the TNT® Coupled Reticulocyte Lysate System (Promega). Postribosomal supernatants were then generated by centrifugation at 200,000 *g* and used for GST pull-down assays.

### GST Pull-down Assays

15 μg of either GST–Eps15-EH or GST–intersectin 1-EH fusion proteins immobilized on glutathione–Sepharose were incubated with *in vitro* translation products or extracts from transfected HeLa cells (0.8 mg of protein) in binding buffer (25 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM MgCl<sub>2</sub>, 1 mM EDTA, protease inhibitors, and 1% [wt/vol] Triton X-100) for 4 h at 4°C. 20 μg of GST–synaptotagmin fusion proteins were incubated as described above, with the exception that the binding buffer used was 50 mM Hepes (pH 7.4), 100 mM NaCl, 1 mM MgCl<sub>2</sub>, protease inhibitors, and 1% (wt/vol) Triton X-100. Beads were spun and washed four times with ice-cold binding buffer and once with ice-cold binding buffer lacking detergent. Bound proteins were analyzed either by immunoblotting or immunoprecipitation–recapture followed by SDS-PAGE and fluorography. For all experiments, GST alone and GST–GGA3–GAE (for nonspecific binding) were used as controls.

### Yeast Culture, Transformation, and Two-Hybrid Assays

The *S. cerevisiae* strain AH109 (CLONTECH Laboratories, Inc.) was transformed by the lithium acetate procedure as described in the instructions for the MATCHMAKER two-hybrid kit (CLONTECH Laboratories, Inc.). For colony growth assays, AH109 transformants were resuspended in water to 0.1 OD<sub>600</sub>/ml, and then 5 μl was spotted on plates lacking leucine, tryptophan, and histidine in the presence or absence of adenine, and containing 10 mM 3-amino-1,2,4-triazole (Fluka Chemie AG) and incubated at 30°C for 4–5 d.

## Results

### Identification of Human Stonin 1 and Stonin 2

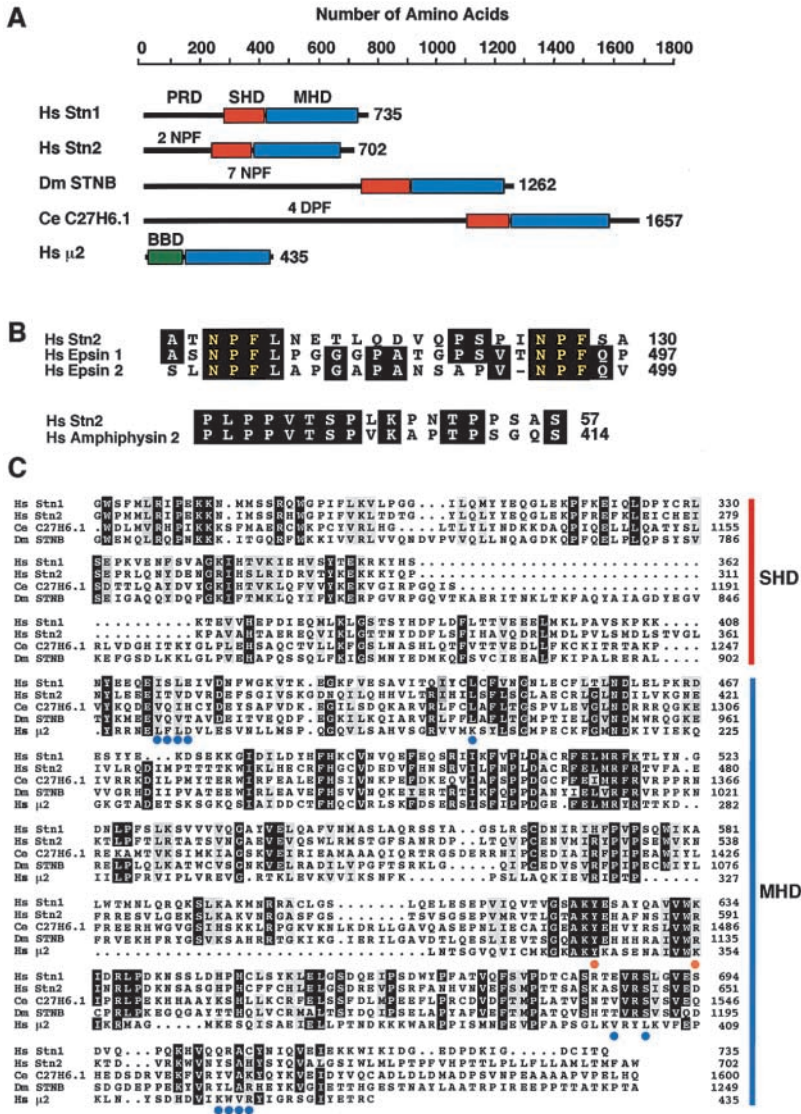
Two distinct human transcripts encoding homologues of the *D. melanogaster* stoned B protein were identified through searches of GenBank. The complete ORFs for these proteins, herein referred to as stonin 1 and stonin 2, were obtained by a combination of 5' and 3' RACE and PCR amplification. Unlike the *D. melanogaster* stoned transcripts, the human stonin transcripts were monocistronic and did not encode stoned A–related products. In fact, our searches did not turn up homologues of *D. melanogaster* stoned A in any other organism. The human stonin 1 transcript encodes a protein of 735 amino acids and a predicted molecular mass of ~83 kD, whereas the stonin 2 transcript encodes a protein of 702 amino acids and a predicted molecular mass of ~79 kD. Residues 1–711 of stonin 1 are almost identical to residues 1–711 of the 1,182–amino acid product of the SALF transcript (Upadhyaya et al., 1999). This transcript has been proposed to encode a protein with an NH<sub>2</sub>-terminal region homologous to the

*D. melanogaster* stoned B protein and a COOH-terminal region homologous to the general transcription factor TFIIAα/β (Upadhyaya et al., 1999). It has been suggested that SALF is a product of two separate but adjacent human genes, which is formed from a pre-mRNA transcript that initiates at an upstream Stoned B–like factor gene and continues into the ALF gene (Han et al., 2001). The stonin 1 transcript reported here differs from the SALF transcript in that it has an inframe termination codon after the stonin 1 sequence and does not contain the TFIIAα/β–homologous sequence. The existence of the stonin 1 transcript was confirmed by RT-PCR from total RNA of multiple cell lines and by PCR amplification from human heart and spleen libraries. Orthologues of both human stonin 1 and stonin 2 were found in the mouse (Martina, J.A., and J.S. Bonifacino, unpublished observations). Single homologues of these proteins exist in *D. melanogaster* (i.e., stoned B) and *C. elegans* (i.e., hypothetical protein C27H6.1), but none in yeast and prokaryotes.

### Domain Organization of Human Stonin 1 and Stonin 2

The two human stonins, as well as their *D. melanogaster* and *C. elegans* counterparts, exhibit a modular structure consisting of an NH<sub>2</sub>-terminal proline- and serine-rich domain (herein referred to as proline-rich domain), a central domain specific to the stonins (stonin homology domain), and a COOH-terminal domain homologous to the signal-binding domain of the μ subunits of AP complexes (μ-homology domain) (Fig. 1 A). The proline-rich domains of the human, *D. melanogaster*, and *C. elegans* homologues are of variable length and exhibit little or no significant sequence homology to one another, except for the abundance of proline and serine residues. The proline-rich domain of *D. melanogaster* stoned B displays seven NPF motifs (Andrews et al., 1996; Stimson et al., 1998), which in general have been shown to mediate interactions with Eps15 homology (EH) domains (Salcini et al., 1997). Despite the lack of overall homology in this region, the proline-rich domain of stonin 2, but not that of stonin 1, has two NPF motifs separated by 13 amino acids (Fig. 1 B). A similar arrangement of NPF motifs is found in epsin 1 and epsin 2 (Fig. 1 B) (Chen et al., 1998; Yamabhai et al., 1998; Hussain et al., 1999; Rosenthal et al., 1999). Not only are the NPF motifs in stonin 2 and the epsins similarly spaced but they also flank an intervening sequence containing additional conserved residues. Most notable is the presence of an L residue after the first NPF motif, a feature that has been shown to enhance interaction with certain EH domains (de Beer et al., 2000). Another short stretch of amino acids in the proline-rich domain of stonin 2 is highly similar to a stretch in the proline-rich region of amphiphysin 2 (Fig. 1 B) (Leprince et al., 1997; Ramjaun et al., 1997; Wigge et al., 1997).

Next to the proline-rich domain lies an ~140–amino acid domain (stonin homology domain) that is conserved in both human stonins, as well as their *D. melanogaster* and *C. elegans* homologues (Fig. 1, A and C). This domain has not been described in other proteins and may thus be unique to members of the stonin family. Immediately adjacent to this domain in all stonin homologues is an ~350–amino acid domain homologous to the YXXØ signal-binding domain of μ2 (Aguilar et al., 1997; Owen and Evans, 1998), a subunit of the heterotetrameric AP-2 complex (Fig. 1, A and C). A



**Figure 1.** Structure of stonins and related proteins. (A) Schematic representation of human stonin 1 (Hs Stn1, GenBank/EMBL/DDBJ accession no. AF255310), human stonin 2 (Hs Stn2, AF255309), *D. melanogaster* stoned B (Dm STNB, U54982), *C. elegans* hypothetical protein C27H6.1 (Ce C27H6.1, Z81042), and the human  $\mu$ 2 subunit of the AP-2 complex (Hs  $\mu$ 2, NM\_004068). Specific domains of homology are color coded and denoted as follows: PRD, proline-rich domain; SHD, stonin homology domain; MHD,  $\mu$ -homology domain; BBD,  $\beta$ -binding domain. Black lines denote regions with no significant sequence homology. Numbers of NPF or DPF motifs in the PRDs are indicated. (B) Alignment of short sequences in the PRD of human stonin 2 with homologous sequences in human (Hs) epsin 1 (AF073727), epsin 2 (AF062084), and amphiphysin 2 (AAB63263). Identical residues are boxed, and NPF motifs are highlighted in yellow. (C) Multiple sequence alignment of the SHD and MHD domains of stonin family members. The black and gray boxes indicate identical and strongly conserved amino acids, respectively. Blue and orange dots indicate amino acids in  $\mu$ 2 that are involved in binding YXX $\phi$ -type signals (Owen and Evans, 1998) and the C2B domain of synaptotagmin I (Hauke et al., 2000), respectively. Numbers denoting the last amino acid in each sequence are indicated on the right.

similar domain is found in the related  $\mu$ 1 (A and B isoforms),  $\mu$ 3 (A and B isoforms), and  $\mu$ 4 subunits of the heterotetrameric AP complexes, AP-1, AP-3, and AP-4, respectively (for review see Hirst and Robinson, 1998; Bonifacino and Dell'Angelica, 1999). These theoretical analyses thus indicate that the stonins share several structural features with known components of the sorting machinery.

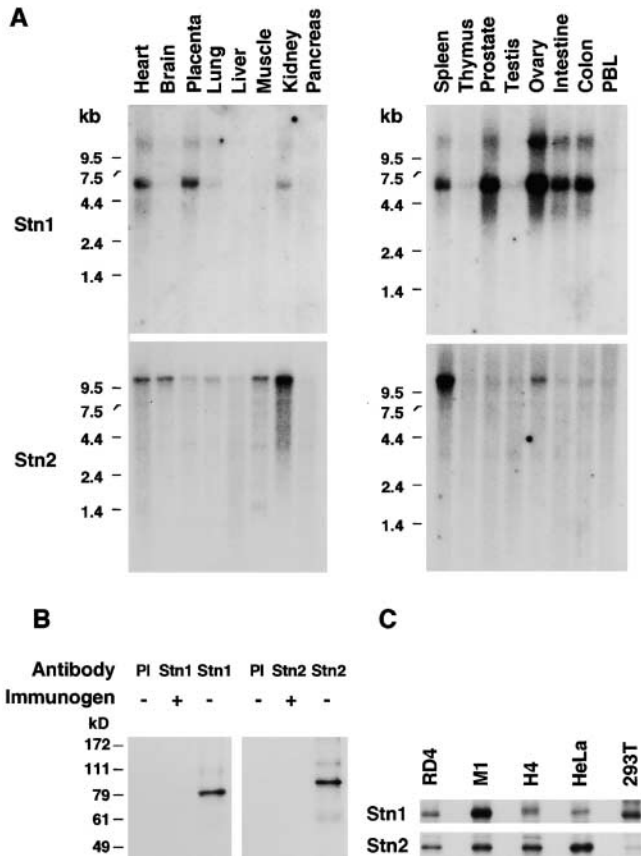
### Ubiquitous Expression of Human Stonin 1 and Stonin 2

Because mutations in the *D. melanogaster* stoned locus result in severe neurological dysfunction (Petrovich et al., 1993; Andrews et al., 1996; Fergestad et al., 1999), it was of interest to determine the pattern of tissue and cell expression of the human stonins. Northern blot analysis revealed expression of a 6.5-kb stonin 1 mRNA and a 11.3-kb stonin 2 mRNA in most human tissues examined (Fig. 2 A). To study expression of the corresponding proteins, we raised polyclonal antibodies to their proline-rich domains. These antibodies were used to identify the proteins by immunoprecipitation–recapture from metabolically labeled cells (Bonifacino and Dell'Angelica, 1998). The antibodies to stonin 1 and stonin 2 were found to immunoprecipitate

major species of  $\sim$ 81 and  $\sim$ 88 kD, respectively, in reasonable agreement with their predicted molecular weights (Fig. 2 B). The bands detected by these antibodies were specific, as they were not detected using preimmune sera (PI) or antisera absorbed with the corresponding immunogens (Fig. 2 B). Use of these antibodies allowed us to demonstrate expression of both stonins in neuronal (H4) as well as nonneuronal (RD4, M1, HeLa, and 293T) human cells (Fig. 2 C). Thus, both stonins are ubiquitously expressed in human tissues, suggesting that their role might not be restricted to the nervous system.

### Both Human Stonins Exist as Cytosolic and Peripheral Membrane Proteins

Fractionation of metabolically labeled HeLa cells showed that stonin 1 was mostly recovered in a cytosolic fraction (C), with a small amount being associated with membranes (M) (Fig. 3 A). Stonin 2 was present in equal amounts in the cytosolic and membrane fractions (Fig. 3 A). The membrane-bound stonin 1 and stonin 2 could be partially extracted with 1 M Tris or NaCl, and totally extracted with 0.2 M  $\text{Na}_2\text{CO}_3$ , pH 11.3 (Fig. 3 B), indicating that they be-

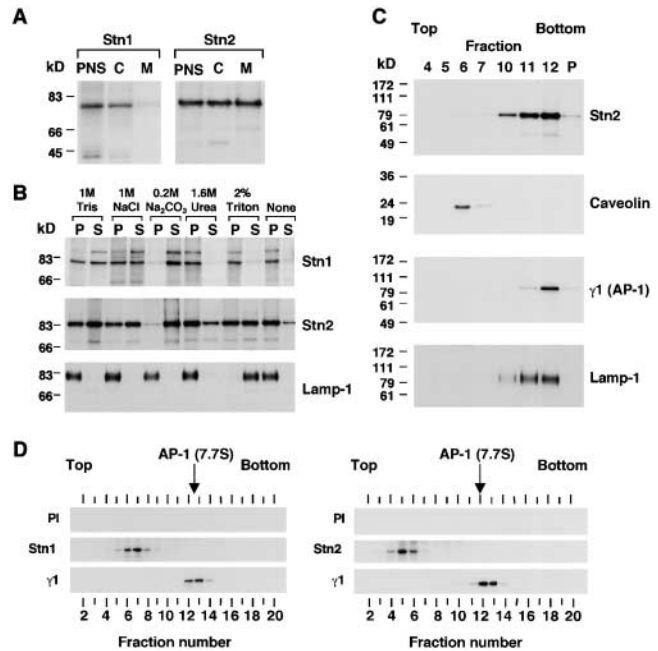


**Figure 2.** Expression of stonins in human tissues and cells. (A) Northern blot analysis of the expression of stonin 1 (Stn1) and stonin 2 (Stn2) mRNAs in human tissues. The positions of RNA molecular size markers (in kb) are indicated on the left. (B) Immunoprecipitation–recapture of endogenous stonin 1 and stonin 2 proteins from [<sup>35</sup>S]methionine-labeled HeLa cells using preimmune serum (PI), specific antibodies to each protein, or specific antibodies preabsorbed with the immunogens. Immunoprecipitates were analyzed by 4–20% SDS-PAGE. The positions of protein molecular mass markers (in kD) are indicated on the left. (C) Expression of endogenous stonin 1 and stonin 2 proteins in different human cell lines. Stonins were isolated from equal quantities of [<sup>35</sup>S]methionine-labeled cell lines using specific antibodies to each protein, as in B.

haved as peripheral membrane proteins. Interestingly, a large fraction of both membrane-bound stonins remained insoluble upon extraction with 2% (wt/vol) Triton X-100 at 4°C (Fig. 3 B). This insoluble stonin 2, however, was not associated with detergent-resistant lipid domains, as determined by its failure to float with caveolin-containing fractions in sucrose density gradients (Fig. 3 C). Thus, the stonins appear to exist in cytosolic and peripheral membrane pools.

#### Human Stonins Are Not Subunits of AP Complexes

As mentioned above, the stonins contain a COOH-terminal domain homologous to the YXXØ signal-binding domain of the  $\mu$  subunits of the heterotetrameric AP complexes, AP-1, AP-2, AP-3, and AP-4 (Aguilar et al., 1997; Owen and Evans, 1998). However, the stonins lack another domain present in the  $\mu$  subunits that mediates assembly with the  $\beta$  subunits of the corresponding AP com-



**Figure 3.** Analysis of the association of stonins with membranes. (A) Immunoprecipitation–recapture of stonin 1 (Stn1) and stonin 2 (Stn2) from a postnuclear supernatant (PNS) of [<sup>35</sup>S]methionine-labeled HeLa cells and from cytosolic (C) and membrane (M) fractions of the PNS after ultracentrifugation. (B) Membrane fractions obtained as in A were extracted for 45 min at 4°C with the solutions indicated on top. Pellet (P) and supernatant (S) fractions were then prepared by ultracentrifugation, and stonin 1, stonin 2, and lamp-1 were isolated by immunoprecipitation–recapture with specific antibodies. (C) [<sup>35</sup>S]methionine-labeled HeLa cells were solubilized in 2% (wt/vol) Triton X-100 on ice for 30 min, and the extract was centrifuged on a discontinuous sucrose gradient. Stonin 2, caveolin, the  $\gamma$ 1 subunit of AP-1 and lamp-1 were then isolated from the indicated gradient fractions using specific antibodies. The positions of molecular mass markers (in kD) are indicated on the left. (D) Analysis of the size of stonins by sedimentation velocity. A cytosolic fraction of [<sup>35</sup>S]methionine-labeled HeLa cells was separated by ultracentrifugation on a 4–20% sucrose gradient. Stonin 1, stonin 2, and  $\gamma$ 1-adaptin (a marker for the position of cytosolic AP-1, 7.7S; Nakagawa et al., 2000) were isolated by immunoprecipitation–recapture from gradient fractions.

plexes ( $\beta$ -binding domain; Fig. 1 C, BBD) (Aguilar et al., 1997). In agreement with this, we did not observe coprecipitation of stoichiometric amounts of either stonin 1 or stonin 2 with subunits of any of the four AP complexes (data not shown). Analysis of the cytosolic stonins by sedimentation velocity analysis on sucrose gradients (Marks, 1998) revealed that both proteins sedimented as if they were much smaller than a typical heterotetrameric AP complex, AP-1 (sedimentation coefficient 7.7S, molecular mass  $\sim$ 270 kD) (Fig. 3 D). In fact, the size of both stonins corresponded to that of monomers or small oligomers.

#### Interaction of Stonin 2 with the EH Domains of Eps15, Eps15R, and Intersectin 1 in the Yeast Two-Hybrid System

To gain insight into the possible function of the human stonins, we sought to identify some of their binding partners. The presence of two NPF motifs in the proline-rich domain

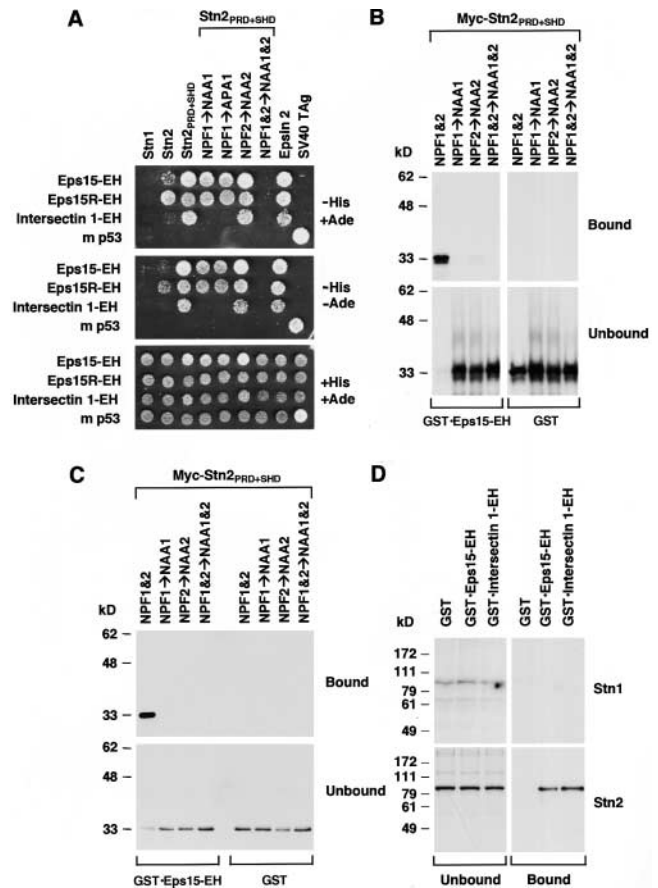


of stonin 2 pointed to possible interactions with proteins containing EH domains. Moreover, the existence of two similarly spaced NPF motifs in epsin 1 and epsin 2, as well as the conservation of additional amino acids in the intervening sequence of these proteins, suggested that the EH-containing binding partners could be shared with the epsins. Both epsin 1 and epsin 2 have been shown to bind Eps15 and intersectin 1 via NPF–EH domain interactions (Chen et al., 1998; Yamabhai et al., 1998; Hussain et al., 1999; Rosenthal et al., 1999; Sengar et al., 1999). To examine if the stonins also interact with these proteins, we performed yeast two-hybrid analyses. GAL4 DNA binding domain (GAL4bd) constructs encoding the three NH<sub>2</sub>-terminal EH domains from Eps15 or the related Eps15R protein, or the two NH<sub>2</sub>-terminal EH domains from intersectin 1 (reviewed in Di Fiore et al., 1997; Mayer, 1999), were coexpressed with GAL4ad constructs encoding stonin 1, stonin 2, or mutants of these proteins (Fig. 4 A). Stonin 1 did not interact with any of the EH domain constructs, as expected from the absence of NPF motifs in this protein (Fig. 4 A). Stonin 2, on the other hand, interacted with the EH domains of Eps15 and Eps15R, and more weakly with those of intersectin 1 (Fig. 4 A). Deletion of the  $\mu$ -homology domain from stonin 2 enhanced growth of the cotransformants (Fig. 4 A, Stn2<sub>PRD+SHD</sub>), probably due to increased expression of the truncated protein (data not shown). Mutation of the first NPF motif to either NAA or APA in the context of the stonin 2<sub>PRD+SHD</sub> construct decreased interactions with the EH domains of Eps15 and Eps15R and completely abrogated interactions with the EH domains of intersectin 1. Mutation of the second NPF motif to NAA, on the other hand, had little effect on the interactions. Finally, mutation of both NPF motifs to NAA completely abolished interactions with all EH domains (Fig. 4 A). All the positive and negative controls yielded the expected results, thus verifying the specificity of the interactions observed. These experiments demonstrated that stonin 2 has the ability to interact with Eps15, Eps15R, and intersectin 1 via the two NPF motifs in its proline-rich region.

### In Vitro and In Vivo Interactions of Stonin 2 with Eps15 and Intersectin 1

The interaction of stonin 2 with Eps15 was confirmed using in vitro binding assays. An in vitro translated, [<sup>35</sup>S]methionine-labeled construct comprising the proline-rich and stonin homology domains of stonin 2 tagged with the Myc epitope was found to bind to a GST fusion protein having the three EH domains from Eps15, but not to GST (Fig. 4 B). Mutation of either or both NPF motifs largely abolished binding. This suggested that both NPF motifs are required for binding in vitro in apparent contradiction with the results of two-hybrid analyses, which showed that the first NPF motif was more important for interactions. This discrepancy could be explained by the greater sensitivity of yeast two-hybrid assays to low-affinity interactions. Similar results were obtained by transiently expressing the same stonin 2 constructs in transfected HeLa cells rather than by in vitro translation (Fig. 4 C). Furthermore, GST fused to the EH domains of Eps15 or intersectin 1 were able to bind endogenous stonin 2, but not stonin 1, from a HeLa cell lysate (Fig. 4 D).

Next, we investigated if the endogenous stonin 2, and Eps15 formed a complex in vivo. This was done by immu-



**Figure 4.** Interactions of stonin 2 (Stn2) with Eps15 and intersectin 1 analyzed using the yeast two-hybrid system (A) and in vitro binding assays (B–D). (A) GAL4 DNA binding domain constructs encoding the three EH domains from Eps15 or the related Eps15R protein, the two EH domains from intersectin 1, or mouse (m) p53 (as a specificity control) were coexpressed with GAL4ad constructs encoding full-length stonin 1, full-length stonin 2, the PRD + SHD domains of stonin 2 (Fig. 1 A) or various mutants of this segment, epsin 2, or the SV40 T antigen (as a specificity control). Interactions were tested by monitoring growth on plates containing –His +Ade, –His –Ade, or +His +Ade (control) media. (B) In vitro translated, [<sup>35</sup>S]methionine-labeled PRD + SHD domains of stonin 2 tagged with the Myc epitope and mutants of this construct were tested for interaction with either a GST fusion protein bearing the three EH domains of Eps15 or with GST using a conventional pull-down assay. Fractions bound and not bound to the glutathione–Sepharose beads are shown. (C) Similar to B, except that stonin 2 constructs were expressed by transient transfection into HeLa cells, extracted with Triton X-100, and bound and unbound proteins detected by immunoblotting with anti-Myc antibody. (D) A Triton X-100 extract of [<sup>35</sup>S]methionine-labeled HeLa cells containing endogenous stonin 1 and stonin 2 was incubated with GST or GST fusion proteins bearing the three EH domains from Eps15 or the two EH domains from intersectin 1. Unbound and bound stonin 1 and stonin 2 were isolated by immunoprecipitation–recapture. The positions of molecular mass markers (in kD) are indicated on the left of B–D.

noprecipitation of HeLa cell lysates with either preimmune sera (PI) or antibodies to stonin 1 or stonin 2, and then blotting for Eps15 (Fig. 5 A). In agreement with the two-hybrid and in vitro binding assays, we could demonstrate association of stonin 2, but not stonin 1, with Eps15

in vivo. A similar experiment could not be performed for intersectin 1 because the antibody to this protein did not work well for immunoblotting. To circumvent this problem, we performed another experiment in which HeLa cells were transfected with plasmids encoding either GFP or GFP-stonin 2. Intersectin 1 was then isolated by immunoprecipitation, and the immunoprecipitates were analyzed by immunoblotting with antibody to GFP (Fig. 5 B). Using this approach, we could demonstrate coprecipitation of intersectin 1 with GFP-stonin 2, but not GFP (Fig. 5 B).

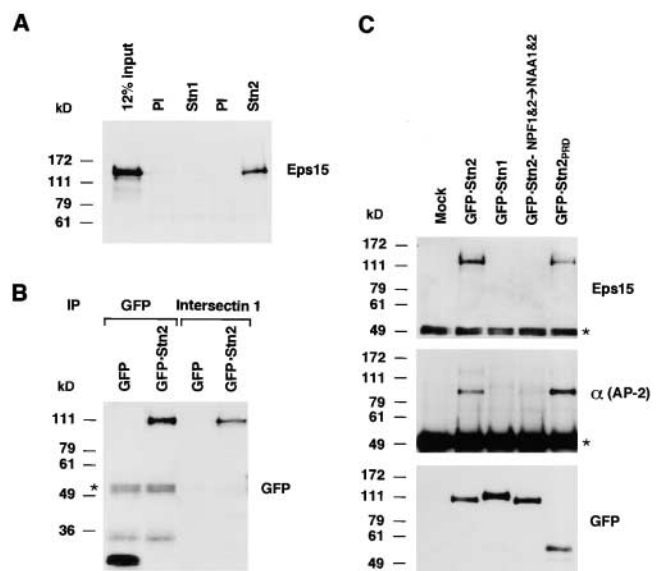
To map the structural determinants for Eps15-stonin 2 interactions in vivo, we transfected HeLa cells with constructs encoding stonin 1, stonin 2, or mutants of stonin 2 fused to GFP (Fig. 5 C). We then immunoprecipitated the GFP fusion proteins and tested for the presence of Eps15 by immunoblotting. Again, we found that Eps15 coprecipitated with the stonin 2, but not stonin 1, fusion protein. This in vivo interaction was mediated by the proline-rich domain of stonin 2 and required the two NPF motifs within this domain (Fig. 5 C). Since Eps15 has multiple DPF motifs in its COOH-terminal region that bind to the  $\alpha$ -adaptin subunit of AP-2 (Benmerah et al., 1996), we also tested for the presence of  $\alpha$ -adaptin in the anti-GFP-stonin immunoprecipitates. As expected, we found that  $\alpha$ -adaptin was present only in the immunoprecipitates that contained Eps15 (Fig. 5 C), suggesting that AP-2 is indirectly associated with stonin 2 via Eps15.

### Interaction of Stonin 2 with Synaptotagmins I and II

Despite the overall homology of the  $\mu$ -homology domain of the stonins to the YXX $\Phi$ -binding domain of AP- $\mu$  subunits, residues involved in interactions of  $\mu$ 2 with YXX $\Phi$  signals (Owen and Evans, 1998) (denoted by blue dots in Fig. 1 C) are not conserved in the stonins. In line with this observation, yeast two-hybrid assays revealed that neither human stonin was able to bind a panel of 10 different YXX $\Phi$  signals (data not shown).  $\mu$ 2 (Haucke et al., 2000) as well as *D. melanogaster* stoned B (Phillips et al., 2000) have been shown to bind to the C2B domain of synaptotagmin I. We found that 4.9–6.5% of Myc-stonin 2 expressed by transient transfection into HeLa cells bound to GST fusion proteins bearing the cytoplasmic domains of synaptotagmins I and II (Fig. 6 A). This binding was judged to be specific, as only a negligible amount of Myc-stonin 2 was recovered on a GST-GGA3 fusion protein (Fig. 6 A) or GST (data not shown). Additional GST pull-down assays revealed that Myc-stonin 2 interacted mainly with the C2B domain of both synaptotagmins (Fig. 6 B).

### Overexpression of Stonin 2 Impairs Receptor-mediated Endocytosis

Although our antibodies to stonin 2 allowed us to immunoprecipitate the endogenous protein, they were not sensitive enough for immunofluorescence microscopy. This prompted us to examine the localization of GFP-stonin 2 expressed by transfection into cells. We observed a heterogeneous distribution including dispersed punctate and juxtannuclear structures superimposed over a diffuse cytoplasmic pattern (Fig. 7, A, D, and G). Some of the punctate structures colocalized with AP-2 (Fig. 7, M–O, inset). At the highest expression levels, we observed a coalescence of



**Figure 5.** Interactions of stonin 2 with Eps15, AP-2, and intersectin 1 in vivo. (A) Coprecipitation of endogenous stonin 2 with Eps15. A Triton X-100 extract of HeLa cells was subjected to immunoprecipitation with preimmune sera (PI) or antibodies to stonin 1 (Stn1) or stonin 2 (Stn2). The presence of coprecipitated Eps15 was detected by immunoblotting. (B) Coprecipitation of transfected GFP-stonin 2 with endogenous intersectin 1. The GFP or GFP-stonin 2 constructs were expressed by transfection into HeLa cells and isolated by immunoprecipitation with anti-GFP or anti-intersectin antibodies from Triton X-100 extracts of the cells. The presence of the GFP proteins was revealed by immunoblotting using anti-GFP antibodies. (C) Coprecipitation of endogenous Eps15 and AP-2 with transfected GFP-stonin constructs. The GFP-stonin fusion proteins indicated in the figure were expressed by transfection into HeLa cells and isolated by immunoprecipitation with anti-GFP antibodies from Triton X-100 extracts of the cells. The presence of Eps15 and the  $\alpha$  subunit of AP-2 were revealed by immunoblotting. The positions of molecular mass markers (in kD) are indicated on the left. \*Indicates the heavy chains of anti-GFP antibody used in B and C.

GFP-stonin 2 in the juxtannuclear area (Fig. 7 M). In these cells, we noticed a depletion of AP-2 from plasma membrane clathrin-coated pits and its colocalization with the juxtannuclear GFP-stonin 2 (Fig. 7, N and O, arrow). This behavior of stonin 2 was similar to that reported for epsin 2 (Rosenthal et al., 1999), consistent with the ability of both proteins to interact directly or indirectly with AP-2. Overexpression of GFP-stonin 2 had no effect on the distribution of two other AP complexes, AP-1 and AP-3 (data not shown), indicating that the effects on AP-2 were specific.

The ability of stonin 2 to interact with several components of the endocytic machinery predicted that overexpression of GFP-stonin 2 could interfere with receptor-mediated endocytosis, as is the case for epsin 2 (Rosenthal et al., 1999). Indeed, we observed that GFP-stonin 2 overexpression inhibited internalization of Cy3-transferrin (Fig. 7, A–C), rhodamine-EGF (D–F), and Dil-LDL (G–I) by their corresponding receptors. Overexpression of GFP, on the other hand, had no effect on internalization of Cy3-transferrin (Fig. 7, J–L) or the other ligands (data not shown). These observations further emphasize the functional connection of stonin 2 to the endocytic machinery.

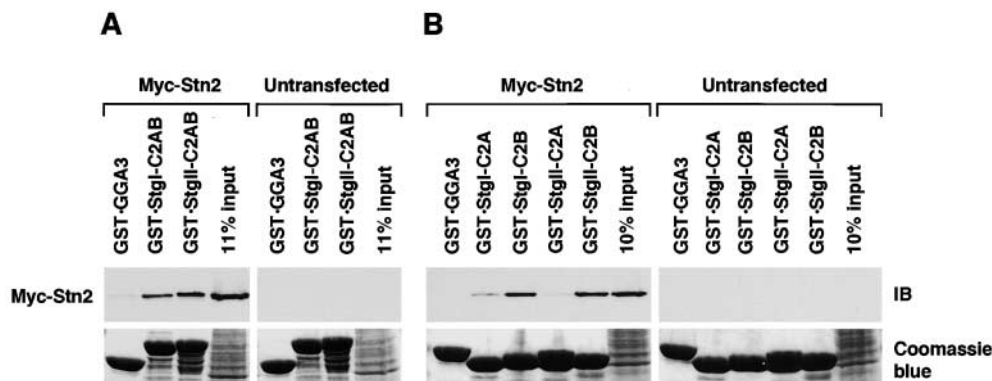


Figure 6. Stonin 2 binds to synaptotagmins I and II. (A) Interaction of stonin 2 with the cytoplasmic (C2AB) domains of synaptotagmins I and II. Triton X-100 extracts of HeLa cells, transiently expressing Myc-Stn2 or from untransfected cells, were incubated with GST fusion proteins comprising the C2A and C2B domains of synaptotagmins I or II, or with GST-GGA3<sub>GAE</sub> (specificity control), using a conventional pull-down assay.

(B) Interaction of stonin 2 with the C2B domain of synaptotagmins I and II. Similar to A, except that the cell extracts were incubated with GST fusion proteins comprising the C2A or C2B domains of synaptotagmins I and II. The presence of Myc-Stn2 in the bound material was revealed by immunoblotting (IB) using monoclonal antibodies to the Myc epitope. Coomassie blue staining of the gels revealed that similar amounts of GST fusion proteins were used.

## Discussion

Here, we report the identification of two ubiquitous human proteins, stonin 1 and stonin 2, which have a modular structure characteristic of components of the endocytic machinery. In all of our protein interaction analyses, we could only demonstrate weak binding of stonin 1 to synaptotagmins I and II. Thus, at present we do not know if the nature of the stonin 1 modules is indicative of a role in endocytosis or other protein sorting processes. We were more successful, however, in identifying several proteins that interact with stonin 2 (i.e., Eps15, Eps15R, intersectin 1, and synaptotagmins I and II). All of these interaction partners have been previously implicated in endocytosis, which makes it likely that stonin 2 is also a component of the endocytic machinery. In support of this notion, overexpression of stonin 2 perturbs the localization of AP-2 to the plasma membrane and inhibits internalization of several endocytic receptors.

The proline-rich domain of stonin 2 contains two NPF motifs that mediate interactions with the EH domains of Eps15, Eps15R, and intersectin 1. This property is shared with other NPF-containing endocytic proteins such as epsin 1 (Chen et al., 1998; Yamabhai et al., 1998), epsin 2 (Yamabhai et al., 1998; Rosenthal et al., 1999), the 170-kD form of synaptojanin (Haffner et al., 1997), nonneuronal members of the AP180/CALM family (Dreyling et al., 1996; Tebar et al., 1999), several SCAMP isoforms (Fernández-Chacón et al., 2000), and Numb (Santolini et al., 2000). Despite the fact that all of these NPF-containing proteins share the same EH-containing partners, interactions between them and Eps15 need not be competitive since Eps15 is a tetramer (Cupers et al., 1997; Tebar et al., 1997) and could thus assemble simultaneously with several NPF-containing proteins. The proline-rich domains of stonin 1 and stonin 2 also contain several PxxP motifs that could potentially interact with SH3 domains of endocytic proteins such as intersectins, amphiphysins, and endophilins (for review see Slepnev and De Camilli, 2000), or with other domains that recognize proline-based motifs (Kay et al., 2000).

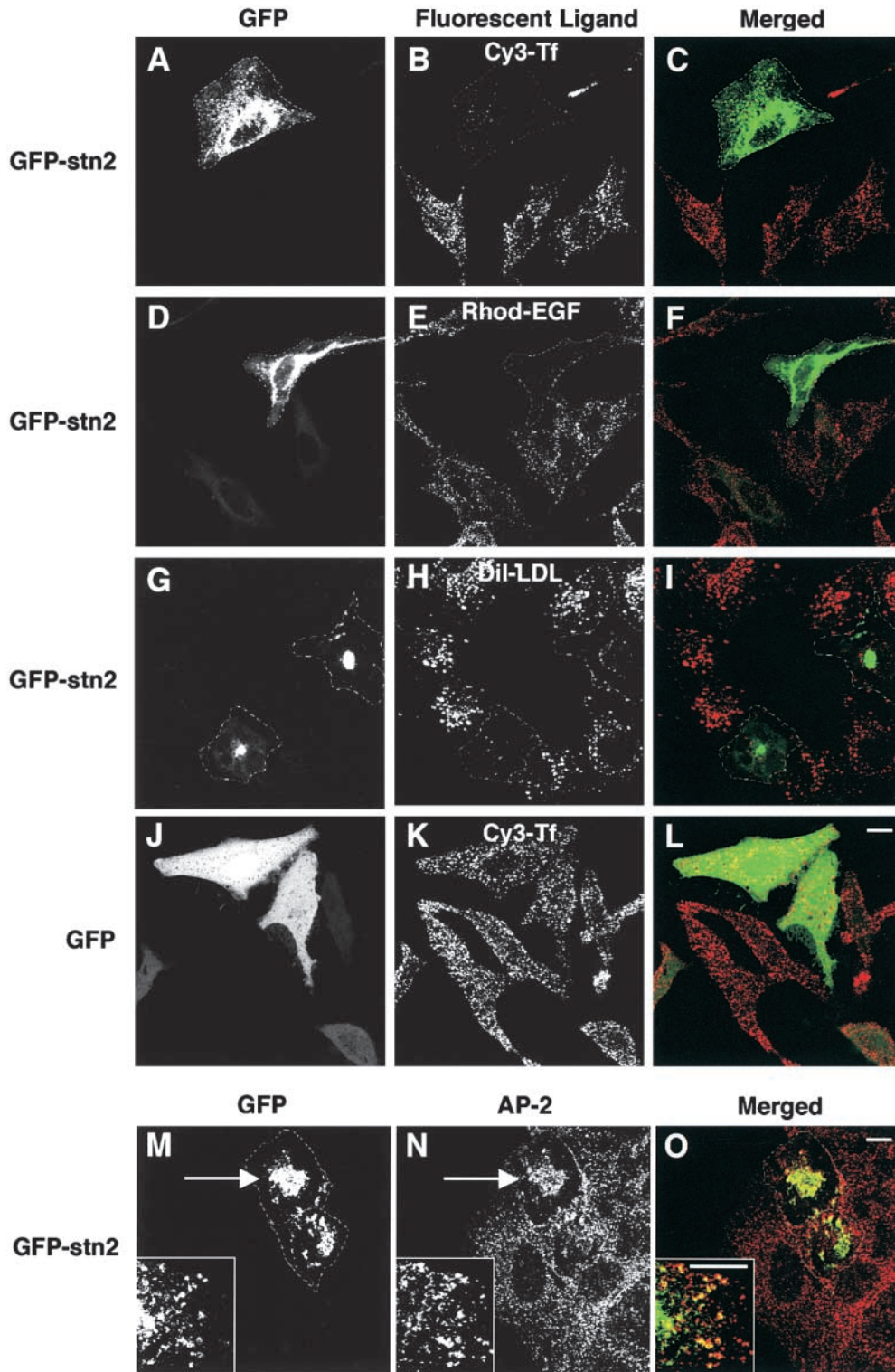
The  $\mu$ -homology domains of stonin 1 and stonin 2 are likely to fold into an all  $\beta$ -sheet structure similar to that of the YXX $\Phi$ -binding domain of the  $\mu$ 2 subunit of AP-2

(Owen and Evans, 1998). This similarity notwithstanding, the human stonins do not appear to bind either tyrosine- or dileucine-based endocytic signals in the yeast two-hybrid system (data not shown). The possibility remains that they could recognize a different type of signal. Nonetheless, the stonins do share with  $\mu$ 2 the ability to bind the C2B domain of synaptotagmins I and II, most likely through a conserved interface distinct from the YXX $\Phi$ -binding site (Haucke et al., 2000). Synaptotagmins have been proposed to function as docking proteins for the AP-2 complex, in what would constitute the first step in the assembly of clathrin coats onto the plasma membrane (Zhang et al., 1994). By analogy, synaptotagmins could also function to recruit stonin 2 to membranes via C2B- $\mu$ -homology domain interactions. The NPF motifs of stonin 2 would then serve as ligands for EH-containing proteins such as Eps15, Eps15R, and intersectin 1. These proteins would, in turn, congregate all of their other binding partners (e.g., epsins, dynamins, synaptojanins, endophilins, and syndapins) at the membrane, and assembly of a clathrin coat and vesicle formation would ensue.

The AP-2 complex is similarly able to bind to synaptotagmin I via its  $\alpha$  and  $\mu$ 2 subunits (Zhang et al., 1994; Haucke et al., 2000) and to Eps15 and Eps15R via its  $\alpha$  subunit (Benmerah et al., 1996; Salcini et al., 1999). It is thus plausible that the role of stonin 2 could be partly redundant or alternative to that of AP-2. Another possibility is that stonin 2 and AP-2 cooperate to stabilize the dynamic network of low-affinity interactions required to assemble a functional endocytic coat. The coprecipitation of endogenous AP-2 with stonin 2, which is likely mediated by Eps15 (Fig. 5), argues in favor of the latter explanation. The fact that mutations in either the  $\alpha$  subunit of AP-2 (Dornan et al., 1997; González-Gaitán and Jackle, 1997) or stoned B (Andrews et al., 1996; Fergestad et al., 1999) in *D. melanogaster* result in defective synaptic vesicle recycling further suggests that both AP-2 and stoned B (of which stonin 2 appears to be the most closely related human homologue) play unique and essential roles in endocytosis.

In addition to providing additional linking potential to the assembling coats, stonin 2 could be involved in the selection of specific cargo molecules for incorporation into the budding vesicles. In favor of this hypothesis is the observation that synaptotagmin is mislocalized in *D. melano-*





**Figure 7.** Effects of over-expression of GFP-stonin 2 on Cy3-Tf, rhodamine-EGF (Rhod-EGF), Dil-LDL internalization, and AP-2 localization. HeLa cells were transfected with plasmids encoding either GFP-stonin 2 (A–I and M–O) or GFP alone (J–L). Live cells were incubated for 15 min in the presence of Cy3-Tf (A–C and J–L), rhodamine-EGF (D–F) or Dil-LDL (G–I) at 37°C. AP-2 (M–O) was detected by immunofluorescence microscopy using antibodies to  $\alpha$ -adaptin (AP.6) followed by Cy3-conjugated donkey anti-mouse IgG. Cells were examined by confocal fluorescence microscopy. (A, D, G, J, and M) GFP fluorescence (green); (B and K) Cy3-Tf (red); (E) Rhodamine-EGF (red); (H) Dil-LDL (red); (N) AP-2 (red). Arrow points to transfected cell exhibiting accumulation of AP-2 in the juxtannuclear area. Bars, 10  $\mu$ m.

*gaster stoned* mutants (Fergestad et al., 1999). Moreover, synaptotagmin appears to be degraded more rapidly in *stoned* mutants (Fergestad et al., 1999). In contrast, the localization and stability of other synaptic vesicle proteins are unaltered in *stoned* mutants (Fergestad et al., 1999). These observations suggest that stoned B could play a role in the specific retrieval of a subset of synaptic vesicle proteins including synaptotagmin. The dual roles of stonin 2 and stoned B in coat assembly and cargo selection need

not be mutually exclusive, as the AP-2 complex is known to participate in both processes.

The neurological defects of *D. melanogaster stoned* mutants are likely due to perturbation of the high endocytic activity of presynaptic terminals. Although less obvious, other endocytic processes in stonin-deficient organisms may be defective as well. The expression of stonin 2 in most human tissues and cells examined, its ability to interact with several widely-expressed proteins, and the over-

expression-induced interference with receptor-mediated endocytosis in nonneuronal cells, all suggest that stonin 2 may play a general role in endocytosis.

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