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Staring, a Novel E3 Ubiquitin-Protein Ligase That Targets Syntaxin 1 for Degradation*

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Lih-Shen Chin‡§, John P. Vavalle§, and Lian Li‡§¶

From the ‡Department of Pharmacology, Center for Neurodegenerative Disease, Emory University School of Medicine, Atlanta, Georgia 30322-3090 and §Department of Pharmacology, School of Medicine, University of North Carolina, Chapel Hill, North Carolina 27599

Syntaxin 1 is an essential component of the neurotransmitter release machinery, and regulation of syntaxin 1 expression levels is thought to contribute to the mechanism underlying learning and memory. However, the molecular events that control the degradation of syntaxin 1 remain undefined. Here we report the identification and characterization of a novel RING finger protein, Staring, that interacts with syntaxin 1. Staring is expressed throughout the brain, where it exists in both cytosolic and membrane-associated pools. Staring binds and recruits the brain-enriched E2 ubiquitin-conjugating enzyme UbcH8 to syntaxin 1 and facilitates the ubiquitination and proteasome-dependent degradation of syntaxin 1. These findings suggest that Staring is a novel E3 ubiquitin-protein ligase that targets syntaxin 1 for degradation by the ubiquitin-proteasome pathway.

Modulation of protein degradation is a major mechanism by which cells regulate the expression levels of specific proteins and consequently the cellular processes that these proteins participate in (1, 2). The ubiquitin-proteasome pathway plays a crucial role in the degradation of proteins involved in a variety of cellular processes, including differentiation, proliferation, and apoptosis. However, the role of the ubiquitin-proteasome pathway in the degradation of presynaptic proteins remains poorly characterized, despite the presence of ubiquitin at nerve terminals (3–5). In the ubiquitin-proteasome pathway, substrates are marked for degradation by covalent linkage to ubiquitin. The ubiquitinated substrate proteins are then recognized and degraded by the 26 S proteasome (1, 2, 6). Ubiquitination involves a highly specific enzyme cascade in which ubiquitin is first activated by an E1[1] ubiquitin-activating enzyme and then transferred to an E2 ubiquitin-conjugating enzyme and finally ligated to the substrate by an E3 ubiquitin-protein ligase (1, 7, 8). The E3 ubiquitin-protein ligase plays an essential role in determining the specificity of ubiquitination and subsequent protein degradation. Consistent with this role, it is estimated that an organism such as a human contains over 100 E3 ubiquitin ligases, in contrast to a single E1 ubiquitin-activating enzyme and about a dozen E2 ubiquitin-conjugating enzymes (9). Despite the importance of E3 ubiquitin-protein ligases in specific protein degradation and the estimated presence of more than 100 E3 ligases in the human genome, only a few E3 ligases have been characterized at the molecular level.

Syntaxin 1 is a neuronal membrane protein that was originally identified as a binding partner for synaptotagmin and the N-type calcium channel (10–12). It is well established that syntaxin 1 functions as a synaptic t-SNARE to mediate synaptic vesicle exocytosis at nerve terminals (13–15). Syntaxin 1 appears early during embryonic development (16, 17), and its expression level is dramatically up-regulated during synapse formation and brain maturation (16–19). Regulation of syntaxin 1 levels may contribute to the mechanism underlying learning and memory, since changes in syntaxin 1 levels have been found to correlate with long term potentiation and various learning and memory behaviors (20–22). Alteration in syntaxin 1 expression levels has been associated with several neurodegenerative diseases and psychiatric disorders, including schizophrenia, Alzheimer's disease, and Creutzfeldt-Jakob disease (23–26).

Despite the importance of the regulation of syntaxin 1 levels in synaptic function and dysfunction, the molecular mechanisms underlying such regulation remain undefined. To identify proteins that regulate syntaxin 1, we carried out a search in rat brain for proteins that interact with syntaxin 1 using yeast two-hybrid screens. Here we report the isolation of a novel syntaxin 1-interacting protein, named Staring, that acts as an E3 ubiquitin-protein ligase to promote the ubiquitination and degradation of syntaxin 1 by the proteasome pathway.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screen and cDNA Cloning—The bait plasmid, pPC97-Syntaxin 1, was constructed by subcloning the cytoplasmic domain (amino acids 5–270) of rat syntaxin 1B (27) into the pPC97 vector (28, 29). For the two-hybrid screen, the yeast strain CG-1945 (CLONTECH) was transformed sequentially with pPC97-Syntaxin 1 and a rat hippocampal/cortical two-hybrid cDNA library (29), using the lithium acetate method (30). Positive clones were selected on 3-aminotriazole (5 mM; Sigma)-containing medium lacking leucine, tryptophan, and histidine and verified with a filter assay for β-galactosidase activity. Prey plasmids were then recovered and retransformed into yeast with pPC97-Syntaxin 1 or various control baits to confirm the specificity of the interaction. For cloning of full-length Staring, a partial Staring cDNA probe from the prey clone (clone 7) was used to screen a rat hippocampal cDNA library in λZAPII (Stratagene) according to the standard procedure (31). The cDNA inserts from positive Staring clones were sequenced multiple times on both strands using an Applied Biosystems 373A DNA sequencer.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF352815.

¶ To whom correspondence should be addressed: Dept. of Pharmacology, Emory University School of Medicine, 1510 Clifton Rd., Atlanta, GA 30322-3090. Tel.: 404-727-5987; Fax: 404-727-0365; E-mail: lianli@pharm.emory.edu.

[1] The abbreviations used are: E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin-protein ligase; GST, glutathione *S*-transferase; DMEM, Dulbecco's modified Eagle's medium; HA, hemagglutinin; SNARE, soluble NSF attachment protein receptors.

Expression Constructs—Conventional molecular biological techniques (31) were used to subclone DNA fragments encoding full-length and truncated forms of Staring into the following vectors: the pPC97 and pPC86 vectors for yeast two-hybrid interaction studies; the prokaryotic expression vectors pGEX-5X-2 (Amersham Biosciences) and pET28c (Novagen) for the production of GST- and His_6-tagged fusion proteins; and the mammalian expression vectors pCDNA3.1(+) (Invitrogen) and pCHA (30) for transfection into HeLa cells. The expression construct pRK5-HA-UbcH5, pRK5-HA-UbcH7, and pRK5-HA-UbcH8 were obtained as generous gifts from Dr. Ted Dawson (32).

Antibodies—Four polyclonal anti-Staring antibodies, two in chicken (CS-N and CS-C) and two in rabbit (RS-N and RS-C), were generated against Staring N-terminal peptide MSGLSNKRAAGDGG and C-terminal peptide AAFGAHDFHRVYIS, respectively. The antibodies were affinity-purified using the immunogen peptide-coupled columns as described previously (30). Other antibodies used in this study include the following: anti-HA (3F10 (Roche Molecular Biochemicals) and HA.11 (Covance)), anti-Myc (9E10.3; Neomarkers), anti-syntaxin 1 (HPC-1; Sigma), anti-actin (C4; Roche Molecular Biochemicals) and secondary antibodies coupled with horseradish peroxidase (Jackson Immunoresearch Laboratories, Inc.).

Northern and Western Blot Analyses—Northern blot analysis of Staring mRNA expression was performed on a rat multiple tissue Northern (MTN™) blot and a human multiple tissue expression (MTE™) array (CLONTECH), using a ^{32}P-labeled Staring cDNA fragment from clone 7 as the probe (31). For Western blot analysis, rat tissues were homogenized in 1% SDS and subjected to SDS-PAGE. The proteins were transferred onto nitrocellulose membranes and probed with anti-Staring and other antibodies. Antibody binding was detected by using the enhanced chemiluminescence system (Amersham Biosciences).

Subcellular Fractionations—Subcellular fractionations of rat brain into cytosol fraction (100,000 × *g* supernatant) and membrane fraction (100,000 × *g* pellet) were preformed as previously described (30). The membrane fractions were subjected to extraction studies as described (30), using 1.5 M NaCl or 4 M urea.

In Vitro Binding Assays—GST-Staring fusion proteins or GST control were immobilized on glutathione-agarose beads (Sigma) and incubated with rat brain homogenates as previously described (30, 33). After extensive washes, bound proteins were eluted by boiling in 2× Laemmli sample buffer and analyzed by SDS-PAGE and immunoblotting with appropriate antibodies.

Cell Transfections and Immunoprecipitations—HeLa or SH-SY5Y cells were transfected with indicated plasmids using LipofectAMINE (Invitrogen) as described by the manufacturer. Cell lysates were prepared and subjected to immunoprecipitation as described previously (34), using anti-HA antibody (3F10), anti-Myc (9E10.3), anti-Staring (RS-N), anti-syntaxin 1 antibody (HPC-1), or control IgG. The immunocomplexes were recovered by incubation with protein G- or protein A-Sepharose beads (Sigma). After extensive washes, the immunocomplexes were dissociated by boiling in the Laemmli sample buffer and analyzed by SDS-PAGE and immunoblotting.

Ubiquitination Assays—HeLa cells were transfected with combinations of the following plasmids: pCHA-syntaxin 1, pcDNA3-Myc-ubiquitin, pFLAG-Staring, and pFLAG-StaringΔR, a C-terminal deletion mutant of Staring that lacks the RING finger motif. SH-SY5Y cells were transfected with pcDNA3-Myc-ubiquitin in combination with pFLAG-Staring or pFLAG-StaringΔR. Twenty-four hours after transfection, the cells were incubated for 8 h with 20 μM MG132 (Calbiochem). The cells were then lysed, and an equal amount of protein from each lysate was immunoprecipitated using antibodies against HA tag or syntaxin 1 (34). Immunoprecipitates were analyzed by SDS-PAGE followed by immunoblotting with an anti-Myc antibody to detect Myc-ubiquitin conjugated to syntaxin 1.

[^{35}S]Methionine Pulse-Chase Experiments—HeLa cells were co-transfected with pCHA-syntaxin 1 and either pFLAG-Staring or pFLAG vector control. SH-SY5Y cells were transfected with pFLAG-Staring or pFLAG vector control. To control for the transfection efficiency, cells were transfected using LipofectAMINE in a 150-mm culture dish. At 24 h post-transfection, the cells were divided into eight 60-mm dishes, each of which was used for a single chase time point. At 48 h post-transfection, cells in the 60-mm dishes were washed and incubated for 30 min with Met/Cys-free DMEM. The medium was then replaced with Met/Cys-free DMEM containing 200 μCi of [^{35}S]Met/Cys (1000 Ci/mmol) express protein labeling mix (PerkinElmer Life Sciences). After incubation for 1 h, the radioactive medium was removed by extensive washes with nonradioactive DMEM. Cells in each 60-mm dish were then incubated for the indicated chase time in nonradioactive DMEM supplemented with 10% fetal bovine serum and 5 times the normal concentration of methionine and cysteine as described previously (35). Cells were then lysed, and an equal amount of protein from each lysate was immunoprecipitated using an anti-HA antibody. Immunoprecipitates were resolved by SDS-PAGE and analyzed by a PhosphorImager (Amersham Biosciences).

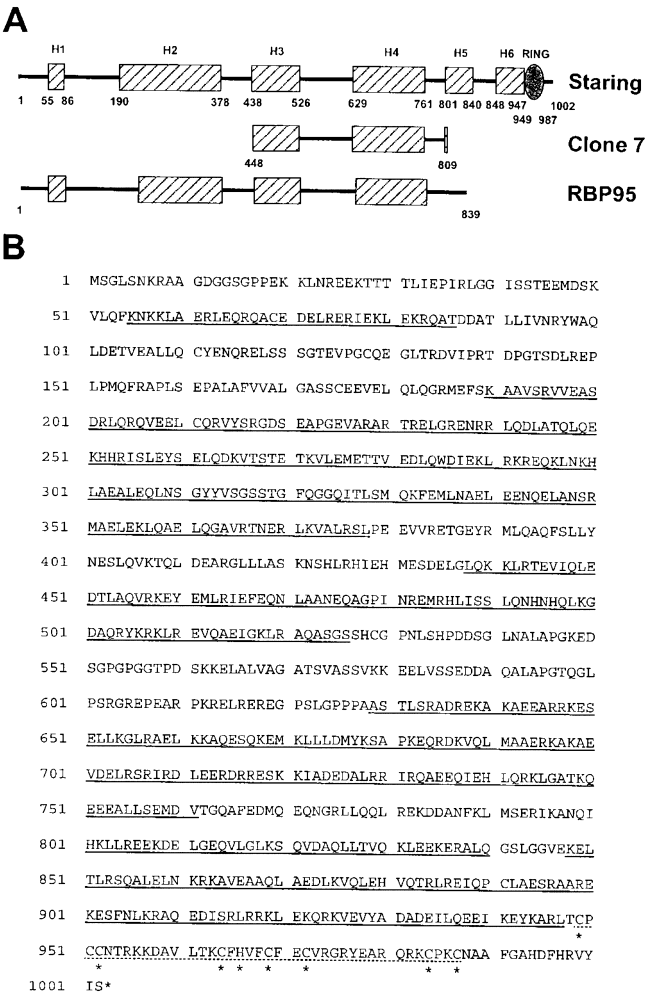

FIG. 1. **Structure of Staring.** *A*, domain structure of Staring. Rat Staring is a 1002-amino acid protein that contains six coiled-coil domains (*H1–H6*) and a RING finger motif at the C terminus. The location of the syntaxin 1-interacting clone (*C7*) isolated from the yeast two-hybrid screen is indicated *below* the domain structure. For comparison, the domain structure of human RBP95 is also shown. *B*, sequence of Staring. The nucleotide sequence of Staring (not shown) was deposited in the GenBank™ with accession number AF352815. The deduced amino acid sequence of Staring is shown in single-letter code and *numbered* on the *left*. Indicated are the predicted coiled-coil domains (*underline)* and the RING finger motif (*dotted line*), in which key cysteine and histidine residues are marked with *asterisks*.

Proteolysis Inhibitor Treatment of Cells—HeLa or SH-SY5Y cells expressing Staring and syntaxin 1 were incubated for 8 h at 37 °C with the proteasome inhibitor MG132 (20 μM; Calbiochem), the cysteine protease inhibitor E-64 (50 μM; Sigma), the lysosomal protease inhibitor NH_4Cl (50 mM) or chloroquine (100 μM; Sigma), or vehicle (Me_2SO; final concentration 0.1%). Cells were then lysed, and the protein concentrations of the lysates were determined by the BCA protein assay (Pierce). An equal amount of protein from each lysate was then analyzed by SDS-PAGE and immunoblotting.

RESULTS

Identification of Staring, a Novel Syntaxin 1-interacting RING Finger Protein—To identify syntaxin 1-interacting proteins, we screened a rat hippocampal/cortical cDNA library by yeast two-hybrid selection using the cytoplasmic domain of rat syntaxin 1B as bait. This screen led to the isolation of several clones encoding SNAP-25, a known syntaxin 1-interacting pro-

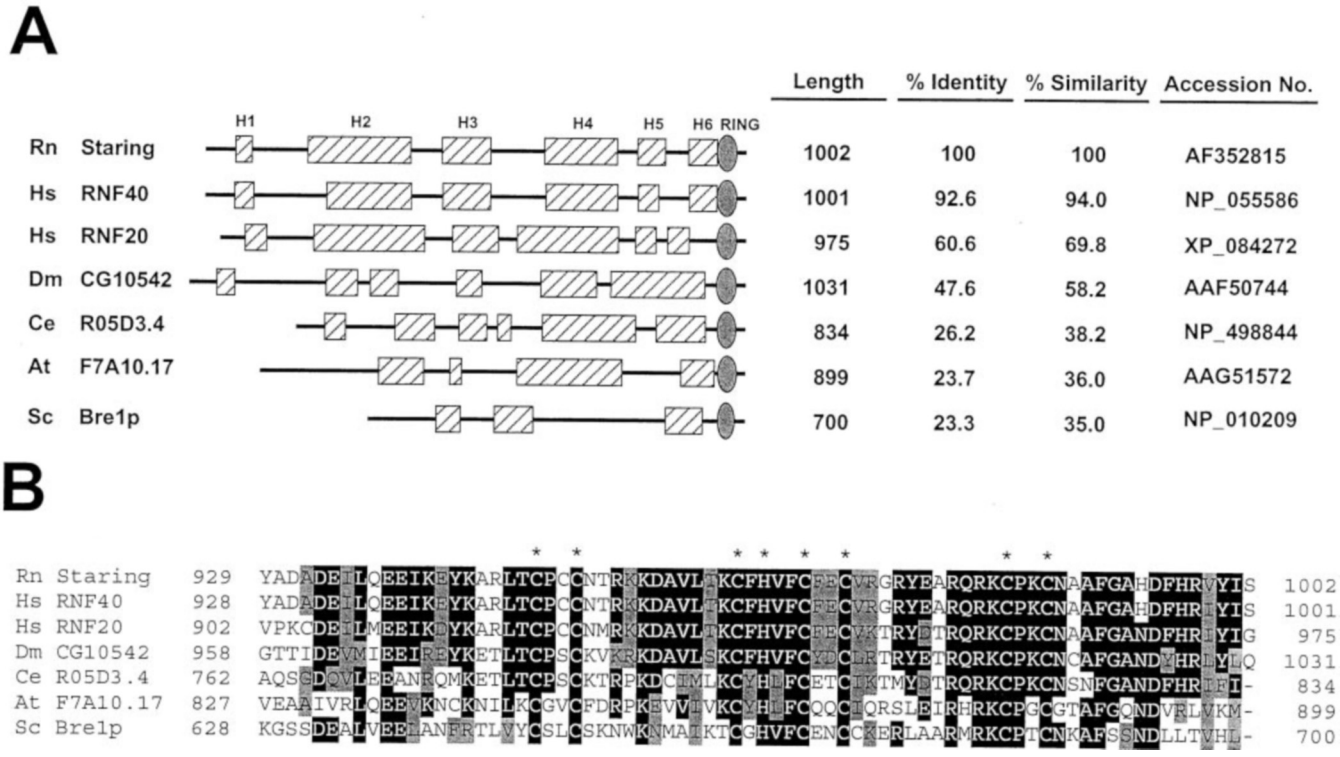

FIG. 2. **Staring is an evolutionarily conserved protein.** *A*, domain structure of the Staring homologues in different species. Predicted coiled-coil domains are shown in *striped boxes*. The names and the lengths of the proteins are indicated along with their accession numbers. The homology of these proteins to Staring is indicated by amino acid identity and similarity of each protein relative to the protein sequence of rat Staring. *B*, alignment of the conserved RING finger motifs in the Staring homologues from different species. *Numbers* indicate the position of the RING finger motif in each sequence. Conserved cysteine and histidine residues are marked with *asterisks*. *Rn*, *Rattus norvegicus*; *Hs*, *Homo sapiens*; *Mm*, *Mus musculus*; *Dm*, *Drosophila melanogaster*; *At*, *Arabidopsis thaliana*; *Ce*, *C. elegans*; *Sc*, *S. cerevisiae*.

tein (data not shown), confirming the validity of the two-hybrid screen. One of the positive clones was shown to encode part of a novel protein that we referred to as Staring (because it is a syntaxin 1-interacting RING finger protein (Fig. 1*A*). Retransformation experiments confirmed that Staring interacts specifically with syntaxin 1A and syntaxin 1B but not with SNAP-25 or SNAP-23 (data not shown). Since SNAP-25 and SNAP-23 contain coiled-coil t-SNARE domains that are homologous to the t-SNARE domain of syntaxin 1 (36), the inability of Staring to interact with SNAP-25 and SNAP-23 further confirms the specificity of the Staring-syntaxin 1 interaction.

Cloning of the full-length Staring cDNA revealed that Staring is a 1002-amino acid protein with a calculated molecular mass of 113.8 kDa. The sequence surrounding the initiator methionine codon of Staring conforms well to the translation initiation consensus sequence (37) and is preceded by an in-frame stop codon in the 5′-untranslated region. Furthermore, the coding sequence of Staring beginning with this methionine initiator can be expressed in mammalian cells to yield a recombinant protein with an apparent molecular weight similar to that of endogenous Staring (data not shown), confirming that the cloned Staring sequence contains the entire coding region. Staring is highly hydrophilic, with a theoretical isoelectric point (pI) of 6.13 and a high percentage (34%) of charged amino acids over the entire length. Staring contains neither a signal sequence nor a potential transmembrane domain.

As shown in Fig. 1, Staring contains six putative coiled-coil domains and a RING finger motif at the C terminus. The RING finger motif is a cysteine/histidine-rich (C_3HC_4), Zn^{2+} binding domain that is found in a number of eukaryotic proteins, some of which have been implicated in vesicular transport (38). Emerging evidence indicates that the RING finger motif may function in protein ubiquitination as a key determinant of the E3 ubiquitin-protein ligase activity (39–41). The N-terminal region of rat Staring is 90% identical to human RBP95, an 838-amino acid protein recently identified from a yeast two-hybrid screen using retinoblastoma protein as bait (42). The expression of endogenous RBP95 protein has not yet been characterized; however, based on EST data base searches, RBP95 seems to be a rare isoform derived from alternative splicing of the gene encoding the uncharacterized human ring finger protein 40 (RNF40, also called KIAA0661) (Fig. 2*A*).

Data base searches reveal the presence of Staring homologues as uncharacterized cDNAs or open reading frames obtained from genome projects in a number of organisms, including humans, mice, *Drosophila*, *Caenorhabditis elegans*, *Arabidopsis*, and yeast (Fig. 2*A*). Sequence comparison analysis indicates that RNF40 is the human orthologue of rat Staring. The human genome also contains a second Staring homologue, RNF20, which is encoded by a gene that is distinct from the RNF40 gene. In *Drosophila*, *C. elegans*, *Arabidopsis*, and yeast, there appears to be only one Staring homologue. Whereas the function of these Staring homologues is unknown, the deletion mutant of the yeast Staring homologue, Bre1p (also called YDL074c), was reported to be sensitive to multiple drugs, including brefeldin A and chlorpromazine (43, 44). Analysis of the deduced proteins from the Staring-homologous sequences reveals that these homologous proteins have a domain structure that is similar to that of Staring (Fig. 2*A*). Most notably, these Staring homologues contain a highly conserved RING finger motif at their C terminus (Fig. 2*B*). The conspicuous homology and conserved domain structure among Staring homologues from different species indicate that Staring is an evolutionarily conserved protein.

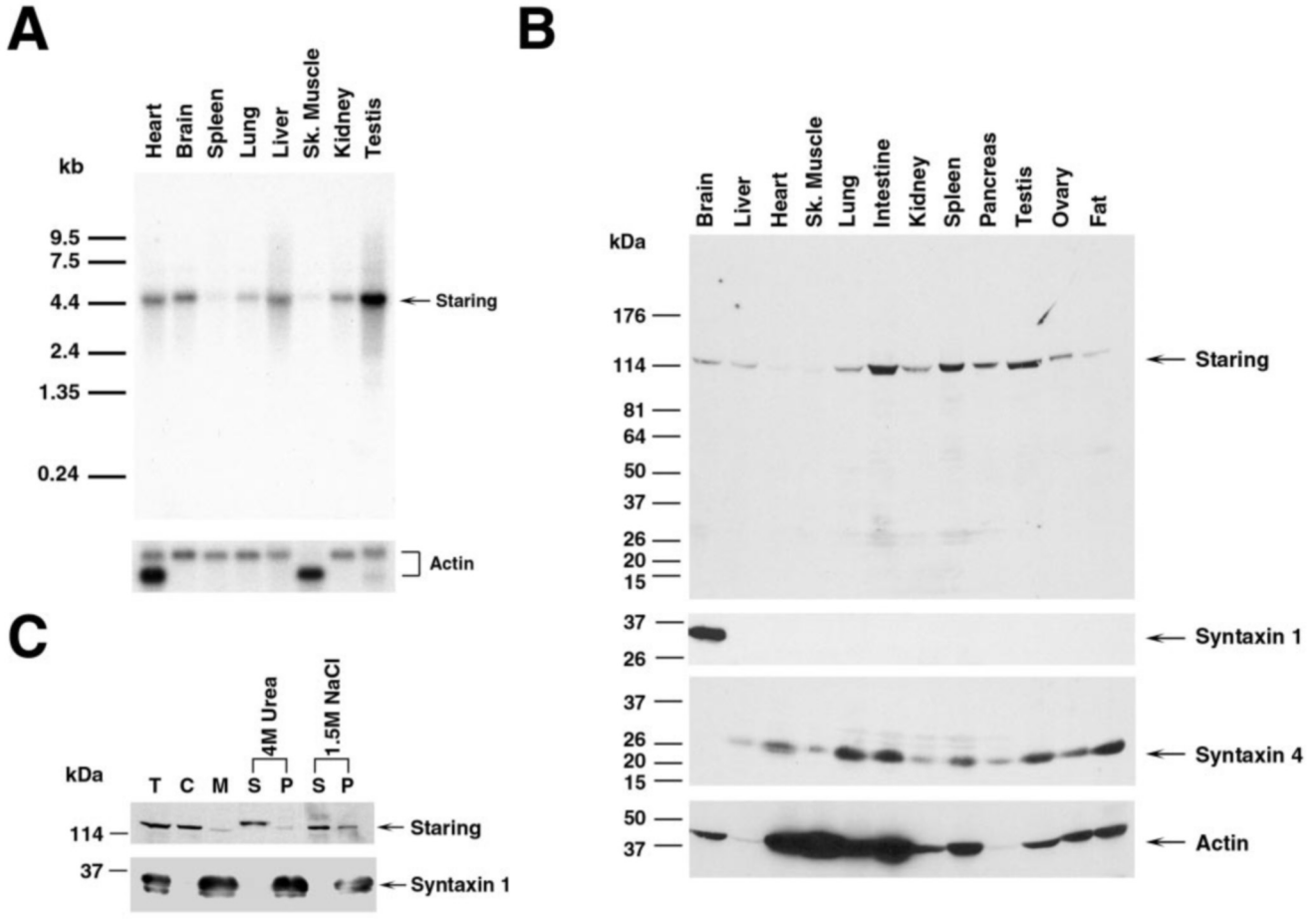

FIG. 3. **Distribution of Staring mRNA and protein expression in rat tissues.** *A*, Northern blot analysis of Staring mRNA expression. A multitissue Northern blot (CLONTECH) was hybridized with a ^{32}P-labeled partial cDNA probe of Staring (*upper panel*). The loading of poly(A)$^+$ RNA in each lane was confirmed by hybridization of the same blot with a ^{32}P-labeled β-actin cDNA probe (*lower panel*). *Sk.*, Skeletal. *B*, Western blot analysis of Staring protein expression. Equal amounts of homogenates (50 μg of protein/lane) from the indicated rat tissues were analyzed by immunoblotting using antibodies against Staring (RS-N), syntaxin 1, syntaxin 4, or actin. *C*, Staring exists in both cytosolic and membrane-bound pools. Postnuclear supernatant (*T*) from rat brain was separated in cytosol (*C*) and membrane (*M*) fractions. The membranes were extracted with 1.5 M NaCl or 4 M urea and then separated into soluble (*S*) and pellet (*P*) fractions. Aliquots representing an equal percentage of each fraction were analyzed by SDS-PAGE and immunoblotting using anti-Staring antibody (RS-C).

Staring is a Ubiquitously Expressed Protein That Exists in Both Cytosolic and Membrane-associated Pools—Northern blot analysis of Staring mRNA expression revealed the presence of a single Staring transcript of 5.1 kb (Fig. 3*A*). The Staring mRNA is relatively abundant in brain, testis, heart, liver, and kidney and expressed at low levels in lung, spleen, and skeletal muscle. Consistent with this result, analysis of human Staring mRNA expression using a multiple tissue expression array showed that Staring mRNA is ubiquitously expressed in various brain regions as well as all fetal and adult human tissues examined (data not shown). The broad tissue distribution of Staring mRNA expression suggests that Staring has a functional role important to many cell types, including neurons.

To characterize Staring at the protein level, we generated four polyclonal anti-Staring antibodies, two in chicken (CS-N and CS-C) and two in rabbit (RS-N and RS-C), against the N- and C-terminal 14-amino acid peptide of rat Staring, respectively. The antibodies (CS-N and RS-N) against the N terminus of Staring are expected to detect both Staring and RBP95 isoforms, whereas the antibodies (CS-C and RS-C) against the C terminus of Staring should only recognize the Staring isoform. Western blot analysis demonstrated that all four anti-Staring antibodies, but not their corresponding preimmune controls, recognized a single endogenous protein band of ~125 kDa (Fig. 3, *B* and *C*; data not shown), indicating that Staring is the predominant isoform expressed in rat. No 95-kDa protein band corresponding to RBP95 could be detected by either chicken (CS-N) or rabbit (RS-N) antibodies against the N terminus of Staring (Fig. 3*B*; data not shown), suggesting that RBP95 is a rare isoform that is either expressed at extremely low levels or not expressed at all. All four anti-Staring antibodies specifically react with recombinant Staring protein expressed in bacterial and mammalian cells (data not shown). Furthermore, preabsorption of these anti-Staring antibodies with recombinant Staring protein completely eliminated their immunoreactivity to recombinant as well as endogenous Staring protein (data not shown), confirming the specificity of these antibodies. In agreement with the result of Northern blot analysis (Fig. 3*A*), Western blot analysis using the anti-Staring antibodies revealed that Staring protein is ubiquitously expressed in all tissues tested, although the expression levels in heart and skeletal muscle are very low (Fig. 3*B*).

To examine the intracellular distribution of endogenous Staring, postnuclear supernatant of rat brain was separated into cytosol and membrane fractions and then subjected to Western blot analysis with anti-Staring antibodies (Fig. 3*C*). Staring immunoreactivity was detected in both cytosol and membrane fraction, although the relative amount of Staring in

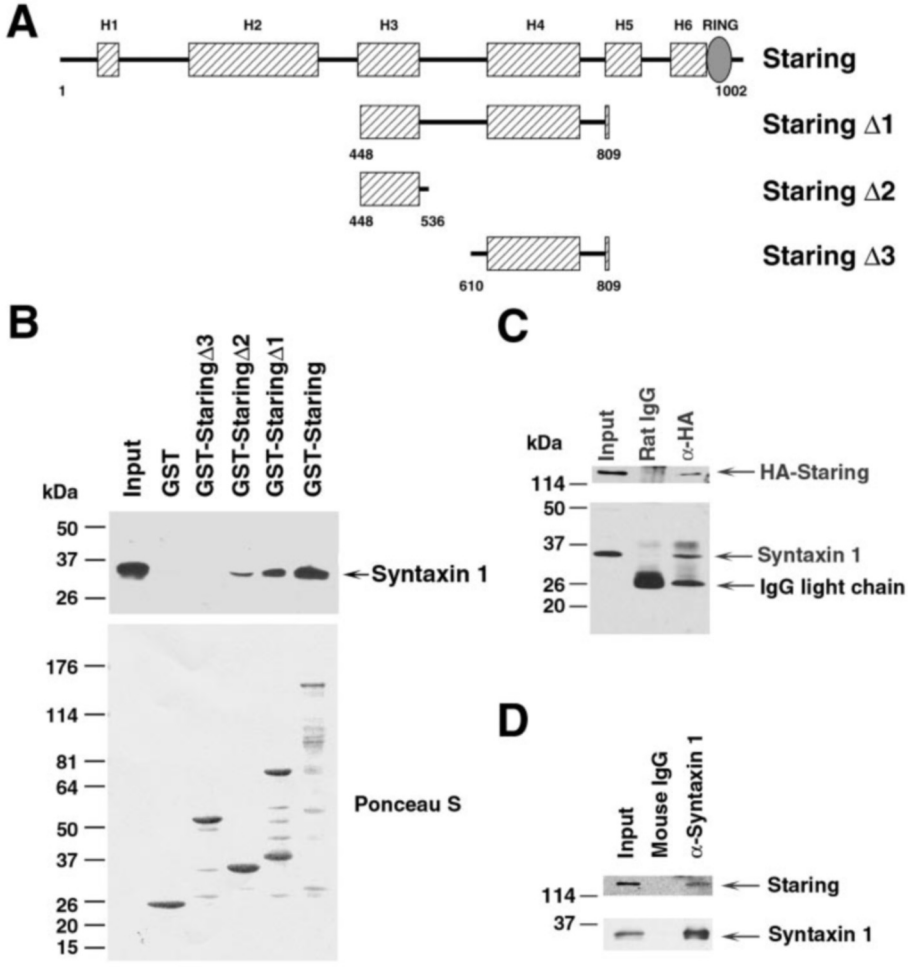

FIG. 4. **Biochemical characterization of the interaction between Staring and syntaxin 1.** *A*, schematic representation of Staring and its deletion mutants encoded by GST fusion cDNA constructs used in the *in vitro* binding assays. *B*, Staring binds syntaxin 1 *in vitro*. GST-Staring fusion proteins were immobilized on glutathione-Sepharose beads and incubated with rat brain homogenate (*Input*). After extensive washes, bound proteins were eluted and analyzed by SDS-PAGE and immunoblotting for syntaxin 1 (*upper panel*). GST-Staring fusion proteins were shown as Ponceau S staining (*lower panel*). *C*, co-immunoprecipitation of Staring with syntaxin 1 from transfected cells. Extracts from HeLa cells co-transfected with pcDNA3-syntaxin 1 and pCHA-Staring were subjected to immunoprecipitation with an anti-HA antibody or control rat IgG. *Input*, 30% of the HeLa extracts used for the immunoprecipitation. *D*, association of endogenous Staring with syntaxin 1 in rat brain. Detergent extracts of rat brain synaptosomes (P2′ fractions) were subjected to immunoprecipitation with anti-syntaxin 1 antibody or control mouse IgG. *Input*, 30% of the brain extracts used for the immunoprecipitation. The immunoprecipitates in *C* and *D* were analyzed by immunoblotting with anti-syntaxin 1 and anti-Staring (RS-C) antibodies. These co-immunoprecipitation experiments were replicated three times with similar results.

the cytosol fraction was severalfold more than that in the membrane fraction. To investigate the nature of Staring association with membranes, the membrane fraction was extracted with 1.5 M NaCl or 4 M urea (Fig. 3*C*). Unlike the integral membrane protein syntaxin 1 that was resistant to extraction by high salt and urea, a majority of Staring was extracted by these treatments, suggesting that Staring is peripherally associated with membranes via hydrophilic interactions.

Staring Interacts with Syntaxin 1 in Vitro and in Vivo—To determine whether the Staring-syntaxin 1 interaction detected in yeast actually takes place *in vitro*, GST fusion proteins containing various portions of Staring (Fig. 4*A*) were immobilized on glutathione beads and used to bind endogenous syntaxin 1 from rat brain homogenate. As shown in Fig. 4*B*, the GST-fusion proteins bearing the full-length Staring or the Staring fragments that contain the predicted coiled-coil domain H3 (Staring Δ1 and Staring Δ2) was able to bind endogenous syntaxin 1. In contrast, the GST-Staring fusion protein containing the coiled-coil domain H4 (Staring Δ3) or GST alone was unable to pull down syntaxin 1, confirming that the observed Staring-syntaxin 1 interaction is specific. These data indicate that the syntaxin 1-binding site of Staring lies within the H3 domain, between amino acid residues 448 and 536. In addition, other parts of Staring seem to also contribute to the interaction with syntaxin 1, since the GST-fusion proteins containing truncated forms of Staring bound much less syntaxin 1 than a similar amount of the full-length GST-Staring (Fig. 4*A*).

To determine whether Staring associates with syntaxin 1 *in vivo*, we first performed co-immunoprecipitation experiments using lysates of HeLa cells expressing exogenous syntaxin 1 and HA-tagged Staring. As shown in Fig. 4*C*, syntaxin 1 and HA-Staring were co-immunoprecipitated by the anti-HA antibody, providing evidence for the association of these two proteins in mammalian cells. By comparison, control IgG was unable to precipitate either syntaxin 1 or Staring. We then performed additional co-immunoprecipitation experiments to examine the association of endogenous Staring and syntaxin 1 in rat brain synaptosomes (Fig. 4*D*). Anti-syntaxin 1 antibody, but not the mouse IgG control, was able to co-immunoprecipitate syntaxin 1 and Staring from solubilized synaptosomes,

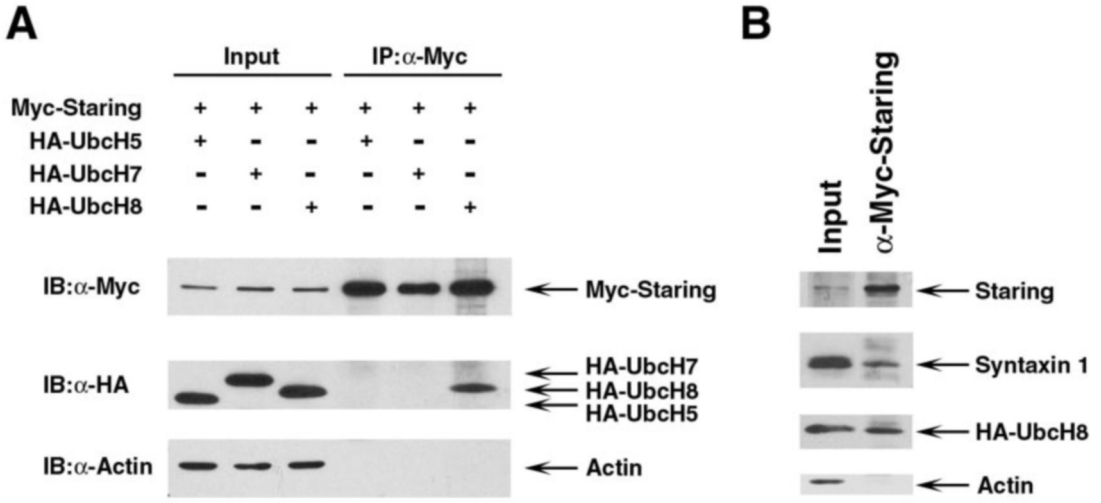

FIG. 5. **Staring binds selectively to UbcH8 and forms a ternary complex with syntaxin 1 and UbcH8.** *A*, specific interaction between Staring and UbcH8. HeLa cells were co-transfected with pcDNA3-Myc-Staring and pRK5-HA-UbcH5, pRK5-HA-UbcH7, or pRK5-HA-UbcH8. Cell lysates of transfected cells were subjected to immunoprecipitation with an anti-Myc antibody. The immunoprecipitates were then analyzed by immunoblotting with antibodies against HA, Myc, or actin. *B*, detection of a protein complex containing Staring, syntaxin 1, and UbcH8. Lysates from HeLa cells co-transfected with pcDNA3-Myc-Staring, pcDNA3-syntaxin 1, and pRK5-HA-UbcH8 were immunoprecipitated with an anti-Myc antibody followed by immunoblotting for Staring, syntaxin 1, HA-UbcH8, and actin.

demonstrating the existence of endogenous Staring-syntaxin 1 complexes at nerve terminals. Under our experiment conditions, ~10% of total endogenous Staring was co-immunoprecipitated with syntaxin 1, indicating that only a fraction of Staring and syntaxin 1 co-exist in the Staring-syntaxin 1 complexes. These results are consistent with previous reports that syntaxin 1 interacts with more than a dozen proteins, including SNAP-25, nSec1/Munc-18, Munc-13, tomosyn, and syntaphilin (14, 45–48). Moreover, as suggested by its multidomain structure, Staring is likely to interact with additional proteins.

Staring Binds and Recruits the E2 Ubiquitin-conjugating Enzyme UbcH8 to Syntaxin 1—The presence of the RING finger motif in Staring raises the possibility that Staring may function as an E3 ubiquitin-protein ligase via interaction with a specific E2 ubiquitin-conjugating enzyme (41). As a first step to test this possibility, Myc-tagged Staring was transiently expressed in HeLa cells along with HA-tagged E2 enzyme UbcH5, UbcH7, or UbcH8, and the interaction of these E2 enzymes with Staring was examined by co-immunoprecipitation analysis (Fig. 5*A*). The results revealed that Staring specifically interacts with UbcH8, a brain-enriched E2 ubiquitin-conjugating enzyme (49). In contrast, no appreciable interaction could be detected between Staring and UbcH5 or UbcH7. These data indicate that UbcH8 is the cognate E2 ubiquitin-conjugating enzyme for Staring. To further determine whether Staring interacts with both UbcH8 and syntaxin 1 to form a ternary complex, co-immunoprecipitation experiments were performed using extracts of HeLa cells co-transfected with epitope-tagged Staring, syntaxin 1, and UbcH8 (Fig. 5*B*). We observed that syntaxin 1 and UbcH8 co-precipitate with Staring in a stable complex, suggesting that Staring has the ability to recruit its cognate E2 enzyme UbcH8 to syntaxin 1.

Staring Promotes the Ubiquitination of Syntaxin 1—Next, we sought to determine whether Staring acts as an E3 ubiquitin-protein ligase to ubiquitinate syntaxin 1 by using a well established *in vivo* ubiquitination assay (50). HA-tagged syntaxin 1 was co-expressed in HeLa cells along with Myc-tagged ubiquitin in the absence or presence of exogenous Staring. Cell lysates were subjected to immunoprecipitation with an anti-HA antibody followed by immunoblotting with an anti-Myc antibody to detect ubiquitin-conjugated syntaxin 1 (Fig. 6). Increased levels of Myc-tagged ubiquitin were detected on syntaxin 1 in the presence of exogenous Staring, indicating that Staring promotes the ubiquitination of syntaxin 1. The Staring-mediated ubiquitination seems to be polyubiquitination, because the ubiquitinated syntaxin 1 was detected as a high molecular weight smear. In addition to the smear, several predominant ubiquitination bands were observed, which may represent predominant ubiquitinated syntaxin 1 species produced *in vivo* as the net result of the ubiquitination and deubiquitination reactions. Similar *in vivo* polyubiquitination patterns have previously been seen with other ubiquitinated proteins, such as synphilin-1 and CDCrel-1 (32, 51). The Myc-

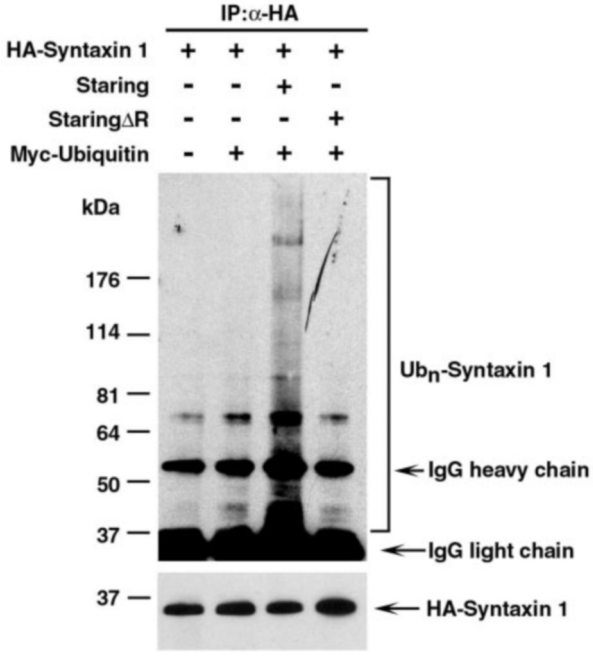

FIG. 6. **Staring is a RING-type E3 ubiquitin-protein ligase that ubiquitinates syntaxin 1.** HeLa cells were transfected with the indicated plasmids and treated with MG132 (20 μM) for 8 h before harvest. Cell lysates were subjected to immunoprecipitation with an anti-HA antibody followed by immunoblotting with an anti-Myc antibody to detect Myc-ubiquitin conjugated to syntaxin 1. The blot was then stripped and reprobed with an anti-syntaxin 1 antibody.

immunoreactive band at ~70 kDa is probably a nonspecific band, because it also exists in the control lane that has no Myc-tagged ubiquitin.

Since the RING finger motif is thought to be essential for the enzymatic activity of a RING-type E3 ubiquitin-protein ligase (40, 41), we examined the effect of deletion of the Staring RING finger motif on the *in vivo* ubiquitination of syntaxin 1. A mutant form of Staring (StaringΔR) that lacks the RING finger motif was generated and analyzed for its ability to ubiquitinate syntaxin 1 using the same *in vivo* ubiquitination assay as described above (Fig. 6). The results revealed that deletion of the RING finger motif abolished the ability of Staring to ubiquitinate syntaxin 1, providing further evidence supporting the role of Staring as a RING-type E3 ubiquitin-protein ligase.

Staring Targets Syntaxin 1 for Degradation by the Proteasome Pathway—Polyubiquitination of a cellular protein generally leads to the degradation of the protein by the proteasome pathway (52). To determine whether Staring-mediated ubiquitination of syntaxin 1 promotes its degradation, we performed pulse-chase experiments to compare the turnover rate of syntaxin 1 protein in the absence and presence of exogenous Staring. As shown in Fig. 7, the half-life of syntaxin 1 was ~16.7 h in the absence of Staring. By comparison, the half-life of syntaxin 1 was reduced to 6.1 h in the presence of Staring, indicating that Staring has the ability to regulate the degradation of syntaxin 1. Consistent with this result, measurement of the syntaxin 1 protein level by Western blot analysis revealed that the steady-state expression level of syntaxin 1 was significantly decreased in the presence of Staring as compared with the syntaxin 1 level in the absence of Staring (Fig. 7*C*). Furthermore, the Staring-induced degradation of syntaxin 1 was blocked by MG132, a potent inhibitor of proteasome function (53), but not by E64, an inhibitor of lysosomal cysteine proteases. Together, these results suggest that Staring promotes the degradation of syntaxin 1 by the proteasome pathway.

Staring Regulates the Ubiquitination and Degradation of Endogenous Syntaxin 1 in SH-SY5Y Cells—We next investigated whether Staring is able to regulate the ubiquitination and degradation of endogenous syntaxin 1. For these studies, we used human neuroblastoma SH-SY5Y cells, a well characterized dopaminergic cell line that expresses endogenous syntaxin 1 and exhibits SNARE-mediated neurotransmitter release (54, 55). Overexpression of Staring in SH-SY5Y cells significantly enhanced the ubiquitination of endogenous syntaxin 1, as indicated by the appearance of a high molecular weight smear containing Myc-ubiquitin-modified syntaxin 1 (Fig. 8*A*). The effect of overexpressing Staring on syntaxin 1 ubiquitination was abolished by the deletion of Staring RING finger motif, demonstrating that Staring promotes the ubiquitination of endogenous syntaxin 1 in a RING finger-dependent manner. In addition, overexpression of Staring significantly accelerated the turnover of endogenous syntaxin 1, as measured by the pulse-chase analyses in the absence and presence of exogenous Staring (Fig. 8*B*). The half-life of endogenous syntaxin 1 was reduced from 19.6 to 8.6 h by overexpression of Staring. The increased turnover rate of syntaxin 1 was accompanied by a decrease in the steady-state expression level of syntaxin 1 in the presence of exogenous Staring (Fig. 8*C*). Moreover, the Staring-induced degradation of endogenous syntaxin 1 was blocked by the proteasome inhibitor MG132 but not by the lysosomal protease inhibitor E-64, NH_4Cl, or chloroquine (Fig. 8*C*). Taken together, these data provide strong support for a role of Staring in regulating the degradation of endogenous syntaxin 1 via the ubiquitin-proteasome pathway.

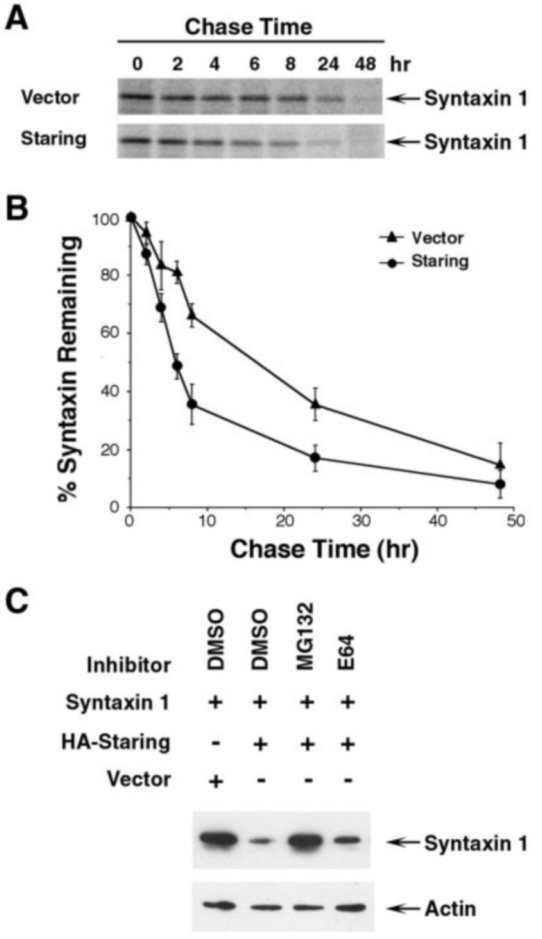

FIG. 7. **Staring accelerates proteasome-mediated degradation of exogenous syntaxin 1 expressed in HeLa cells.** *A*, degradation of syntaxin 1 in the presence or absence of exogenous Staring. HeLa cells were co-transfected with pCHA-syntaxin 1 and either pFLAG vector or pFLAG-Staring. Forty-eight hours after transfection, the cells were pulse-labeled for 1 h with DMEM containing [^{35}S]Met/Cys, chased with nonradioactive Met/Cys for the indicated time, and then lysed. ^{35}S-Labeled syntaxin 1 was immunoprecipitated with a monoclonal anti-HA antibody and detected by SDS-PAGE and autoradiography. *B*, levels of syntaxin 1 were measured by quantification of the intensity of the 35-kDa syntaxin 1 band using a PhosphorImager and plotted relative to the amount of syntaxin 1 present at 0 h. Data are shown as mean ± S.E. (*error bar*) of the results from three independent experiments. *C*, effect of Staring on syntaxin 1 degradation was blocked by proteasome inhibitors. HeLa cells were co-transfected with pcDNA3-syntaxin 1 and pCHA-Staring or pCHA vector control. Twenty-four hours later, cells were incubated for 8 h at 37 °C with Me_2SO (*DMSO*), proteasome inhibitor MG132 (20 μM in Me_2SO), or cysteine protease inhibitor E64 (50 μM). Cells were then lysed, and an equal amount of protein from each lysate was analyzed by immunoblotting for syntaxin 1 and actin.

DISCUSSION

Whereas it is becoming increasingly clear that the ubiquitin-proteasome proteolytic pathway is involved in synaptic function and neurodegeneration (56–58), very little is known about neuronal protein substrates that are targeted by this pathway. On the other hand, alterations in the expression levels of presynaptic proteins have been linked to synaptic plasticity and neurodegeneration, yet it is not understood whether and how the ubiquitin-proteasome proteolytic pathway regulates the degradation of these proteins at nerve terminals. In this study, we have identified and characterized Staring, a novel RING finger protein that interacts with syntaxin 1, an essential component of neurotransmitter release machinery. Our data suggest that Staring may function as an E3 ubiquitin-protein

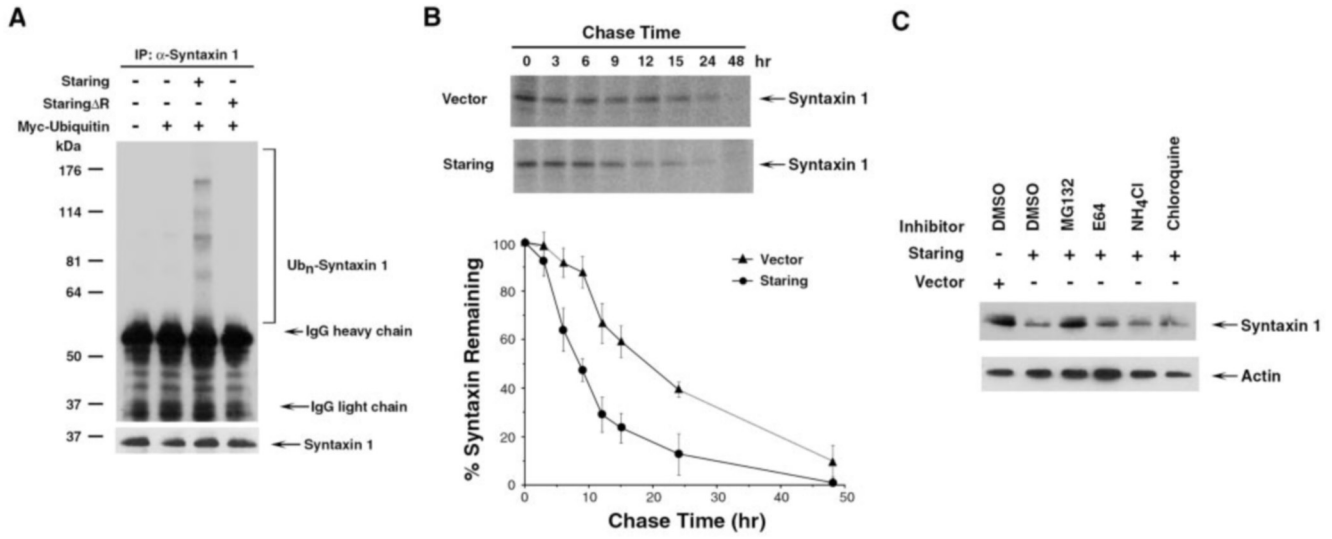

FIG. 8. **Overexpression of Staring promotes the ubiquitination and degradation of endogenous syntaxin 1 in SH-SY5Y cells.** *A*, Staring ubiquitinates endogenous syntaxin 1 *in vivo*. Lysates of SH-SY5Y cells transfected with indicated plasmids were subjected to immunoprecipitation with an anti-syntaxin 1 antibody followed by immunoblotting with an anti-Myc (*upper panel*) or anti-syntaxin 1 (*lower panel*) antibodies. *B*, Staring accelerates the turnover of endogenous syntaxin 1. SH-SY5Y cells transfected with pFLAG-Staring or pFLAG vector were pulse-labeled for 1 h with [^{35}S]Met/Cys and chased with nonradioactive medium for indicated times. ^{35}S-Labeled syntaxin 1 was immunoprecipitated with anti-HA antibody, detected by autoradiography (*upper panel*), and quantified with a PhosphorImager (*lower panel*). Data are shown as mean ± S.E. (*error bar*) of the results from three independent experiments. *C*, Staring-induced degradation of syntaxin 1 is sensitive to proteasome inhibition. SH-SY5Y cells transfected with pCHA-Staring or pCHA vector control were treated for 8 h before harvest with 0.1% Me_2SO, 20 μM MG132, 50 μM E64, 50 mM NH_4Cl, or 100 μM chloroquine. An equal amount of protein from each lysate was analyzed by immunoblotting for syntaxin 1 and actin.

ligase to promote the ubiquitination and degradation of syntaxin 1 by the proteasome pathway.

Traditionally, it is thought that the lysosomal pathway is used for the degradation of transmembrane proteins like syntaxin 1, whereas the ubiquitin-proteasome pathway is involved in the degradation of cytosolic and nuclear proteins. However, several recent papers reported the involvement of the ubiquitin-proteasome pathway in the degradation of transmembrane proteins, such as $GABA_A$ receptor, Netrin-1 receptor DCC (deleted in colorectal cancer), amiloride-sensitive epithelial Na^+ channels, and the Epstein-Barr virus latent membrane protein 1 (LMP1) (59–63). In addition, our recent work has demonstrated that the degradation of synaptic vesicle membrane protein synaptophysin is regulated by the E3 ubiquitin-protein ligase Siah in a proteasome-dependent manner (35). The evidence presented here suggests that the ubiquitin-proteasome pathway also regulates the degradation of the plasma membrane t-SNARE syntaxin 1. These findings implicate an important role for the ubiquitin-proteasome pathway in the degradation of both cytosolic and membrane proteins at nerve terminals. Since in neuron the lysosome is localized in the cell body instead of the nerve terminal, degradation by the ubiquitin-proteasome pathway may provide a more efficient means for the regulation of presynaptic protein levels locally at the nerve terminal.

Staring is a novel member of a growing family of RING finger proteins that function as E3 ubiquitin-protein ligases. The RING type E3 ligases consist of two classes: single-subunit RING E3 ligases and multisubunit RING E3 ligases. A single subunit RING E3 ligase contains the substrate-binding domain and the RING finger motif in the same molecule. In contrast, a multisubunit RING E3 ligase is a multiprotein complex that contains the RING finger protein as one subunit and a separate protein for substrate recognition (41, 64). Our data indicate that Staring is a single-subunit RING E3 ligase that uses its coiled-coil domain H3 to bind substrate syntaxin 1. In addition to the H3 domain, Staring also contains five other coiled-coil domains, suggesting that Staring may bind and ubiquitinate additional substrates.

Among the molecular components of the ubiquitin-proteasome pathway, the E3 ubiquitin-protein ligase is perhaps the most important player, because it binds specific substrates and determines their ubiquitination and subsequent degradation. Furthermore, regulation of the E3-mediated substrate recognition and ubiquitination is a major mechanism for modulation of specific protein degradation (52, 64). Aberrations in the E3 ubiquitin-protein ligase activities have been implicated in the pathogenesis of several human diseases (64). For example, mutations or exon deletions in the gene encoding the E3 ubiquitin-protein ligase parkin are a major cause of autosomal recessive juvenile parkinsonism (58, 65, 66). Interestingly, parkin is widely expressed in many tissues, yet autosomal recessive juvenile parkinsonism is characterized by a selective degeneration of nigral neurons, suggesting that accumulation of a neuron-specific substrate(s) may be involved in the pathogenesis (65, 66). Similar to parkin, Staring is also ubiquitously expressed. We have found that Staring selectively interacts with the brain-enriched E2 ubiquitin-conjugating enzyme UbcH8 and promotes the ubiquitination and degradation of the neuron-specific protein syntaxin 1. Since altered syntaxin 1 levels have been associated with several neurodegenerative diseases, it would be tempting to speculate that Staring-mediated syntaxin 1 degradation contributes to the pathophysiology of these diseases. Further studies of the molecular mechanism that controls the degradation of presynaptic proteins will not only enhance our understanding of neurodegeneration but will also advance our knowledge of synaptic plasticity such as learning and memory.

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REFERENCES

1. Schwartz, A. L., and Ciechanover, A. (1999) *Annu. Rev. Med.* **50,** 57–74
2. Ciechanover, A. (2001) *Isr. Med. Assoc. J.* **3,** 319–327
3. Chapman, A. P., Courtney, S. C., Smith, S. J., Rider, C. C., and Beesley, P. W. (1992) *Biochem. Soc. Trans.* **20,** 155S
4. Chapman, A. P., Smith, S. J., Rider, C. C., and Beesley, P. W. (1994) *Neurosci. Lett.* **168,** 238–242
5. Flann, S., Hawkes, R. B., Riederer, B. M., Rider, C. C., and Beesley, P. W. (1997) *Neuroscience* **81,** 173–187
6. Bochtler, M., Ditzel, L., Groll, M., Hartmann, C., and Huber, R. (1999) *Annu. Rev. Biophys. Biomol. Struct.* **28,** 295–317
7. Bonifacino, J. S., and Weissman, A. M. (1998) *Annu. Rev. Cell Dev. Biol.* **14,** 19–57
8. Ciechanover, A. (1998) *EMBO J.* **17,** 7151–7160
9. Hershko, A., and Ciechanover, A. (1998) *Annu. Rev. Biochem.* **67,** 425–479
10. Bennett, M. K., Calakos, N., and Scheller, R. H. (1992) *Science* **257,** 255–259
11. Inoue, A., and Akagawa, K. (1992) *Biochem. Biophys. Res. Commun.* **187,** 1144–1150
12. Yoshida, A., Oho, C., Omori, A., Kuwahara, R., Ito, T., and Takahashi, M. (1992) *J. Biol. Chem.* **267,** 24925–24928
13. Rothman, J. E. (1994) *Nature* **372,** 55–63
14. Jahn, R., and Sudhof, T. C. (1999) *Annu. Rev. Biochem.* **68,** 863–911
15. Lin, R. C., and Scheller, R. H. (2000) *Annu. Rev. Cell Dev. Biol.* **16,** 19–49
16. Kushima, Y., Fujiwara, T., Sanada, M., and Akagawa, K. (1997) *J. Mol. Neurosci.* **8,** 19–27
17. Noakes, P. G., Chin, D., Kim, S. S., Liang, S., and Phillips, W. D. (1999) *J. Comp. Neurol.* **410,** 531–540
18. Miya, F., Yamamoto, A., Akagawa, K., Kawamoto, K., and Tashiro, Y. (1996) *Cell Struct. Funct.* **21,** 525–532
19. Veeranna, Grant, P., and Pant, H. C. (1997) *Dev. Neurosci.* **19,** 172–183
20. Davis, S., Rodger, J., Hicks, A., Mallet, J., and Laroche, S. (1996) *Eur. J. Neurosci.* **8,** 2068–2074
21. Hicks, A., Davis, S., Rodger, J., Helme-Guizon, A., Laroche, S., and Mallet, J. (1997) *Neuroscience* **79,** 329–340
22. Richter-Levin, G., Thomas, K. L., Hunt, S. P., and Bliss, T. V. (1998) *Neurosci. Lett.* **251,** 41–44
23. Gabriel, S. M., Haroutunian, V., Powchik, P., Honer, W. G., Davidson, M., Davies, P., and Davis, K. L. (1997) *Arch. Gen. Psychiatry* **54,** 559–566
24. Shimohama, S., Kamiya, S., Taniguchi, T., Akagawa, K., and Kimura, J. (1997) *Biochem. Biophys. Res. Commun.* **236,** 239–242
25. Honer, W. G., Falkai, P., Young, C., Wang, T., Xie, J., Bonner, J., Hu, L., Boulianne, G. L., Luo, Z., and Trimble, W. S. (1997) *Neuroscience* **78,** 99–110
26. Ferrer, I., Rivera, R., Blanco, R., and Marti, E. (1999) *Neurobiol. Dis.* **6,** 92–100
27. Kwong, J., Roundabush, F. L., Moore, P. H., Montague, M., Oldham, W., Li, Y., Chin, L., and Li, L. (2000) *J. Cell Sci.* **113,** 2273–2284
28. Chevray, P. M., and Nathans, D. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 5789–5793
29. Li, X. J., Li, S. H., Sharp, A. H., Nucifora, F. C., Jr., Schilling, G., Lanahan, A., Worley, P., Snyder, S. H., and Ross, C. A. (1995) *Nature* **378,** 398–402
30. Chin, L. S., Nugent, R. D., Raynor, M. C., Vavalle, J. P., and Li, L. (2000) *J. Biol. Chem.* **275,** 1191–1200
31. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
32. Zhang, Y., Gao, J., Chung, K. K., Huang, H., Dawson, V. L., and Dawson, T. M. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 13354–13359
33. Li, Y., Chin, L. S., Weigel, C., and Li, L. (2001) *J. Biol. Chem.* **276,** 40824–40833
34. Chin, L.-S., Raynor, M. C., Wei, X., Chen, H., and Li, L. (2001) *J. Biol. Chem.* **276,** 7069–7078
35. Wheeler, T. C., Chin, L. S., Li, Y., Roudabush, F. L., and Li, L. (2002) *J. Biol. Chem.* **277,** 10273–10282
36. Weimbs, T., Low, S. H., Chapin, S. J., Mostov, K. E., Bucher, P., and Hofmann, K. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 3046–3051
37. Kozak, M. (1987) *Nucleic Acids Res.* **15,** 8125–8148
38. Saurin, A. J., Borden, K. L., Boddy, M. N., and Freemont, P. S. (1996) *Trends Biochem. Sci.* **21,** 208–214
39. Borden, K. L. (2000) *J. Mol. Biol.* **295,** 1103–1112
40. Joazeiro, C. A., and Weissman, A. M. (2000) *Cell* **102,** 549–552
41. Pickart, C. M. (2001) *Annu. Rev. Biochem.* **70,** 503–533
42. Wen, H., and Ao, S. (2000) *Biochem. Biophys. Res. Commun.* **275,** 141–148
43. Rieger, K. J., El-Alama, M., Stein, G., Bradshaw, C., Slonimski, P. P., and Maundrell, K. (1999) *Yeast* **15,** 973–986
44. Muren, E., Oyen, M., Barmark, G., and Ronne, H. (2001) *Yeast* **18,** 163–172
45. Hata, Y., Slaughter, C. A., and Sudhof, T. C. (1993) *Nature* **366,** 347–351
46. Betz, A., Okamoto, M., Benseler, F., and Brose, N. (1997) *J. Biol. Chem.* **272,** 2520–2526
47. Fujita, Y., Shirataki, H., Sakisaka, T., Asakura, T., Ohya, T., Kotani, H., Yokoyama, S., Nishioka, H., Matsuura, Y., Mizoguchi, A., Scheller, R. H., and Takai, Y. (1998) *Neuron* **20,** 905–915
48. Lao, G., Scheuss, V., Gerwin, C. M., Su, Q., Mochida, S., Rettig, J., and Sheng, Z. H. (2000) *Neuron* **25,** 191–201
49. Kimura, M., Hattori, T., Matsuda, Y., Yoshioka, T., Sumi, N., Umeda, Y., Nakashima, S., and Okano, Y. (1997) *Cytogenet. Cell Genet.* **78,** 107–111
50. Ellison, M. J., and Hochstrasser, M. (1991) *J. Biol. Chem.* **266,** 21150–21157
51. Chung, K. K., Zhang, Y., Lim, K. L., Tanaka, Y., Huang, H., Gao, J., Ross, C. A., Dawson, V. L., and Dawson, T. M. (2001) *Nat. Med.* **7,** 1144–1150
52. Kornitzer, D., and Ciechanover, A. (2000) *J. Cell. Physiol.* **182,** 1–11
53. Lee, D. H., and Goldberg, A. L. (1998) *Trends Cell Biol.* **8,** 397–403
54. Goodall, A. R., Danks, K., Walker, J. H., Ball, S. G., and Vaughan, P. F. (1997) *J. Neurochem.* **68,** 1542–1552
55. Purkiss, J. R., Friis, L. M., Doward, S., and Quinn, C. P. (2001) *Neurotoxicology* **22,** 447–453
56. Wan, H. I., DiAntonio, A., Fetter, R. D., Bergstrom, K., Strauss, R., and Goodman, C. S. (2000) *Neuron* **26,** 313–329
57. DiAntonio, A., Haghighi, A. P., Portman, S. L., Lee, J. D., Amaranto, A. M., and Goodman, C. S. (2001) *Nature* **412,** 449–452
58. Giasson, B. I., and Lee, V. M. (2001) *Neuron* **31,** 885–888
59. Hu, G., Zhang, S., Vidal, M., Baer, J. L., Xu, T., and Fearon, E. R. (1997) *Genes Dev.* **11,** 2701–2714
60. Hicke, L. (1999) *Trends Cell Biol.* **9,** 107–112
61. Aviel, S., Winberg, G., Massucci, M., and Ciechanover, A. (2000) *J. Biol. Chem.* **275,** 23491–23499
62. Malik, B., Schlanger, L., Al-Khalili, O., Bao, H. F., Yue, G., Price, S. R., Mitch, W. E., and Eaton, D. C. (2001) *J. Biol. Chem.* **276,** 12903–12910
63. Bedford, F. K., Kittler, J. T., Muller, E., Thomas, P., Uren, J. M., Merlo, D., Wisden, W., Triller, A., Smart, T. G., and Moss, S. J. (2001) *Nat. Neurosci.* **4,** 908–916
64. Glickman, M. H., and Ciechanover, A. (2002) *Physiol. Rev.* **82,** 373–428
65. Kitada, T., Asakawa, S., Hattori, N., Matsumine, H., Yamamura, Y., Minoshima, S., Yokochi, M., Mizuno, Y., and Shimizu, N. (1998) *Nature* **392,** 605–608
66. Tanaka, K., Suzuki, T., Chiba, T., Shimura, H., Hattori, N., and Mizuno, Y. (2001) *J. Mol. Med.* **79,** 482–494