A Neural-specific F-box Protein Fbs1 Functions as a Chaperone Suppressing Glycoprotein Aggregation*

Yukiko Yoshida†§†, Arisa Murakami†§, Kazuhiro Iwai†§, and Keiji Tanaka§

From the †Tokyo Metropolitan Institute of Medical Science, 3-18-22 Honkomagome, Bunkyo-ku, Tokyo 113-8613, §CREST, Japan Science and Technology Corporation (JST), Saitama 332-0012, and the ‡Department of Molecular Cell Biology, Graduate School of Medicine, Osaka City University, 1-4-3 Asahi-cho, Abeno-ku, Osaka 545-8585, Japan

Fbs1 is an F-box protein present abundantly in the nervous system. Similar to the ubiquitously expressed Fbs2, Fbs1 recognizes N-glycans at the innermost position as a signal for unfolded glycoproteins, probably in the endoplasmic reticulum-associated degradation pathway. Here, we show that the in vivo majority of Fbs1 is present as Fbs1-Skp1 heterodimers or Fbs1 monomers but not SCF<sup>Fbs1</sup> complex. The inefficient SCF complex formation of Fbs1 and the restricted presence of SCF<sup>Fbs1</sup> bound on the endoplasmic reticulum membrane were due to the short linker sequence between the F-box domain and the sugar-binding domain. In vitro, Fbs1 prevented the aggregation of the glycoprotein through the N-terminal unique sequence of Fbs1. Our results suggest that Fbs1 assists clearance of aberrant glycoproteins in neuronal cells by suppressing aggregates formation, independent of ubiquitin ligase activity, and thus functions as a unique chaperone for those proteins.

The SCF (Skp1/Cul1/F-box protein) complex, the largest known class of sophisticated E3<sup>2</sup> ubiquitin ligases, consists of common components, Skp1, Cul1, and Roc1/Rbx1, as well as variable components known as F-box proteins that bind the substrates (1, 2). In this complex, the scaffold protein Cul1 (alias cullin1) interacts at the N terminus with the adaptor subunit Skp1 and at the C terminus with the RING-finger protein Roc1/Rbx1 that recruits a specific ubiquitin-activating enzyme (E2) for ubiquitylation. F-box proteins, interacting with Skp1 through the ~40 amino acid F-box motif, play an indispensable role in the selection of target proteins for degradation because each distinct F-box protein usually binds a protein substrate(s) with a degree of selectivity for ubiquitylation through C-terminal protein-protein interaction domains (3). The human genome contains 69 genes for F-box proteins and a large number of F-box proteins function in the specific ubiquitylation of a wide range of substrates. The F-box proteins are divided into three classes according to the type of substrate-binding domains. The two classes of binding domains are WD40 repeats and leucine-rich repeats, which are named Fbw (or FBXW) and Fbl (or FBXL) families, respectively (4). The third class of F-box proteins is the Fbx (or FBXO) family that does not contain any of these domains.

It has been reported that a subfamily under the Fbx family consists of at least five homologous F-box proteins containing a conserved FBA motif (5, 6). Among them, Fbs1/Fbx2/NFB42/Fbg1 and Fbs2/Fbx6b/Fbg2 can bind to proteins with high mannose oligosaccharides modification that occurs in the endoplasmic reticulum (ER) (7). Experiments using a fully reconstituted system showed that both Fbs1 and Fbs2 can form SCF-type ubiquitin ligase complexes specific for N-linked glycoproteins (7, 8). Overexpression of the Fbs1 or Fbs2 dominant-negative form or decrease of endogenous Fbs2 by small interfering RNA resulted in inhibition of degradation of endoplasmic reticulum-associated degradation (ERAD) substrates, suggesting the involvement of SCF<sup>Fbs1</sup> and SCF<sup>Fbs2</sup> in the ERAD pathway. Interestingly, x-ray crystallographic and NMR studies of the substrate-binding domain of Fbs1 have revealed that Fbs1 interacts with the innermost chitobiose in N-glycans of glycoproteins by a small hydrophobic pocket located at the top of the β-sandwich, indicating that both Fbs1 and Fbs2 efficiently recognize the inner chitobiose structure in Man<sub>α</sub>3–9GlcNAc<sub>2</sub> glycans (9). Indeed, the introduction of point mutation into the residues in the pocket impaired the binding activity toward its glycoprotein substrates. In general, the internal chitobiase structure of N-glycans in many native glycoproteins is not accessible by macromolecules. Fbs1 interacted with denatured glycoproteins more efficiently than native proteins, indicating that the innermost position of N-glycans becomes exposed upon protein denaturation and used as a signal of unfolded glycoproteins to be recognized by Fbs1 (10).

Of the Fbs family proteins, whereas Fbs2 is distributed ubiquitously in a variety of cells and tissues, Fbs1 is expressed only in neurons (7). In considering the involvement of these F-box proteins in the ERAD pathway in general, the restricted expression of Fbs1 in neurons remains a mystery. In this study, we found that the major population of Fbs1 protein did not form the SCF<sup>Fbs1</sup> complex in cells although Fbs1 is known to act as a component of SCF-type ubiquitin ligase (8). Moreover, the results showed that the sequence of the intervening segment between the F-box domain and the substrate-binding domain of the Fbs1 hampered the assembly of the SCF<sup>Fbs1</sup> complex in the cytosol without affecting the association with Skp1. The Skp1-Fbs1 heterodimers as well as SCF<sup>Fbs1</sup> complex effectively prevented the aggregation of the glycoprotein in vitro, and this
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activity was dependent on the presence of the N-terminal domain and the substrate-binding domain of Fbs1. Our data thus imply that Skp1 and Fbs1 may function in both SCF and non-SCF complexes.

**EXPERIMENTAL PROCEDURES**

**Affinity Purification and Immunoprecipitation of Brain Lysate**—The preparation of lysates from mouse brains and purification of Fbs1 by using a ribonuclease B (RNaseB) column were performed as described previously (10). For immunoprecipitation, we used polyclonal antibody to Fbs1 as described previously (11). For immunoblotting, we used rabbit polyclonal antibodies against Fbs1, Cul1 (Zymed Laboratories Inc., San Francisco, CA) and Skp1 (Santa Cruz Biotechnology, Santa Cruz, CA), and horseradish peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) for Fbs1 and Skp1 blots or horseradish peroxidase-conjugated goat anti-rabbit IgG light chain (Jackson ImmunoResearch Laboratories) for Cul1 blots. Lectin blotting was performed using horseradish peroxidase-conjugated ConA (Seikagaku-kogyo, Japan) as described previously (11).

**Glycerol Gradient Analysis**—The fraction eluted with 0.1 M chitobiose from the RNaseB resin was prepared from 0.5 ml of lysates (14 mg/ml) from mouse brains. The eluate was dialyzed against TBS. The resultant fraction and a 1-mg lysate of brains were used for glycerol gradient analysis. Samples and molecular weight markers (Amersham Biosciences) were fractionated by linear glycerol density gradient centrifugation (22 h, 100,000 × g) as described previously (12).

**Cell Culture and Immunological Analysis**—PC12 cells were grown in RPMI medium 1640 (Invitrogen) supplemented with 10% horse serum and 5% fetal bovine serum. For neuronal differentiation, PC12 cells were treated with 10–20 ng/ml nerve growth factor (Invitrogen) on collagen-coated plates. 293T and HeLa cells were grown in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% fetal bovine serum and were transfected as described previously (8). FLAG-tagged Fbs1 mutant vectors consisting of Fbs1 and Fbs2 fragments were generated by PCR, and those sequences were verified. Whole cell lysates were prepared with 20 mM Tris-HCl (pH 7.5), 150 mM NaCl (TBS) containing 0.5% Nonidet P-40. The supernatant and precipitate fractions were prepared by ultracentrifugation of the supernatant that was prepared by centrifugation of freezing-and-thawing cell lysates in TBS at 8,000 × g for 20 min and at 100,000 × g for 60 min. The precipitate fraction was solubilized with Triton X-100. Each immunoprecipitation analysis was performed for whole cell lysates or subcellular fraction of cells by using the same amount of proteins. Monoclonal antibodies to calnexin and rhodopsin were purchased from BD Transduction Laboratories and Affinity Bioreagents (Golden, CO), respectively. Antibodies to FLAG, HA, and fetuin have been described previously (8).

**Pulse-chase Analysis**—The expression plasmid for P23H rhodopsin was kindly provided by M. E. Cheetham (University College London). Pulse-chase experiments were performed as described previously (7). Briefly, 293T cells were transfected with 1 μg of P23H rhodopsin expression plasmid and 1 μg of FLAG-tagged Fbs1 derivatives or pcDNA3-FLAG plasmid. Twenty-four hours after transfection, the cells were starved for 30 min and labeled for 1 h with 150 μCi of Pro-MixL-35S in vitro cell labeling mix (Amersham Biosciences) per milliliter. After washing, the cells were chased with complete Dulbecco’s modified Eagle’s medium supplemented with fetal bovine serum in the presence or in the absence of 50 μg/ml MG132 (Peptide Institute, Tokyo, Japan) for the indicated time intervals. After the harvested cells were lysed by TBS containing 0.1% SDS and 1% Nonidet P-40, immunoprecipitation was performed with anti-rhodopsin and FLAG antibodies.

**Preparation of Recombinant Proteins and in Vitro Ubiquitylation Assay**—The His-tagged Fbs1 ΔF, Fbs1 ΔP baculovirus were produced by Bac-to-Bac baculovirus expression system (Invitrogen). The SCF/Fbs1, Skp1-Fbs1 dimers, Fbs1 ΔF, Fbs1 ΔP, Skp1-ΔP dimers, and Fbs1 ΔN were obtained by baculovirus-infected HighFive cells as described previously (10). These proteins were purified by affinity chromatography using RNaseB-immobilized beads as a ligand and chitobiose as an eluent, and the eluates were dialyzed to 1,000 volumes of TBS three times. In vitro ubiquitylation assays were performed as described previously (10).

**Aggregation Assay**—Jack bean α-mannosidase (Sigma) was desalted using a NAP-25 gel filtration column (Amersham Biosciences) equilibrated in 10 mM Tris-HCl (pH 8.0). The desalted protein was lyophilized and redissolved at 21.7 μM in 0.1 M Tris-HCl (pH 8.0) and 6 M GdnHCl as described previously (13). After denaturation for 60 min at room temperature, samples were diluted to 0.3 μM in 1 ml of TBS containing various concentrations of bovine serum albumin or recombinant Fbs1 derivatives. Protein aggregation was monitored at 25 °C over a period of 60 min by measuring absorbance at 360 nm.

**RESULTS**

**Multiple States of Fbs1 in Brain**—Fbs1 has been found in the fraction eluted with di-N-acetyl-d-glucosamine (thereafter referred to as chitobiose) from GlcNAc-terminated fetuin of lysates prepared from mouse brain (8). Fbs1 and Skp1 proteins were detected in the eluted fraction with Coomassie Brilliant Blue staining, but we could not detect the apparent band of Cul1. However, the formation of the SCF/Fbs1 complex was confirmed by reciprocal immunoprecipitation experiments in 293T cells but also by reconstitution of baculovirally expressed recombinant SCF/Fbs1 proteins. To address these contradictory observations, we tested whether endogenous Fbs1 in the mouse brain forms the SCF complex by examining the interaction of Fbs1 with Cul1 (Fig. 1A). Fbs1 can be easily purified by affinity chromatography using RNaseB that contains a high mannose oligosaccharide as a ligand and chitobiose as an eluent (10). Since Fbs1 contains a single binding domain toward an N-glycan, it seems likely that the eluted Fbs1 protein or its complex from the RNaseB-immobilized resin is free from its substrates. Indeed, the glycoproteins modified with high mannose oligosaccharides were not included in the eluates by chitobiose (Fig. 1B). Although Skp1 was effectively co-immunoprecipitated with Fbs1 from the lysate of mouse brain, the amount of Skp1 that was eluted with Fbs1 from the RNaseB resin was small (Fig. 1A). Despite the difference in the quantities of Skp1 bound to Fbs1 in the fractions between eluates from
the RNaseB resin and immunoprecipitation with an anti-Fbs1 antibody, almost the same and small quantities of Cul1 were detected in these fractions. These results suggest that major populations of substrate-free Fbs1 and substrate-binding Fbs1 are present as Fbs1 monomers and Fbs1-Skp1 dimers, respectively, and the binding of substrates to Fbs1 does not influence the weak SCF complex formation.

To examine the behavior of endogenous Fbs1 in more detail, eluates from the RNaseB resin and lysates from the mouse brain were separated by a 4–17% glycerol density gradient centrifugation (Fig. 1C). The distribution of Fbs1 (~42 kDa) in the chitobiose eluates corresponded to the position of Fbs1 monomers (fraction 4) and Skp1-Fbs1 dimers (~63 kDa) (fraction 6). Although Cul1 was not detected in any fractions, the peak of Skp1 in eluates from the RNaseB resin was in the position of the Skp1-Fbs1 dimer. On the other hand, Fbs1 protein in brain lysate was detected in a broad range of fractions mainly larger than Fbs1-Skp1 dimers, indicating that most Fbs1, if not all, is associated with various glycoprotein substrates; i.e. Fbs1-Skp1 dimers maintain the association with glycoproteins in vivo. Cul1 (~90 kDa) in brain lysate was distributed broadly in higher density fractions, indicating its association with various other SCF-components.

Minor Population of Fbs1 Forms SCF Complex on ER—We next examined FLAG-tagged F-box proteins alone or their combination with HA-tagged Skp1 in 293T cells and immunoprecipitation with anti-FLAG and anti-HA antibodies (Fig. 2A). The expression of HA-tagged Skp1 increased the amount of exogenous F-box proteins, suggesting that Skp1 stabilizes F-box proteins (lanes 1–8). Cul1 was co-immunoprecipitated with Fbs2 and Fbg3, which are highly homologous with Fbs1, or βTrCP1/Fbw1, one of the Fbw family members (lanes 11–16). The interaction between Cul1 and these F-box proteins increased upon co-expression of Skp1. However, unlike these F-box proteins, Fbs1 was almost undetectable in the immune complex with Cul1, regardless of the overexpression of exogenous Skp1 (lanes 9 and 10), although Fbs1 was co-immunoprecipitated with Skp1 as well as other F-box proteins (lanes 18, 20, 22, and 24 in the αFlag panel). Moreover, the amount of Cul1 associated with exogenous Skp1 was lower in the presence of Fbs1 than in those of other F-box proteins, suggesting that expression of Fbs1 prevents forming other SCF complexes by dimerizing with Skp1 (lanes 18, 20, 22, and 24 in the αCul1 panel). These results suggest that Fbs1 can strongly bind Skp1 but is weak in forming the SCF(Fbs1) complex.

We have recently reported that Fbs1 is a cytosolic protein but that part of Fbs1 associates with the ER membrane through interaction with p97/VCP (valosin-containing protein) (11). We next examined whether the ER membrane-associated Fbs1 formed the SCF complex. Lysates of 293T and HeLa cells expressing FLAG-tagged Fbs1 were fractionated into the 100,000 × g supernatant and precipitate fractions excluding the 8,000 × g precipitate, and then Fbs1 was immunoprecipitated from these fractions by anti-FLAG antibody. As shown in Fig. 2B, Cul1 was co-immunoprecipitated with Fbs1 mainly from the precipitate (p) fraction (lanes 4 and 8). Although the association of Fbs1 with Skp1 occurred more effectively in the supernatant (s) fraction, the formation of the SCF complex, including Cul1, was hardly detected in the supernatant fraction (lanes 3 and 7). Moreover, we examined whether endogenous Fbs1 formed the SCF complex in the precipitate fraction using nerve growth factor-treated PC12 cells, which endogenously express Fbs1 (14). As shown in Fig. 2C, part of Cul1 was co-immunoprecipitated with Fbs1 from the precipitate (p) fraction. These results indicate that the major population of endogenous Fbs1 is present as the Fbs1-Skp1 heterodimers or the Fbs1 monomers in the cytosol, and a minor population of Fbs1 forms the SCF complex bound on the ER membrane.

Linker Sequence of Fbs1 Prevents SCF Complex Formation—Although the SCF complex formation of Fbs1 was inefficient, Fbs2 formed the SCF complex effectively (Fig. 2A). To identify the region(s) of Fbs1 that impedes SCF complex formation, we examined the ability of various fusion proteins containing Fbs1 and Fbs2 fragments to form the complex and compared these findings with the full-length proteins in co-immunoprecipitation assay (Fig. 3A). Fbs1 ΔF was used as negative control that did not bind to Skp1 (Fig. 3B, lane 3). Fbs1 YW and Fbs1 ΔC, both of which are deficient in substrate binding, could not restore SCF complex formation, indicating that the interaction
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![Image](image_url)

**FIGURE 2. Major population of Fbs1 forms non-SCF complex in vivo.** A, 293T cells were transfected with plasmids encoding various FLAG-tagged F-box proteins (Fbs1, Fbs2, Fbg3, and βTrCP1 (βTr)) combination with empty HA plasmids (−) or plasmids encoding HA-tagged Skp1 (+). Whole cell lysates were subjected to immunoprecipitation (IP) with antibodies to FLAG and HA, and lysates (15 μg each) and one half of the resulting precipitates were analyzed by immunoblotting with antibodies to Cul1, FLAG, and Skp1. Asterisks show Ig heavy and light chains. B, 293T and HeLa cells were transfected with FLAG-tagged Fbs1. Cell lysates were fractionated by ultracentrifugation, and FLAG-Fbs1 was immunoprecipitated with an antibody to FLAG from the same amount of proteins of 100,000 × g supernatant (s) and precipitate (p) fractions. The total amount of protein of the supernatant fraction was 2–3 times larger than that of the precipitate fraction. Lysates (15 μg each) and immunoprecipitates were analyzed by immunoblotting with antibodies to Cul1, FLAG, and Skp1. Asterisks show Ig heavy and light chains. To control for the fractionation, immunoblotting with an antibody to calnexin was performed. C, endogenous Fbs1 was immunoprecipitated with an antibody to Fbs1 from 100,000 × g supernatant, and precipitate fractions of differentiated PC12 cells were treated with nerve growth factor. Lysates (15 μg each) and immunoprecipitates were analyzed by immunoblotting. The immunoblotting analysis for separated supernatant and precipitate fractions was conducted as for B.

between Fbs1 and its substrates does not affect the complex formation (lanes 4 and 5). The N-terminal sequence of Fbs1 called the P domain is unique and is not seen in other F-box proteins, but the removal of this domain from Fbs1 or the addition to Fbs2 did not affect the complex formation (Fbs1 ΔP and Fbs2 ΔP; lanes 6 and 13). Exchange of the F-box domains between Fbs1 and Fbs2 caused the loss of the Skp1 binding activity, probably due to the incorrect folding (Fbs1 F2, Fbs1 ΔPF2, Fbs2 F1, and Fbs2 PF1: lanes 7, 8, 10, and 11, respectively). However, the replacement of the Fbs1 N-terminal region (which contains P and F-box domains and linker sequence) with the Fbs2 N-terminal region rescued the complex formation (Fbs2-2N1C: lane 9). In contrast, the addition of the Fbs1 N-terminal region instead of the Fbs2 N-terminal region markedly reduced the activity of Fbs2 to form the SCF complex but did not affect the Skp1 binding (Fbs-1N2C: lane 12). The linker sequences of the intervening segments between the F-box domain and the substrate-binding domain showed lower homology than other portions between Fbs1 and Fbs2, suggesting that the Fbs1 linker sequence is responsible for impeding the SCF<sup>F<sub>Fbs1</sub></sup> complex formation. Indeed, only the Fbs1 mutant protein that contained the Fbs2 linker sequence could form the SCF complex, but the efficiency of the SCF complex formation was less than that of Fbs2-2N1C (Fbs1 I2: lane 15). On the other hand, Fbs2 protein containing the Fbs1 linker sequence and the Fbs1 protein without its linker sequence did not seem to show the correct folding for Skp1 binding (Fbs2 I1 and Fbs1 ΔI: lanes 16 and 17). The Fbs1 mutant in which the F-box domain and the linker sequence are replaced with those of Fbs2 forms the SCF complex effectively. Thus, we conclude that the Fbs1 linker sequence between the F-box and substrate-binding domains hampers the SCF<sup>F<sub>Fbs1</sub></sup> complex formation.

We next compared the localization of Fbs1, Fbs2, and the mutant Fbs1 proteins capable of forming the SCF complex: Fbs-2N1C and Fbs1 I2 (Fig. 3C). Although a minor population of Fbs1 in the precipitate (p) fraction formed the SCF complex, most Fbs2 formed the SCF<sup>F<sub>Fbs2</sub></sup> complex in the supernatant (s) fraction as well as the precipitate fraction. Fbs-2N1C could form the SCF complex mainly in the supernatant fraction (Fig. 3C). Moreover, the amount of Cul1 co-immunoprecipi-
Fbs-2N1C could bind to P23H, although its binding to P23H seemed weaker than that of wild-type Fbs1 (Fig. 4A, left panel). Since the activity to bind RNaseB was not different between Fbs1 and Fbs-2N1C (data not shown), it seems likely that the SCF^{Fbs-2N1C} causes degradation of P23H through its ubiquitylation. Interestingly, the quantity of P23H decreased upon Fbs-2N1C expression (Fig. 4A, right panel). It has been reported that the degradation of P23H was suppressed by MG132 treatment (15, 16). The quantities of both P23H associated with Fbs-2N1C and the P23H protein were recovered by the addition of MG132. Moreover, we performed pulse-chase analysis using 293T cells co-expressing the P23H mutant and FLAG-tagged Fbs-2N1C.

**FIGURE 3.** Linker sequence between F-box and substrate-binding domains of Fbs1 hampers SCFFbs1 complex formation. A, schematic representation of constructs of fusion proteins consisting of Fbs1 and Fbs2 fragments. The fragments derived from Fbs1 and Fbs2 appear in gray and white boxes, respectively. The numbers above the constructs represent the amino acid position of Fbs1. The vertical bars represent identical amino acids between Fbs1 and Fbs2. P and F-box domains, linker sequence, and sugar-binding domain are represented by P, F-box, linker, and SBD, respectively. The binding activities of these constructs toward Cul1 and Skp1 shown in B are summarized on the right, with + representing strong binding, + representing weak binding, and − representing no binding. B, 293T cells were transfected with plasmids encoding the FLAG-tagged mutants F-box proteins represented in A. Whole cell lysates were subjected to immunoprecipitation (IP) with an antibody to FLAG, and the resulting precipitates were analyzed by immunoblotting with antibodies to Cul1, Skp1, and FLAG. Asterisks show Ig heavy and light chains.

**FIGURE 4.** Expression of Fbs-2N1C promotes substrate degradation. A, 293T cells were transfected with plasmids encoding FLAG-tagged empty vector (V), Fbs1 (W), Fbs-2N1C (2N), or Fbs1 YW (YW) and combination with rhodopsin P23H mutant. Some cells were treated with 10 μM MG132 for 16 h. Whole cell lysates were subjected to immunoprecipitation (IP) with antibodies to FLAG and rhodopsin, and the resulting precipitates were analyzed by immunoblotting with an antibody to rhodopsin. Asterisks show Ig heavy and light chains. B, rhodopsin P23H was co-transfected with FLAG-tagged empty vector, Fbs1, Fbs-2N1C, or Fbs1 YW. Twenty-four hours after transfection, 293T cells were pulse-labeled with [35S]Met/Cys for 1 h and chased for the indicated time intervals. Rhodopsin P23H and Fbs1 derivatives were immunoprecipitated with antibodies to rhodopsin and FLAG, respectively. The plotted data at the bottom show a quantification analysis of the stability of rhodopsin P23H over time in the upper panels. Data are the mean ± S.D. of three independent experiments. WT, wild type.
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Fbs1 derivatives. The degradation of P23H was suppressed by MG132 treatment as reported previously (Fig. 4B). Although wild-type Fbs1 or the YW mutant did not influence the kinetics of P23H degradation, co-expression of Fbs1-2N1C efficiently promoted its degradation. On the other hand, like wild-type Fbs1, Fbs1 I2, could associate with P23H, but its expression did not influence both the amount of P23H and the kinetics of P23H degradation (data not shown). These results demonstrate that the non-SCF complex of Fbs1 can be converted to an active E3 ligase by introducing the complex-forming activity mapped to the Skp1-Fbs1 domain of Fbs1 is required for this aggregation suppressing activity.

Fbs1 Suppresses Aggregation of Denatured Glycoprotein in Vitro—We reported previously that the expression of Fbs1 inhibits aggresome formation in Cos7 cells (8). Furthermore, since Fbs1 interacts with denatured glycoproteins more efficiently than native glycoproteins, we examined whether Fbs1 functions as a molecular chaperone for glycoproteins in vitro. To this end, we prepared recombinant SCFFbs1, Skp1-Fbs1 dimers, Fbs1, Fbs1 ΔF, Fbs1 ΔP, Skp1-ΔP dimers, and Fbs1 ΔN, all of which were produced by using a baculovirus system (Fig. 5A). To obtain highly purified recombinant proteins, we purified them by using the affinity for the RNaseB resin but not nickel resin toward His tag. The purified SCFFbs1 but not Skp1-Fbs1 dimers could ubiquitylate GlcNAc-terminated fetuin (GST-Ub) effectively (Fig. 5B). We next assessed the ability of these proteins or their complexes to suppress the aggregation by using denatured α-mannosidase that contains high mannose type oligosaccharides, a typical substrate for the glycoprotein aggregation assay (17). Although Fbs1 alone suppressed the aggregation of denatured α-mannosidase in a concentration-dependent manner, the Fbs1-Skp1 dimers as well as the SCFFbs1 complex suppressed the aggregation much more effectively than Fbs1 alone (Fig. 5C). Although the addition of half-molar of Fbs1 ΔF did not affect the aggregation of denatured α-mannosidase, ΔF was also active to suppress the aggregation at a level similar to that of Fbs1 alone in an equal molar ratio, suggesting that the partial suppression by Fbs1 is independent of the hydrophobic F-box domain. On the other hand, ΔN, consisting of substrate-binding domain alone, enhanced its aggregation. Both the aggregation-suppressing activity of SCFFbs1, Skp1-Fbs1 dimers, or Fbs1 and the aggregation-enhancing activity of ΔN were inhibited by chitobiose (Fig. 5D and not shown). In contrast, these recombinant Fbs1 protein complexes had no effect on the aggregation of non-glycosylated proteins such as citrate synthase and luciferase (data not shown). Importantly, ΔP as well as the Skp1-ΔP dimers could not suppress the aggregation of α-mannosidase in an equal molar ratio (Fig. 5C, E, and F, and not shown). These results indicate that the Skp1-Fbs1 dimers effectively suppress the aggregation of denatured glycoproteins by recognizing the exposed chitobiose in N-glycans and that the P domain of Fbs1 is required for this aggregation suppressing activity.
DISCUSSION

The F-box family of proteins, which are the substrate-recognition subunits of the SCF ubiquitin ligase, play important roles in ubiquitin-dependent proteolysis in eukaryotes (18, 19). However, it is not clear whether all F-box proteins indeed function as receptor subunits of SCF complexes. For example, it has been reported that at least two F-box proteins, Ctf3 and Rcy1, out of 11 F-box proteins in Saccharomyces cerevisiae, do not form SCF complexes (20–22). Since not all RING-finger proteins are ubiquitin ligases, it is possible that non-canonical F-box proteins that fail to form the SCF complex play some important roles other than ubiquitin ligase activity. In the present study, we showed that the SCF complex formation of Fbs1, which recognizes N-glycans, is not efficient and that the intervening segment between the F-box domain and the sugar-binding domain of Fbs1 suppresses the formation of the SCF complex. The major population of Fbs1 is present as Fbs1-Skp1 heterodimers or Fbs1 monomers, which can inhibit the aggregation of the glycoproteins. Our results show that Fbs1 contributes to a chaperone function in addition to the role of the SCFFbs1 ubiquitin ligase, opening new perspectives for cellular activities of F-box proteins.

Although most endogenous Fbs1 was not assembled into the SCFFbs1 complex, a minor population of Fbs1 was capable of forming the SCFFbs1 complex in cells. Moreover, the SCFFbs1 complex could be produced in insect cells by infection with the baculovirus, indicating that Fbs1 can intrinsically form the SCF complex. It is worth noting that the SCFFbs1 was mainly present in the 100,000 × g precipitate fraction including the microsome (Fig. 2, B and C). It is not clear why the SCFFbs1 is bound to the ER membrane, although it is plausible that it plays a pivotal role in the ERAD pathway. To examine how the SCF complex formation of Fbs1 was promoted in vivo, we treated Fbs1-expressing cells with ER stress inducers, such as thapsigargin and dithiothreitol or a proteasome inhibitor MG132. These treatments, however, did not affect the SCF complex formation (data not shown). Furthermore, although the interaction between Fbs1 and its substrate glycoproteins did not affect the SCF complex formation (Figs. 1A and 3B), we examined the effects of overexpression of p97/VCP, Fbs1 substrates, Skp1, or Cul1. No protein other than Cul1 accelerated the SCFFbs1 formation not only in the 100,000 × g precipitate fraction but also in the cytosol (Fig. 2A and data not shown). Intriguingly, whereas Fbs1 and Skp1 were mainly located in the cytosol (100,000 × g supernatant fraction), Cul1 was detected not only in the cytosol but also in the 100,000 × g precipitate fraction (Figs. 2, B and C, and 3C), suggesting that Cul1 is recruited to the microsome membrane where the SCFFbs1 complex will be assembled to ubiquitylate efficiently the N-linked glycosylated ERAD substrates.

Fbs1 belongs to a subfamily consisting of at least five homologous F-box proteins that contain a conserved FBA motif in their C termini (5, 6). Among them, at least Fbs2 recognizes high mannosse oligosaccharides as well as Fbs1 and forms an SCF-type ubiquitin ligase. The Fbs1 protein sequence shows highly homologous to that of Fbs2 other than the P domain of Fbs1 and C-terminal part of Fbs2, but the linker sequence between the F-box and FBA domains shows lower homology than other portions (Fig. 3A). As shown in Fig. 3B, the difference in the ability for assembling into the SCF complex between Fbs1 and Fbs2 is ascribed to the short linker sequence (92–117 amino acids of Fbs1). Although Fbs2 formed the SCF complex efficiently in the cytosol as well as the 100,000 × g precipitate fraction, the SCFFbs1 formation was mainly present bound on the ER membrane (Fig. 3C). Although it is not clear whether the linker sequence of Fbs1 prevents the SCFFbs1 from being in the cytosol or causes the formation of the SCFFbs1 bound on the ER membrane, this limited localization of SCFFbs1 is also due to the linker sequence (Fig. 3C). Crystal structure and mutational analyses of Cdc4 and βTrCP1 revealed the importance of orientation and rigidity in the linker sequence between F-box and WD40 domains for their in vivo function (23, 24). The linker sequences of Cdc4 and βTrCP1 are longer than that of Fbs1 and form three or four helix globular domains. On the other hand, the linker sequence of Fbs1 is an unstructured domain that consists of a flexible linker loop and an α-helix and is too far from Cul1 to influence directly the SCF complex formation.3 The information of the structure of Skp1-Fbs1 suggests that the prevention of the SCF complex formation by this unstructured linker sequence can be cancelled by binding to the membrane or unidentified proteins on the ER.

In this study, we demonstrated that the Fbs1 could suppress the aggregation of denatured glycoproteins. This activity is due to the N-terminal P domain that is not seen in other F-box proteins. This N-terminal domain has been reported as a PEST sequence rich in proline, glutamic acid, serine, and threonine, which are often found in short-lived proteins (25). The N-terminal sequence in Fbs1, however, did not seem to act as a general PEST because the deletion of the P domain from Fbs1 or the addition to Fbs2 did not affect the protein stability (Fig. 3). More recently, it has been reported that U-box type E3 CHIP (C terminus of Hsc-70-interacting protein) is associated with Fbs1 through the P domain (26). Although we did not detect the E3 activity of the Skp1-Fbs1 dimers produced in the insect cells toward glycoprotein substrate GlcNAc-terminated fetuin (Fig. 5B), it is possible that an unknown chaperone molecule of insect cells was bound to the P domain of Fbs1. Skp1-Fbs1 dimers and Fbs1 monomers as well as the SCFFbs1 complex showed activity to suppress the denatured glycoprotein aggregation, suggesting that the majority of Fbs1 is present as Skp1-Fbs1 dimers or Fbs1 monomers in cells and is not an intermediate prior to assembly of the SCFFbs1 complex, but rather, a novel functional unit.

It is predicted that more than 30% of eukaryotic proteins contain substantial regions of disordered structure (27). One feature of intrinsically disordered proteins is their rapid degradation. Intracellular protein quality control, especially the degradation of proteins with aberrant structures, is thought to be important particularly in quiescent cells such as neurons (28). Fbs1 is expressed mainly in neuronal cells in the adult brain (14). Recently, it has been reported (29, 30) that loss of autophagy leads to neurodegeneration even in the absence of any.

3 Mizushima, T., Yoshida, Y., Kumanomidou, T., Hasegawa, Y., Suzuki, A., Yamane, T., and Tanaka, K., unpublished data.
aggregation-prone mutant proteins. Moreover, these reports have shown that the primary role of autophagy under normal conditions is the turnover of diffused cytosolic proteins, rather than direct elimination of inclusion bodies (29, 30). Our study suggests that Fbs1 contributes to the clearance of such cytosolic proteins by constitutive autophagy, like other chaperone systems, to suppress the aggregation of abnormal glycoproteins in neurons. For this, the N-terminal unique sequence of Fbs1, the P domain, having chaperone function, may have been made up during evolution. Since Cul1 is a common component of the SCF complexes and Fbs1 is abundant in neuronal cells, Fbs1 may also evolutionally acquire the linker sequence that supplies Cul1 toward other F-box proteins. It also seems possible that Fbs1 functions as a chaperone to keep the solubility of a particular glycoprotein(s) in the cytosol in neuronal cells. Further studies are needed to identify the Fbs1 target glycoproteins in neuronal cells, which may reveal the role of Fbs1 in maintaining homeostasis of neuronal cells.

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