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# Glycoprotein-specific ubiquitin ligases recognize *N*-glycans in unfolded substrates

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**Misfolded or unassembled polypeptides in the endoplasmic reticulum (ER) are retro-translocated into the cytosol and degraded by the ubiquitin–proteasome system. We reported previously that the SCF$^{Fbs1,2}$ ubiquitin-ligase complexes that contribute to ubiquitination of glycoproteins are involved in the ER-associated degradation pathway. Here we investigated how the SCF$^{Fbs1,2}$ complexes interact with unfolded glycoproteins. The SCF$^{Fbs1}$ complex was associated with p97/VCP AAA ATPase and bound to integrin-β1, one of the SCF$^{Fbs1}$ substrates, in the cytosol in a manner dependent on p97 ATPase activity. Both Fbs1 and Fbs2 proteins interacted with denatured glycoproteins, which were modified with not only high-mannose but also complex-type oligosaccharides, more efficiently than native proteins. Given that Fbs proteins interact with innermost chitobiose in *N*-glycans, we propose that Fbs proteins distinguish native from unfolded glycoproteins by sensing the exposed chitobiose structure.**

Keywords: ubiquitin ligase; glycoprotein; unfold; *N*-glycan

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## INTRODUCTION

Most secretory and membrane proteins are co-translationally translocated into the lumen of the endoplasmic reticulum (ER). In the ER, these proteins are modified by *N*-linked oligosaccharides and subjected to 'quality control' in which aberrant proteins are distinguished from properly folded proteins (Ellgaard & Helenius, 2003). When the improperly folded or incompletely assembled proteins fail to restore their functional states, they are degraded by the ER-associated degradation (ERAD) system, which involves retrograde transfer of proteins from the ER to the cytosol followed by degradation by the proteasome. p97/VCP, a cytosolic ATPase member of the AAA ATPase family, may have several roles in the ERAD pathway. It has been suggested that the p97–Ufd1–Npl4 complex is required for the extraction of misfolded proteins from the ER into the cytosol (Tsai *et al*, 2002). Such a retro-translocation step would most probably be mediated by dual recognition of the substrates by p97, with the complex binding both the nonubiquitinated segment of a substrate and the attached polyubiquitin chain (Ye *et al*, 2003). The p97 complex, which associates with the ER membrane proteins VIMP and Derlin-1 through the amino-terminal domain of p97, is thought to be required for the extraction of misfolded proteins from the ER (Lilley & Ploegh, 2004; Ye *et al*, 2004). However, p97 has also been proposed to be important for the release of ERAD substrates after their export from the ER, and thereby for their accessibility to the proteasome (Elkabetz *et al*, 2004). The role of each of these functions remains to be established.

In the ubiquitin system, the ubiquitin ligase 'E3' has an important role in the selection of target proteins for ubiquitination. At present, several E3s have been identified in the ERAD pathway, such as Hrd1 (Bays *et al*, 2001) and Doa10 (Swanson *et al*, 2001) in yeast, and gp78 (Fang *et al*, 2001), CHIP (Meacham *et al*, 2001) and Parkin (Imai *et al*, 2001) in mammals. In addition, we have recently identified a new member of the ERAD-linked E3 family, SCF$^{Fbs}$, which participates in ERAD for selective elimination of glycoproteins (Yoshida *et al*, 2002, 2003). Whereas Hrd1, Doa10 and gp78 are localized in the ER, SCF$^{Fbs}$ complexes are localized in the cytosol similar to CHIP and Parkin.

The SCF is composed of Cullin1/Cdc53, Skp1, Roc1/Rbx1, and one member of the large family of F-box proteins, which are involved in trapping target proteins (Deshaies, 1999). Fbs1 and Fbs2 (F-box protein that recognizes sugar chains) interact with glycoproteins containing high-mannose oligosaccharides, protein modification of which occurs in the ER. Our recent X-ray crystallographic and nuclear magnetic resonance (NMR) studies of the substrate-binding domain of Fbs1 have shown that Fbs1 recognizes the inner chitobiose of high-mannose oligosaccharides by a small hydrophobic pocket located at the top of the β-barrel (Mizushima *et al*, 2004).

In this study, we examined whether Fbs proteins discriminate between folded and unfolded glycoproteins because ERAD substrates are thought to be unfolded. Both Fbs1 and Fbs2 preferably bind to denatured proteins that contain not only

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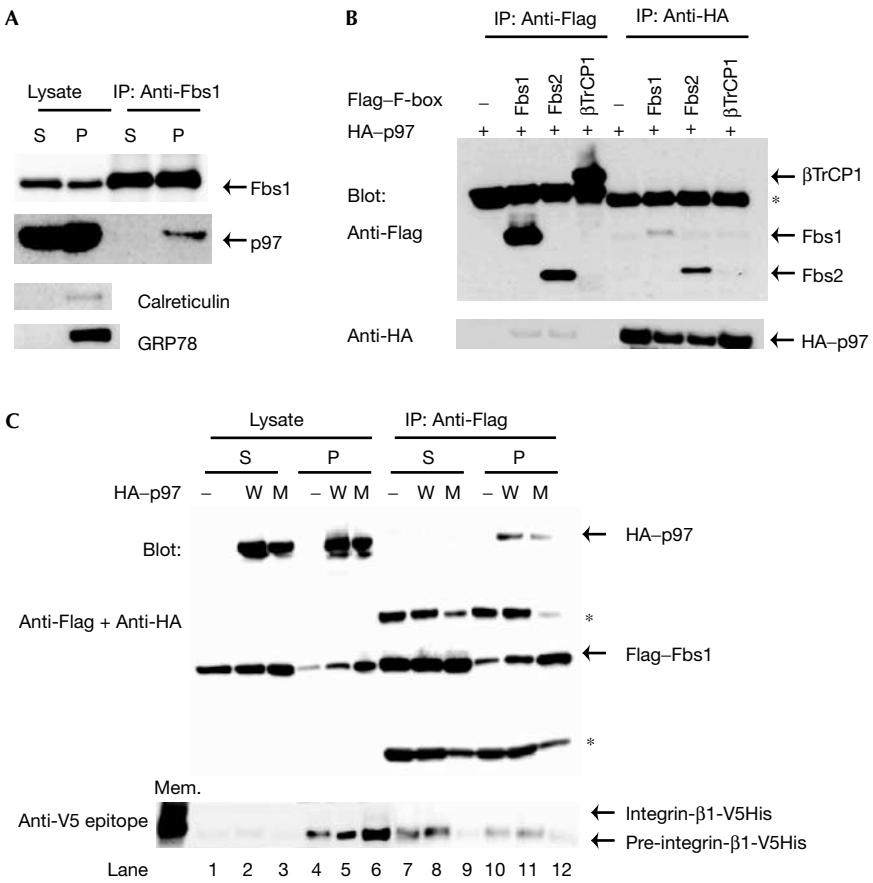


**Fig 1** | Fbs1 binds to integrin-β1 dependent on p97 ATPase activity. (**A**) Fbs1 associates with p97 in microsomal fraction. Endogenous Fbs1 was immunoprecipitated from 100,000*g* supernatant (S) and precipitate (P) fractions of brains of adult mice. Lysate (15 μg each) and immunoprecipitates were analysed by immunoblotting with antibodies against Fbs1, p97, calreticulin and GRP78. (**B**) Interaction of Fbs proteins with p97. Lysates of 293T cells transiently expressing Flag-tagged F-box proteins (−, empty vector) and HA-tagged p97 were subjected to immunoprecipitation, and the resulting precipitates were analysed by immunoblotting. The asterisk shows immunoglobulin heavy chains. (**C**) Fbs1 binding to integrin-β1 in the cytosol depends on p97 ATPase activity. 293T cells were transfected with Flag-tagged Fbs1, V5-tagged integrin-β1 and HA-tagged p97 (−, empty vector; W, wild-type p97; M, mutant p97 (K524A)). Fbs1 was immunoprecipitated from supernatant (S) and precipitate (P) fractions. Expressions of p97, Fbs1 and integrin-β1 in fractionated lysates (5 μg each) and the amount of integrin-β1 associated with Fbs1 were analysed by immunoblotting using anti-V5 antibody. The membranous fraction (Mem.) was prepared from 24,000*g* precipitate. Asterisks show immunoglobulin heavy and light chains.

high-mannose but also complex-type oligosaccharides over native counterparts. The results showed that these F-box proteins probably interact with the innermost chitobiose in *N*-glycans in only unfolded glycoprotein in the ERAD pathway, considering that chitobiose moieties are usually masked by the folded polypeptide.

## RESULTS

### SCF$^{Fbs}$ associates with the p97 complex

We have isolated Fbs1 from mouse brain cytosol as a novel sugar-binding protein that functions as a substrate-binding subunit in SCF-type E3 for ERAD (Yoshida *et al*, 2002), but the ubiquitination machinery for ERAD is probably associated with the ER membrane. To test the localization of Fbs1 proteins in mouse brain, we prepared anti-Fbs1 polyclonal antibodies. Lysates from adult mouse brain were fractionated into 100,000*g* supernatant (S) and precipitate (P) fractions excluding 24,000*g* precipitate, and the presence of Fbs1 was analysed by immunoblotting (Fig 1A). Fbs1 was detected in the P as well as the S fractions, suggesting that Fbs1 interacts with proteins that associate with the ER membrane. As p97/VCP is thought to be involved in the retro-transport of ERAD substrates (Tsai *et al*, 2002), we examined the interaction of Fbs1 with p97. As shown in Fig 1A, Fbs1 was co-immunoprecipitated with p97 from the P but not from the S fraction. To determine whether other F-box proteins interact with p97, the Flag-tagged F-box proteins were expressed, together with haemagglutinin (HA)-tagged p97, in 293T cells, immunoprecipitated and analysed by immunoblotting (Fig 1B). Both Fbs1 and Fbs2 but not βTrCP1, an F-box protein with WD repeats for substrate recognition, were co-immunoprecipitated with p97. These results suggest that a part of Fbs proteins binds specifically to the p97-containing complex at the ER.

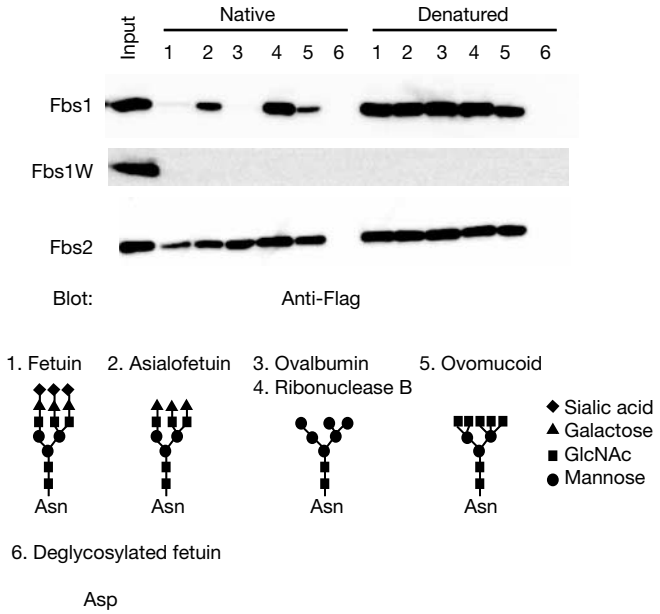


**Fig 2** | Pull-down analysis of the interactions of Fbs1, Fbs1W and Fbs2 with native and denatured *N*-glycoproteins. Extracts of cells expressing Flag-tagged Fbs1, the W280A mutant of Fbs1 (Fbs1W) and Fbs2 were incubated with native or guanidine-HCl-treated (denatured) glycoproteins (lanes 1–5) or deglycosylated fetuin (lane 6)-immobilized beads. The beads were washed and then boiled with sample buffer. Lysates (7.5 μg) and bound proteins were analysed by immunoblotting using anti-Flag antibody (top) and the structures of *N*-glycans in the glycoproteins tested are shown at the bottom.

## Fbs1 binds to integrin-β1 dependent on p97 activity

We identified pre-integrin-β1, which was modified with high-mannose oligosaccharides, as one of the Fbs1 substrates (Yoshida *et al*, 2002). As the Fbs1–pre-integrin-β1 interaction occurs in the cytosol, retro-translocation of integrin-β1 from the ER into the cytosol is required for Fbs1 binding. To analyse the involvement of p97 in the interaction between Fbs1 and pre-integrin-β1, 293T cells were transfected with expression plasmids encoding Flag-tagged Fbs1, V5-tagged integrin-β1 together with HA-tagged p97 or its mutant, and treated with the proteasome inhibitor MG132 for 1.5 h before collecting cells. Immunoprecipitates with anti-Flag antibody of S and P fractions of transfected 293T cells were immunoblotted with anti-V5 antibody to detect co-immunoprecipitated integrin-β1 (Fig 1C). Most of pre-integrin-β1 was localized in the P fractions, and the expression of the ATPase-defective p97 mutant (K524A) increased the amount of total pre-integrin-β1 in the P fraction (lanes 4–6). The amount of pre-integrin-β1 associated with Fbs1 was greater in wild-type p97-expressing cells than in mutant p97-expressing cells in both S and P fractions (lanes 7–12). These results suggest that Fbs proteins recognize and ubiquitinate pre-integrin-β1 retro-translocated by p97, and this modification may facilitate pre-intergrin-β1 binding to the p97–Ufd1–Npl4 complex, as well as its extraction from the ER.

## Fbs interacts with denatured *N*-glycoproteins

Glycoproteins retro-translocated from the ER are not native proteins. Therefore, to examine whether Fbs proteins recognize denatured glycoproteins better than native proteins, first we carried out a pull-down assay using several *N*-glycoproteins (Fig 2). We have reported that both Fbs1 and Fbs2 recognize the innermost chitobiose structure in high-mannose oligosaccharides (Yoshida *et al*, 2003). Although both ovalbumin and ribonuclease B (RNaseB) contain high-mannose oligosaccharides, Fbs1 effectively bound to RNaseB alone (see Discussion). Fbs1 could bind to asialofetuin and ovomucoid, but could hardly bind to fetuin and ovalbumin. The ability of Fbs2 to bind to asialofetuin, fetuin and ovomucoid was weaker than that to proteins attached to the high-mannose oligosaccharides. Interestingly, both Fbs proteins could bind to all the denatured *N*-glycoproteins tested but not to denatured deglycosylated proteins (lane 6), whereas the W280A mutant of Fbs1 (Fbs1W) that fails to interact with the innermost GlcNAc moiety in *N*-glycan (Mizushima *et al*, 2004) could not bind to any native or denatured glycoproteins, suggesting that the denaturation of glycoproteins increases the accessibility to the innermost chitobiose of *N*-glycans by Fbs proteins.

We next examined whether the binding potency and substrate specificity of Fbs proteins are influenced by denaturation of cellular glycoproteins. Lysates from the mouse brain or Neuro2a cells were treated with or without 6 M guanidine-HCl, diluted ten times with the lysis buffer and incubated with the His-tagged Fbs proteins produced by *Escherichia coli* (Fig 3; supplementary information 1 online). Guanidine-HCl at 0.6 M had no influence on Fbs binding to glycoproteins (supplementary information 2 online). The glycoproteins bound to Fbs were isolated using Ni-NTA affinity chromatography and detected by lectin blotting (Fig 3). Blotting with GNA, a lectin that binds to high-mannose oligosaccharide, showed that denaturation markedly increased the number of proteins bound to Fbs. The spectrum of Fbs1-bound protein bands in the brain detected by WGA, a lectin specific for terminal GlcNAc or sialic acids, was similar to those detected by GNA, suggesting that these proteins are modified by both high-mannose and complex-type oligosaccharides. Conversely, the proteins detected by RCA120, a lectin that binds to terminal galactose-β1-4GlcNAc, were different to those detected by GNA. Both the quantities and species of RCA120-reactive proteins recognized by Fbs1 were also considerably increased by denaturation. Treatment of denatured proteins with peptide: *N*-glycanase (PNGase F) almost diminished their binding to Fbs. Furthermore, Fbs1W could hardly bind these glycoproteins. These results strongly suggest that both Fbs1 and Fbs2 bind to the innermost GlcNAc moiety irrespective of the terminal sugar moieties, and that the accessibility of Fbs proteins to the innermost GlcNAc moiety is enhanced by denaturation of the substrate glycoproteins. As all *N*-linked oligosaccharides contain innermost chitobiose structure, Fbs proteins seem to be capable of binding most *N*-glycoproteins when denatured.

## $SCF^{Fbs1}$ ubiquitinates denatured glycoproteins

To see whether $SCF^{Fbs}$ ubiquitinates denatured glycoproteins more efficiently than native counterparts, we performed an *in vitro* ubiquitination assay using purified components including recombinant $SCF^{Fbs1}$ proteins. Efficient ubiquitination of GlcNAc-terminated fetuin (GTF), which is an *in vitro* substrate for $SCF^{Fbs1}$ (Yoshida *et al*, 2002), was detected by immunoblotting using an anti-fetuin antibody. When an excess amount of substrates existed, denatured asialofetuin was efficiently ubiquitinated,

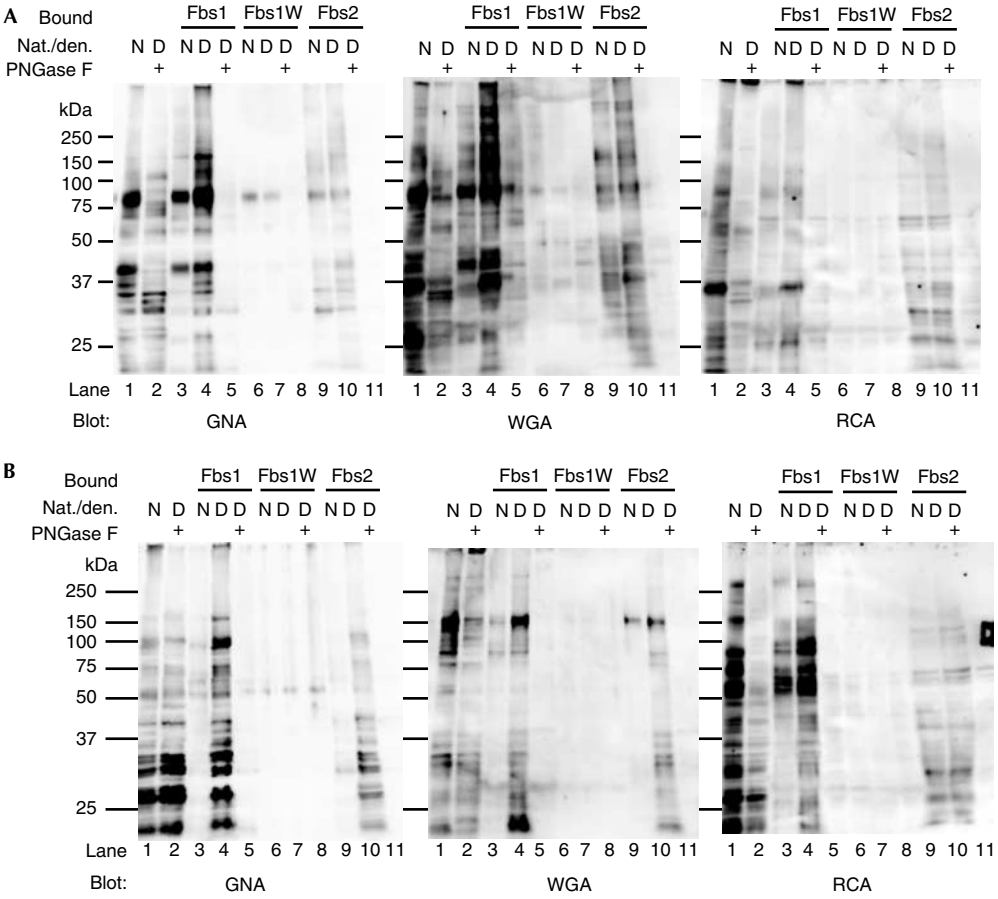


**Fig 3** | Interactions of Fbs1 and Fbs2 with cellular glycoproteins containing *N*-linked oligosaccharides in native and denatured states. Native (N), denatured (D) or PNGase-treated proteins prepared form mouse brain (**A**) or Neuro2a cells (**B**) were incubated with recombinant Fbs1-, Fbs1W- and Fbs2-immobilized beads. Native and PNGase-treated lysates (15 μg each; lanes 1 and 2) and proteins bound to Fbs1, Fbs1W or Fbs2 were analysed by lectin blotting using HRP-labelled GNA, WGA and RCA.

whereas ubiquitination of native asialofetuin was marginal (Fig 4; supplementary information 3 online). No ubiquitination of GTF or denatured asialofetuin was detected in the absence of E1, E2, ATP or substrate, and $SCF^{Fbs1W}$ (Mizushima *et al*, 2004) failed to ubiquitinate these substrates (supplementary information 4 online). These results demonstrate that the higher affinity of Fbs1 for denatured *N*-glycoproteins results in a more efficient ubiquitination of the denatured substrates than native counterparts.

## DISCUSSION

In the early secretory pathway, *N*-glycosylation facilitates conformational maturation by promoting the glycoprotein-folding machinery, and functions as tags for ER retention and targeting to the ERAD pathway. The calnexin–calreticulin cycle, consisting of two homologous lectins, calnexin and calreticulin, which interact with monoglucosylated *N*-glycans, in concert with UDP-glucose:glycoprotein glucosyltransferase (GT) and glucosidase II, has a central role in folding and ER retention. Conversely, it is shown that α-mannosidase I and EDEM have a pivotal role in selective disposal of misfolded glycoproteins (Ellgaard & Helenius, 2003; Yoshida, 2003). Among these oligosaccharide-related molecules in the ER, only GT has been shown to recognize incompletely folded proteins (Parodi, 2000). In the cytosol, the *N*-glycans in proteins extracted from the ER are removed before proteolysis by PNGase. In addition, PNGase can discriminate between non-native and folded glycoproteins, favouring the former (Hirsch *et al*, 2004). In this study, we showed that the Fbs proteins preferentially bind to denatured glycoproteins over properly folded proteins. As the retro-translocated proteins in the cytosol from the ER are misfolded, it is conceivable that *N*-glycan recognition proteins in the cytosol can sense misfolded states.

Although GT and PNGase can distinguish the folding states of substrates, the structural elements required for identification of their targets are not fully understood. *In vitro* studies have shown that GT also preferentially re-glucosylates glycoproteins in partially folded, molten globule conformations (Caramelo *et al*, 2003), and that an important feature for recognition is the exposure of hydrophobic clusters and innermost GlcNAc residue (Sousa & Parodi, 1995). Fbs1 interacts with the inner chitobiose in *N*-glycans of glycoproteins by a specific binding surface located at one tip of the β-sandwich of its substrate-binding domain (Mizushima *et al*, 2004). The intramolecular interactions of

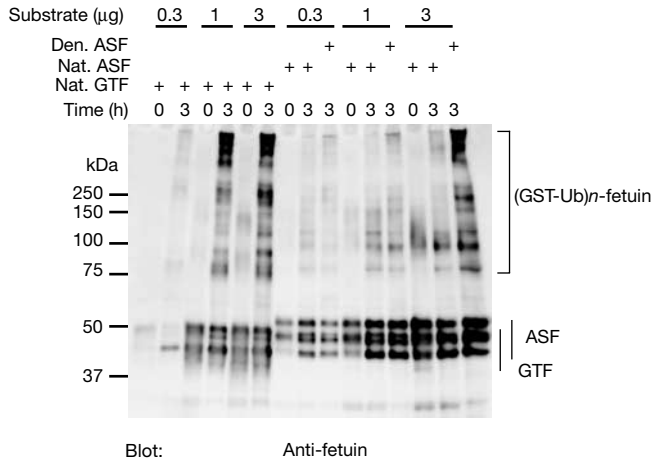


**Fig 4** | *In vitro* ubiquitination of native GlcNAc-terminated fetuin (GTF), asialofetuin (ASF) and denatured ASF by $SCF^{Fbs1}$ ligase. The high-molecular-mass ubiquitinated fetuin ((GST-Ub)*n*-fetuin) was detected by immunoblotting with anti-fetuin antibody.

innermost GlcNAc residue and the polypeptide moiety generally hamper the binding of Fbs1 to the chitobiose portions of glycoproteins as a result of steric hindrance in their native states. Therefore, Fbs1 recognizes the innermost position of *N*-glycans as a signal for unfolded glycoproteins. Conversely, as RNaseB contains an oligosaccharide that does not contact the polypeptide chain except at the covalent attachment point (Williams *et al*, 1987), it is likely that RNaseB interacts with Fbs1 even in the native form probably due to the exceptional freedom of the innermost chitobiose portion (Fig 2). The present results confirmed that Fbs proteins bind to denatured glycoproteins.

Considering that ubiquitination of ERAD substrates is linked to retro-translocation and rapid degradation, ubiquitin ligases for ERAD might be associated with the ER membrane. Moreover, as many glycoproteins are efficiently deglycosylated by PNGase after retrograde transfer into the cytosol (Blom *et al*, 2004), the rapid recognition of substrate by Fbs proteins before deglycosylation is critical. We found a part of Fbs proteins in association with p97 in the microsomal fractions, and extraction of the substrate of $SCF^{Fbs1}$ was dependent on the ATPase activity of p97 followed by association with Fbs1. Thus, $SCF^{Fbs}$ seems to be positioned in the ER membrane in such a way that it can ubiquitinate substrates immediately after retro-translocation to the cytosol.

Fbs proteins can bind not only high-mannose oligosaccharides but also various types of *N*-glycans in glycoproteins. These modified glycoproteins other than the high-mannose oligosaccharides are not ERAD substrates. Therefore, our finding suggests that $SCF^{Fbs}$ mediates ubiquitination of exogenous or membrane proteins endocytosed into the cells. This is not unusual, because it is well known that extracellular proteins incorporated by phagocytosis into dendritic cells are presented to MHC class I molecules after proteasomal degradation (Castellino *et al*, 2000). Other studies demonstrated the transfer of endocytosed proteins into the cytosol by unknown mechanisms before their proteasomal processing and/or destruction (Kovacsovics-Bankowski & Rock, 1995). Further functional analysis of Fbs family proteins can shed light on the degradation of endocytosed proteins.

## METHODS

**Transfection, plasmids, antibodies, immunoprecipitation and immunoblotting.** 293T cells were transfected as described previously (Yoshida *et al*, 2002). HA–p97-expressing plasmid was a kind gift from S. Khochbin (INSERM, France). HA–p97 (K524A)-expressing plasmid was constructed by site-directed mutagenesis. Human integrin-β1 complementary DNA was cloned from cDNA clone (ATCC 988953) in pTracer-EF-V5His vector (Invitrogen, Carlsbad, CA, USA). The anti-mouse Fbs1 serum was generated in rabbits by standard procedures using a synthetic peptide corresponding to residues 1–14 (MDGDGDPFSVSHPE) of the predicted protein coupled to keyhole limpet haemocyanin. Monoclonal antibodies to p97 and V5 epitope were purchased from Progen (Heidelberg, Germany) and Invitrogen, respectively, and polyclonal antibodies to calreticulin and GRP78 were from Affinity Bioreagents (Exeter, Devon, UK). Antibodies to Flag, HA and fetuin have been described previously (Yoshida *et al*, 2003). Immunoprecipitation from whole-cell extracts or subcellular fractionation of cells and immunoblotting were performed in TBS-T (1% Triton X-100, 50 mM Tris–HCl pH 7.5, 150 mM NaCl and protease inhibitors) as described previously (Yoshida *et al*, 2002).

**Pull-down assay.** Fetuin, asialofetuin, ovalbumin, RNaseB and ovomucoid were purchased from Sigma-Aldrich (St Louis, MO, USA). For preparation of deglycosylated fetuin (DGF), 10 mg of asialofetuin was incubated with 200 U of PNGase F (Roche, Mannheim, Germany) in 50 mM phosphate buffer, pH 7.2, at 37 °C for 24 h. The enzyme-treated proteins were loaded onto successive WGA and RCA-lectin agarose columns. The flow-through fraction from both columns was used as DGF. Each 10 mg glycoprotein was immobilized to 0.5 ml of Affi-gel 10 or 15 (Bio-Rad, Richmond, CA, USA). For preparation of denatured-glycoprotein-immobilized beads, after each half of glycoprotein-immobilized beads was incubated in 6 M guanidine-HCl for 2 h, the beads were washed five times with ten volumes of 20 mM Tris–HCl (pH 7.5)/150 mM NaCl (TBS) containing 0.5% NP-40 (TBS-N). Each cell extract prepared with TBS-N from Flag-tagged Fbs1, Fbs1 W280A mutant or Fbs2-expressing 293T cells (30 μg) was incubated with 15 μl of various glycoprotein-immobilized beads. Bound proteins were eluted by boiling with SDS sample buffer and were analysed by immunoblotting.

**Binding assay and lectin blotting.** The substrate-binding domain of mouse Fbs1 (117–297) and its W280A mutant were cloned into pET15b (Mizushima *et al*, 2004), that of mouse Fbs2 (46–295) was cloned into pET33b, and expressed in *E. coli*. The His-tagged Fbs proteins were bound to Ni-NTA agarose beads (Qiagen, Hilden, Germany). Mouse brains and Neuro2a cells were homogenized in TBS-N and protease inhibitors. After centrifugation of the homogenate at 15,000*g* for 30 min, guanidine-HCl was dissolved with one-third of the supernatant (protein concentration 5 mg $ml^{-1}$) up to 6 M. Guanidine-HCl-treated and untreated lysates were diluted ten times with TBS-N. Another aliquot was treated with PNGase F subsequent to denaturation by heating for 5 min at 100 °C in the presence of 1% SDS and was then diluted ten times with TBS-N. The dilutes and PNGase-treated lysates were precleared with Ni-NTA agarose and then the flow-through fractions were incubated with the Fbs-protein-bound beads for 18 h at 4 °C. The beads were washed with TBS-N containing 20 mM imidazole. The adsorbed proteins were eluted by 0.2 M

imidazole in TBS-N. Eluted proteins were separated by SDS–PAGE, and blotted onto a membrane (Immobilon). After the blotted membranes were blocked with 3% bovine serum albumin in PBS, lectin blotting was performed using horseradish peroxidase (HRP)-labelled GNA (EY Laboratories), RCA120 and WGA (Seikagaku-kogyo, Japan).

***In vitro* ubiquitination assays.** Preparation of GTF and *in vitro* ubiquitination assays were performed as described previously (Yoshida *et al*, 2002). Denatured asialofetuin was prepared by 200 times dilution of 20 mg ml$^{-1}$ asialofetuin treated with 6 M guanidine-HCl. Details of the assay condition are described in supplementary information 5 online.

**Supplementary information** is available at *EMBO reports* online (http://www.emboreports.org).

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