from RNase B by several oligosaccharides (Fig. 5) (7). All *N*-glycans containing a diacetylchitobiose structure with mannose residues, $Man_9GlcNAc_2$, $Man_8GlcNAc_2$, $Man_5GlcNAc_2$, and $Man_3GlcNAc_2$ were found to cause elution of Fbs2 from RNase B at similar concentrations (Fig. 5*A*, *lanes 1–16*). In addition, the presence of the asparagine residue did not affect the efficiency of the elution (*lanes 13–20*). However, the amount of Fbs2 eluted by $Man_5GlcNAc_1$ or chitobiose was 3–4 orders of magnitude lower than that by $Man_{3-9}GlcNAc_2$, indicating that both the inner diacetylchitobiose structure and terminal mannose residues are required for efficient Fbs2 binding (compare *lanes 13–16* with *lanes 21–28*). The addition of GlcNAc residues on mannose residues in $Man_3GlcNAc_2$ reduced $\sim 10^2$ the elution efficiency, and core-fucosylation caused further reduction (*lanes 9–12* and *lanes 29–34*, Fig. 5*A*). The tendency of elution of Fbs1 bound to RNase B by oligosaccharides was similar to that of Fbs2 (Fig. 5*B*). Compared with Fbs2, however, all oligosaccharides tested were found to elute Fbs1 at almost 10^2 lower concentrations. These data suggest that $Man_{3-9}GlcNAc_2$ is required for the efficient binding of both Fbs1 and Fbs2 and that the dissociation of Fbs2 from the *N*-glycans is lower than that of Fbs1.

Involvement of Fbs2 in the ERAD Pathway—To investigate the role of Fbs2 in the ERAD pathway, we used the TCRα subunit as a substrate for ERAD. TCRα is a type I glycoprotein attached to four *N*-glycans, and unassembled TCRα chains are degraded by proteasomes following their dislocation from the ER membrane (26). To examine whether the interaction of Fbs2 with TCRα mediates *N*-glycans, TCRα-expressing cells were labeled in the presence of tunicamycin, a glycosylation inhibitor, and immunoprecipitated. After tunicamycin treatment, 38-kDa glycosylated TCRα-HA protein shifted to 28 kDa. Full-length Fbs2 and ΔF could interact with glycosylated TCRα, whereas they failed to interact with deglycosylated TCRα (Fig. 6*A*).

We performed pulse-chase analysis using 293T cells co-expressing HA-tagged TCRα (TCRα-HA) and FLAG-tagged Fbs2 derivatives (Fig. 6*B*). Although wild-type Fbs2 did not influence the kinetics of TCRα degradation, co-expression of ΔF efficiently suppressed the decay of TCRα (Fig. 6*B*). To confirm the involvement of Fbs2 in the ERAD pathway, we performed further experiments aimed at reducing the level of endogenous Fbs2 using synthetic siRNA (Fig. 6, *C* and *D*). Although a nonspecific control duplex (designated *N* in Fig. 6, *C* and *D*) did not affect the expression of Fbs2, Fbs2-specific siRNA (285 or 754) decreased the Fbs2 expression (Fig. 6*C*). In the pulse-chase analysis of TCRα, although reduction of TCRα expression still occurred with siRNAs both specific and nonspecific to Fbs2, only Fbs2-specific siRNAs, not the nonspecific ones, decreased the rate of TCRα degradation (Fig. 6*D*). These results point to the involvement of Fbs2 in the ERAD pathway for misfolded and unassembled glycoproteins through its interaction with *N*-glycans.

DISCUSSION

Most proteins in the secretory pathway, such as membrane proteins and secretory proteins, are modified with *N*-glycans in the ER. In the early secretory pathway, *N*-glycosylation facilitates conformational maturation by promoting the glycoprotein-folding machinery consisting of two homologous lectins, calnexin and calreticulin, which interact with monoglucosylated *N*-linked core glycans in concert with UDP-glucose:glycoprotein glucosyltransferase (UGGT) and glucosidase II (10, 27). Recent studies suggest that mannosidase I and EDEM, an ER-degradation-enhancing α-mannosidase-like lectin, play a pivotal role in the selective disposal of misfolded glycoproteins (14–16) and that the misfolded proteins are accepted from calnexin by EDEM (28, 29). In the present study, we report that Fbs2, in addition to Fbs1, formed an SCF-type ubiquitin ligase that is responsible for the ubiquitylation of *N*-linked glycoproteins in the ERAD pathway. These findings indicate that the *N*-glycan of misfolded proteins functions as a covalent tag for recognition by not only folding but also by degradation machinery.

Our data indicate that both Fbs1 and Fbs2 recognize *N*-linked high mannose oligosaccharides, especially their internal diacetylchitobiose structure. In many native glycoproteins, the internal diacetylchitobiose is not accessible to macromolecules such as peptide *N*-glycanase (PNGase), and cleavage of oligosaccharides requires denaturation of the glycoproteins *in vitro*. On the other hand, it has been suggested that UGGT, which is responsible for re-glucosylation of the substrate so that it can reassociate with calnexin or calreticulin in the ER, recognizes both the innermost GlcNAc unit of the oligosaccharide and protein domains with hydrophobic patches exposed in the substrates (30). As it is well known that re-glucosylation by UGGT occurs only if the glycoprotein is incompletely folded, UGGT serves as a sensor for misfolded proteins in the ER (27). Because Fbs proteins are involved in the ERAD pathway, the inner chitobiose residues of the target glycoproteins for Fbs may be exposed to outside molecules generated through protein denaturation. Thus, it is possible that Fbs proteins recognize inner chitobiose in a manner similar to peptide *N*-glycanase or UGGT.

We also found that Fbs2 as well as Fbs1 could recognize not only high mannose oligosaccharides but also other type *N*-glycans (Fig. 4), suggesting the general role of Fbs-related SCF in glycoprotein clearance in the cytosol. It is also possible that these ubiquitin ligases mediate ubiquitylation of exogenous or membrane proteins endocytosed, but leaked in the cytosol, into the cells. This is not unusual, because it is well known that extracellular proteins incorporated by phagocytosis into dendritic cells are presented to major histocompatibility complex class I molecules after proteasomal degradation (31). There are also other studies that demonstrate that the transfer of endocytosed proteins into the cytosol by unknown mechanisms prior to their proteasomal processing and/or destruction is sensitive to proteasome inhibitors (32).

Although Fbs proteins recognize high mannose *N*-glycans, their affinities for oligosaccharides seem to be different. An overlay assay for the glycoproteins using labeled Fbs proteins revealed that the strength of the binding ability of Fbs2 was weaker than that of Fbs1 (Fig. 1). Furthermore, using several oligosaccharides from *N*-linked glycoproteins, we showed that the dissociation of Fbs2 was lower than that of Fbs1 (Fig. 5). Although the *in vitro* ubiquitylation of GTF by SCF^{Fbs1} and the interaction of glycoproteins with Fbs1 in the overlay assay were inhibited by chitobiose, the addition of chitobiose inhibited neither ubiquitylation by SCF^{Fbs2} nor the interaction with

Fbs2-specific siRNAs (siRNA$^{285-304}$ and siRNA$^{754-773}$, here named simply *285* and *754*, respectively) on the relative level of the Fbs2 transcript in 293T cells. mRNAs prepared from cells were converted to cDNAs, and then the same amount of each cDNA (as estimated with 25 cycles of PCR for the glyceroaldehyde-3-phosphate dehydrogenase (*G3PDH*) gene) was subjected to 35 cycles of PCR. *D*, effect of siRNA-Fbs2 on the stability of TCRα. TCRα-HA was co-transfected with an empty vector (*Vector*), 60 nM siRNA directed against Fbs2 (*285* and *754*), or 60 nM nonspecific siRNA (*N*). 293T cells were pulse-labeled with [^{35}S]Met/Cys and chased for the indicated times. The relative intensities were quantified as the plotted data to show the stability of TCRα over time.

Fbs2 (data not shown). In addition, whereas Fbs1 interacted strongly with pre-integrin β1 (7), Fbs2 did so only weakly (data not shown). On the other hand, Fbs2 bound strongly to TCRα, relative to Fbs1, in 293T cells, and the degradation of TCRα was more markedly suppressed by the dominant negative form of Fbs2 than by that of Fbs1 (Fig. 6, and data not shown). In addition to these properties, the Fbs2 transcript is widely expressed in various tissues in contrast to the limited expression of Fbs1 in the brain and testis (Fig. 2), suggesting that these Fbs proteins have distinct substrates or roles *in vivo*.

It has been reported that Fbs1 belongs to a subfamily consisting of at least five homologous F-box proteins (23). Among them, the FBG3 protein exhibits 75% identity with Fbs2, and the identity of Fbs1 and Fbs2 is similar to that of Fbs1 and FBG3 (23). Interestingly, *Fbs1*, *FBG3*, and *Fbs2* genes are located in tandem on chromosome (23), and the expression of FBG3 is observed ubiquitously but strongly in the brain and testis (data not shown). It is anticipated that FBG3 can recognize high mannose *N*-glycans because of their high homology. However, we could not detect any sugar-binding activity for FBG3 even though several assay systems were used in our studies. One possibility is that FBG3 suffers a negative modification that prevents it from accessing glycoproteins or that some modification of FBG3 is needed for the target recognition. Alternatively, this could be simply due to different substrate specificities. On the other hand, the expression of FBG4/Fbx17 and FBG5 transcripts is restricted, compared with those of Fbs2 and FBG3 (23), and the FBG4 protein shows a high homology with FBG5 (23), suggesting that they are another subfamily of F-box proteins that recognizes other sugar chains or other modifications.

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