

# Diversity in Tissue Expression, Substrate Binding, and SCF Complex Formation for a Lectin Family of Ubiquitin Ligases\*<sup>§</sup>

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Post-translational modification of proteins regulates many cellular processes. Some modifications, including *N*-linked glycosylation, serve multiple functions. For example, the attachment of *N*-linked glycans to nascent proteins in the endoplasmic reticulum facilitates proper folding, whereas retention of high mannose glycans on misfolded glycoproteins serves as a signal for retrotranslocation and ubiquitin-mediated proteasomal degradation. Here we examine the substrate specificity of the only family of ubiquitin ligase subunits thought to target glycoproteins through their attached glycans. The five proteins comprising this FBA family (FBXO2, FBXO6, FBXO17, FBXO27, and FBXO44) contain a conserved G domain that mediates substrate binding. Using a variety of complementary approaches, including glycan arrays, we show that each family member has differing specificity for glycosylated substrates. Collectively, the F-box proteins in the FBA family bind high mannose and sulfated glycoproteins, with one FBA protein, FBXO44, failing to bind any glycans on the tested arrays. Site-directed mutagenesis of two aromatic amino acids in the G domain demonstrated that the hydrophobic pocket created by these amino acids is necessary for high affinity glycan binding. All FBA proteins co-precipitated components of the canonical SCF complex (Skp1, Cullin1, and Rbx1), yet FBXO2 bound very little Cullin1, suggesting that FBXO2 may exist primarily as a heterodimer with Skp1. Using subunit-specific antibodies, we further demonstrate marked divergence in tissue distribution and developmental expression. These differences in substrate recognition, SCF complex formation, and tissue distribution suggest that FBA proteins play diverse roles in glycoprotein quality control.

Careful maintenance of properly folded glycoproteins is crucial for cellular homeostasis. Under physiological conditions, however, some glycoproteins fail to fold or assemble correctly

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and must be degraded (1, 2). Many secreted and membrane proteins are glycosylated in the endoplasmic reticulum (ER),<sup>2</sup> where correct biosynthesis of the nascent glycoprotein is aided by interactions with ER resident lectin-like proteins that bind glycan moieties on glycoproteins. ER resident lectins such as calnexin and calreticulin act as chaperones to ensure that nascent glycoproteins fold properly. Here we examine a novel group of lectin-like subunits of ubiquitin ligase complexes, the FBA family of F-box proteins, which have been proposed to target misfolded glycoproteins for degradation by the proteasome (3).

In the ER lumen, *N*-linked glycans are attached to newly synthesized proteins to facilitate proper protein folding and assembly (4–6). Nascent glycoproteins undergo successive rounds of folding and glycan trimming, during which ER resident lectins monitor their folding state (4, 5, 7). The retention of high mannose glycans on misfolded or unassembled glycoproteins serves as a key signal for recognition by ER lectins such as EDEM (8) and subsequent retrotranslocation from the ER and proteasomal degradation. This process is known as glycoprotein ER-associated degradation or GERAD (9).

The targeting of misfolded proteins to the proteasome is accomplished in part by ubiquitin ligases that recognize and ubiquitinate specific substrates (10). ER proteins marked for degradation by GERAD can be ubiquitinated through at least two pathways. One relies on the ER membrane-bound protein complex Hrd1/Der3 (11, 12), and the second relies on cytoplasmic ubiquitin ligases (13), including members of the large class of cytoplasmic ubiquitin-protein isopeptide ligases known as Skp/Cullin/F-box (SCF) complexes (14). SCF ubiquitin ligases contain three core components (Skp1, Cul, and Rbx1) and any one of dozens of F-box proteins (10, 15, 16). Through its substrate binding domain, the F-box protein confers substrate specificity onto the SCF complex in which it resides (15, 17, 18).

Although the human genome may encode over 500 ubiquitin ligases (14) and as many as 70 F-box proteins, only two F-box proteins have been shown to target glycoproteins in a lectin-like manner (3, 19). Both belong to the small family of F-box proteins known as the F-box associated (FBA) family. Intriguingly, FBA family members were recently shown to co-immunopre-

<sup>2</sup> The abbreviations used are: ER, endoplasmic reticulum; GST, glutathione *S*-transferase; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; IP, immunoprecipitation; PNGase, peptide:*N*-glycosidase; RFU, relative fluorescent unit; GN, glycan number; ConA, concanavalin A; AGP,  $\alpha$ 1-acid glycoprotein.

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**TABLE 1**

**Primers used in cloning FBA family and generating Null mutants**

Gene column lists the HUGO designation of the gene and reference GenBank™ accession numbers. Location refers to nucleotide in reference gene or, in the case of the Null mutants, the location from the FBXO start site. Expected products indicate length of gene cloned or, in the case of the Null mutants, the size of the entire plasmid obtained using QuikChange mutagenesis. F is forward, and R is reverse.

Gene	Forward and reverse primers	Location	Expected product
			<i>kb</i>
<i>hFBXO2</i>	F, GCTAAGCTTGATGGACGGAGACGGTGACCCAG	75–97	0.9
BC025233.1	R, CTGAATTCAGGGTTCACCCACACGCTGC	966–944	
<i>hFBXO6</i>	F, GGAGACAGCTTCAGGACACG	76–95	1
NM_018438.4	R, GACCCAGACACAGGATGGAC	1042–1023	
<i>hFBXO44</i>	F, AATTGCGGCCGCAAGAAGCCACAAGCCATGGCTG	113–133	0.8
NM_033182.5	R, CAACGGATCCTGTTTACCAGTCAGGGTTCAG	936–916	
<i>hFBXO17</i>	F, CGAATTCATCAAGCAAGGACTCTGG	105–123	0.9
NM_148169.1	R, GTCGGTACCCTAGGACAGACGGATCTCTG	968–951	
<i>hFBXO27</i>	F, CGAATTCATGGGGCGCTCGGTCTCC	81–100	0.9
BC014527.2	R, GGTAGATCTGGACTAGGACAGACG	939–922	
<i>mFbxo2</i>	F, GCTAAGCTTGGATGGAGATGGTGTATCCAGAGA	24–47	0.9
BC046586.1	R, CTATCGATGGTACCGTCCAGGGTTCACCCAC	916–900	
<i>mFbxo44</i>	F, GCTAAGCTTGGCAGTAGGCAACATCAACGAGC	168–191	0.6
BC028884.1	R, CTATCGATGGTACCGTACATGAGACCCCTCCC	803–786	
<i>mFbxo27</i>	F, GCTAAGCTTGGGTGCCGTGGATATCCAGGACCC	102–125	0.7
BQ922154.1	R, TCGATGGTACCGCTACTGCAGGTAGATATCATT	786–804	
<i>hFBXO2 Null</i>	F, CCGGGGGCAGGACTCCGTCGCGCTAAGGGCTGGTTCGGGGCCCGGGTG	812–860	6.8
	R, CACCCGGGCCCAACCAGCCCTTAGCCGGCAGCGAGTCTGCCCCCGG	860–812	
<i>hFBXO6 Null</i>	F, CAGCATGGGGCCATACCAGCCTGCAGCGGCTGGGTGTCCTGCCCCATGCTG	715–768	6.8
	R, GACTCGGGGCCATACCAGCCTGCAGCGGCTGGGTGTCCTGCCCCATGCTG	768–715	
<i>hFBXO44 Null</i>	F, GCACGGCCGGCTGGACACTCATGCCGCTGCCGCTGGTACCGGCCGAGGGTC	676–727	6.8
	R, GACCTCGGGCCGTACCAGCCGGCAGCGCATGAGTGTCCACGCCCCGCTGC	727–676	
<i>hFBXO17 Null</i>	F, CCGGAGAGACGTGAGTGCCTGGTGGGGCACTACGGCGC	752–790	6.8
	R, GCGCGTAGTGCCCAACCGCGGCTCAGTCTCTCCCG	790–752	
<i>hFBXO27 Null</i>	F, CGAACACCGGGCCAGGACACACAGCCCGGCTGGCCACTATGGAGCCCGTG	819–871	6.8
	R, CACGGGCTCCATAGTGCCAGCCCGGCTGTGTCTGGCCCCGGTGTTCG	871–819	

<sup>a</sup> All primers are presented 5' to 3'.

cupitate with Hrd1 from the ER membrane-bound protein complex, Hrd1/Der3 (20). Hence, this FBA family of ubiquitin ligases may serve multiple roles in GERAD.

The five F-box proteins comprising the FBA family are predicted to bind glycoprotein substrates through their conserved C-terminal domain, known as the FBA or G domain (21, 22). This domain is structurally homologous to the galactan binding domains of two other mammalian lectins, galectin (23) and PNGase F (24). The five FBA proteins, termed FBGs 1–5 by some researchers (19, 21), are formally designated by HUGO as FBXO2, FBXO6, FBXO44, FBXO17, and FBXO27, respectively (25). Two members of this FBA family, FBXO2 and FBXO6, have been shown to bind high mannose *N*-linked glycoproteins and function as ubiquitin ligase subunits (3, 19, 26–29). Whether the other members of the family share this lectin-like ubiquitin ligase activity is unknown.

The discovery that FBXO2 and FBXO6 recognize high mannose glycoproteins was an important advance in understanding the ubiquitin-proteasome components that mediate GERAD. But it is unknown whether the entire FBA family of F-box proteins participates in GERAD, or whether they all function as ubiquitin ligases. To answer these questions we examined the substrate binding, the ability to form SCF complexes, and the tissue distribution of all five FBA proteins.

## MATERIALS AND METHODS

### Cloning and Plasmid Constructs

Primers used in the construction of the human and mouse FBA clones are listed in Table 1.

**Human FBAs**—FBXO2 was amplified from IMAGE clone 5090925 and FBXO27 from IMAGE clone 3841901. FBXO6, FBXO44, and FBXO17 were cloned from heart and brain cDNA

libraries, a kind gift from Dr. Bento Soares (Northwestern University). All FBA clones were sequenced; any PCR mutations were corrected with QuikChange mutagenesis (Invitrogen) and the resultant cDNAs ligated into pFLAG-CMV-6b (Sigma).

**Mouse FBAs**—*mFbxo2* was cloned from IMAGE clone 6487759; *mFbxo44* was cloned from IMAGE clone 4165174, and *mFbxo27* was cloned from IMAGE clone 6468333.

*MFbxo6* and *mFbxo17* in pCMV-FLAG were a kind gift from Dr. Yukiko Yoshida (Tokyo Institute of Medical Science).

**GST Fusions**—Using primers in Table 1, human FLAG-FBA proteins were amplified and then subcloned into the polylinker of pET41C (Novagen, Madison, WI). FBXO2 was also cloned into pGEX-6P1 as described previously (27) to generate a cleavable GST fusion protein. All FBA clones were sequenced.

**Null Mutants**—Using primers listed in Table 1, GST-FBA fusions were constructed to replace two critical amino acids in the G domain hydrophobic pocket with alanines (Fig. 1, A and B). QuikChange mutagenesis was used with complementary primers spanning the insertion substitution sites.

### Cell Culture and Transfections

COS-7 cells (American Type Tissue Collection (ATCC), Manassas, VA) were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (Invitrogen). Cell lines were incubated at 37 °C, 5% CO<sub>2</sub>. For transient transfections, 80% confluent COS-7 cells were transfected with 6 μg of DNA/10-cm plate with Lipofectamine-Plus reagent (Invitrogen) in Opti-MEM (Invitrogen) according to the manufacturer's protocol for COS-7 cells. Co-transfections were performed as above with equimolar concentrations of DNA, and the total DNA did not exceed 6 μg of DNA/10-cm plate. For SCF complex isolation, equimolar amounts of FBXO and Skp1, Cull1,

and Rbx1 were transfected as described previously (30). Cells were harvested 48 h after transfection.

### Cell Lysates

Cells were rinsed with ice-cold PBS and harvested in Laemmli buffer (50 mM Tris, pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol). Cell lysates were then heated to 95 °C for 5 min, sonicated for 30 s, and centrifuged at  $16,000 \times g$ , 4 °C for 15 min to pellet debris. Nondenatured lysates were prepared by rinsing cells with PBS, incubating on ice with FLAG Lysis Buffer (FLB: 50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 containing EDTA-free protease inhibitors (Complete, Roche Applied Science) for 10 min, then scraping cells off the plate, and incubating cells on ice for a further 30 min, vortexing each 5 min. Lysates were cleared by centrifugation at  $16,000 \times g$  for 15 min.

### GST Fusion Purification

BL21(DE3)RP cells (Stratagene, La Jolla Ca) were transfected with the indicated GST fusion plasmids. Single colonies were grown overnight in LB broth with kanamycin (10 mg/liter) at 37 °C. The culture was diluted 1:50 in Terrific Broth-Kan, grown at 37 °C to an  $A_{600\text{ nm}} = 0.6$ , and then induced for 2.5 h with 0.4 mM isopropyl 1-thio- $\beta$ -D-galactopyranoside. The cells were pelleted and lysed for 30 min in 30 ml of BugBuster (Novagen)/500 ml of cell culture with protease inhibitors (Complete, Roche Applied Science), in accordance with the manufacturer's protocol. The supernatant was collected after centrifugation at  $16,000 \times g$  for 20 min at 4 °C. PBS-equilibrated glutathione-Sepharose 4B beads (1.0 ml slurry/500 ml cell culture) (Amersham Biosciences) were added, and the lysates were rocked for 30 min at room temperature. The beads were pelleted at  $500 \times g$  for 5 min and washed with 10 bed volumes of PBS. Purified GST fusion protein was eluted with 700  $\mu$ l of reduced glutathione (10 mM) for 10 min at room temperature. Size filtration was used to concentrate the GST fusion proteins using a Microcon YM-100 filter (Millipore, Bedford, MA) and resuspended in TSM buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM CaCl<sub>2</sub>, 2 mM MgCl<sub>2</sub>, 0.05% Tween 20). Protein concentration was determined at an  $A_{280\text{ nm}}$ , using absorbance of 1 = 0.5 mg/ml. Aliquots containing 25–100 mg of proteins were separated on SDS-polyacrylamide gels, transferred to polyvinylidene difluoride membranes, and stained with 0.8 mg/ml Direct Blue 71 (Sigma) in 40% EtOH, 10% acetic acid as described by Hong *et al.* (31). 600  $\mu$ g of purified GST-FBA protein was shipped on dry ice for analysis by the glycan array. Although multiple bands were observed on Direct Blue staining (supplemental Fig. 2A), the absence of any significant glycan binding to either GST alone or GST fusion null mutations demonstrates that the presence of truncated fusion proteins does not confound interpretation of the glycan array or pulldown results.

### Glycan Array

Because of variation in protein expression, we needed 1.0 liter of cell culture for FBXO2, 10 liters for FBXO6, FBXO27, and FBXO44, and 15 liters for FBXO17 for analysis by the Consortium for Functional Glycomics sponsored glycan array (32). Details of glycan array procedures and full data sets for all gly-

can array studies are available at the Consortium for Functional Glycomics web site. Purified proteins were added to the arrays at 30  $\mu$ g/ml in TSM buffer. FBXO2 cleaved from GST (27) was applied to printed array version 1 (PA\_v1). Rabbit anti-FBXO2 was used at 100  $\mu$ g/ml followed by goat anti-rabbit IgG-Alexa 488 antibody at 25  $\mu$ g/ml. GST-FBXO2, GST, and all other GST fusion proteins used goat anti-GST fluorescein isothiocyanate at 20  $\mu$ g/ml. GST alone was analyzed on plate array version 3.5. GST-FBXO2 was analyzed with PA\_v1. FBXO6 and FBXO17 were analyzed with PA\_v2.1. FBXO27 was analyzed with PA\_v2. Finally, FBXO44 was analyzed in both PA\_v1 and PA\_v2.1. Image intensities were detected with a ScanArray confocal scanner. After consulting with the consortium, and viewing previously generated data (available on the consortium web site), we set cutoff values to establish significance of binding. These values were set at 1,700 relative fluorescent units (RFUs) for the array used with FBXO2, and 15,000 RFUs for the later generation arrays used to assess FBXO6, FBXO44, FBXO17, and FBXO27 binding.

### Pulldown Assay

RNase B (R7884), AGP (G9885), lactoferrin (L9507), heparin sulfate (H4784), and chondroitin sulfate B (C3788) were purchased from Sigma. 10 mg of each glycoprotein was conjugated to 1 ml of Affi-Gel 10 (Bio-Rad). Denatured glycoprotein-conjugated beads were prepared by the addition of a 1:1 ratio of 6 M guanidine HCl and were rotated end over end at 4 °C for 2 h. Beads were subsequently washed five times with 10 bed volumes of TBS + 0.5% Nonidet P-40. 10  $\mu$ g of recombinant GST fusion protein was applied to 30  $\mu$ l of the different glycoprotein-conjugated beads. Samples were rotated at room temperature for 30 min, and beads were then transferred to a Handee Micro-Spin column (Pierce) and washed by spin filtration twice with 200  $\mu$ l of TBS + 0.5% Nonidet P-40. Bound GST fusion proteins were eluted with Laemmli buffer and incubated at room temperature for 15 min.

### Tissue Lysates

Unless otherwise specified, adult tissues were obtained from male and female mice at least 8 weeks old. Embryonic tissues at day 7 and 15 were processed from eight embryos. The animals were anesthetized with Xylocaine/procaine and perfused with cold PBS plus protease inhibitors. The organs were quickly removed, placed on ice, dissociated with a Dounce homogenizer on ice in 2  $\mu$ l of RIPA buffer/mg of dry weight with protease inhibitors. Denaturation of lysates was achieved by adding an equal volume of  $2 \times$  Laemmli buffer, heating the samples to 95 °C for 5 min, and pelleting debris by centrifugation at  $16,000 \times g$ , 4 °C for 15 min. Mouse tissue preparations from a commercial supplier (BioChain, Hayward, CA) produced similar results.

### Immunoprecipitation (IP)

Nondenatured lysates from 10-cm plates were incubated with 50  $\mu$ l of equilibrated FLAG-conjugated agarose beads (Sigma) for 1 h at 4 °C. The beads were pelleted by centrifugation for 30 s at  $8,200 \times g$  and washed four times with FLB. After the final wash, bound proteins were eluted from beads with

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Laemmli buffer without dithiothreitol. Beads were pelleted, and the supernatant containing the eluted proteins was resolved in 4–15% gradient SDS-PAGE (Bio-Rad).

For GST fusion IPs, recombinant GST-FBXO fusion proteins were purified from OverExpress C41 cells (Lucigen, Middleton, WI), an *Escherichia coli* strain selected for tolerance to toxic proteins, grown in MagicMedia (Invitrogen), and harvested as described above. Nondenatured lysates prepared from COS-7 cells were incubated with 50  $\mu$ g of purified GST fusion proteins and 15  $\mu$ l of glutathione-Sepharose beads for 30 min at room temperature. The beads were then transferred to Handee Micro-Spin columns, washed four times with 200  $\mu$ l of FLAG lysis buffer, and eluted with 30  $\mu$ l of Laemmli buffer at room temperature for 15 min.

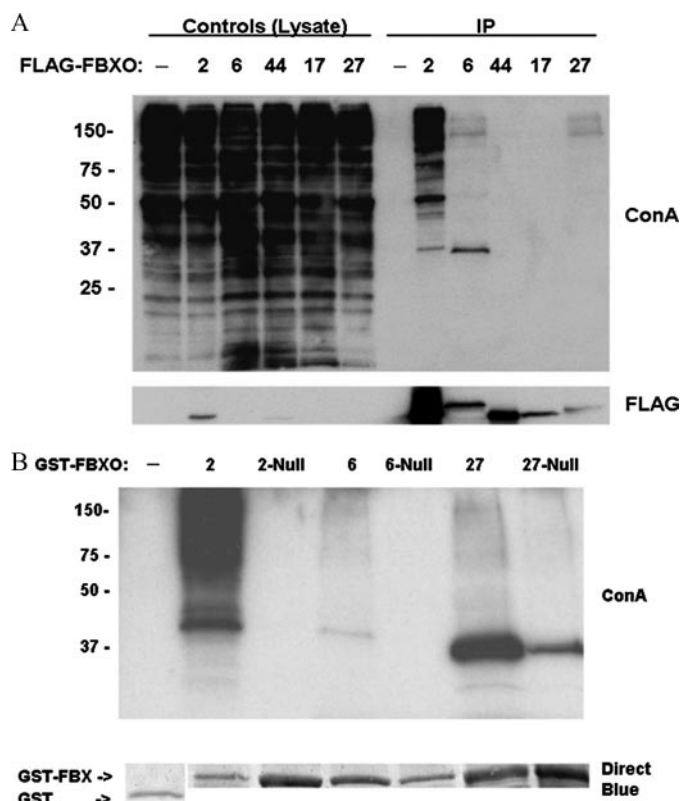
### Antibodies

FBA-specific antibodies were generated (Sigma Genosys) by inoculating New Zealand White rabbits with the following key-hole limpet hemocyanin-conjugated peptides. An underlined C indicates cysteine added for conjugation. Numbers in parentheses indicate amino acid position of the peptide in the mouse sequence. While this work was in progress a rabbit polyclonal antibody to FBXO2 was generated (29) using a sequence similar to that described below. The following peptides were used: Fbxo2 (1–19), MDGDGDPEVSHPEEASPEC; Fbxo6 (103–119), CKVETLPGSCGTSFPDNK; Fbxo44 (185–202), CHAPLGTFQPDPVMIQKKS; Fbxo17 (88–106) DADADGNRHDEF-PFCALAR; and Fbxo27 (8–25), TRVPTPEPDPQEVLDLSR.

For affinity purification, immune serum was incubated overnight at 4 °C with the appropriate peptide conjugated to agarose beads. Beads were then pelleted and washed with TBS (25 mM Tris, pH 7.4, 2.7 mM KCl, 14 mM NaCl) until the  $A_{280\text{ nm}}$  returned to base line. The purified antibody was eluted with four successive bead volumes of glycine, pH 2, and aliquots containing antibody were pooled. FBXO2 (1:5,000), FBXO6 (1:200), FBXO44 (1:500), FBXO17 (1:100), and FBXO27 (1:100) antibodies, pre-immune and pre-adsorbed serum (both used at 1:1,000) were diluted in 5% nonfat dry milk, and antibody specificity was confirmed with Western analysis (supplemental Fig. 3). FLAG M5 (1:1,000), mouse  $\alpha$ -tubulin (1:2,000), antibodies, and peroxidase-conjugated ConA (6  $\mu$ g/30 ml) were purchased from Sigma. Skp1 mouse monoclonal antibody (BD Transduction Laboratories, catalog number 610530) was used at 1:5000. Rbx1 used at 1:100 (NeoMarkers, catalog number RB-9287), and Cullin1 was used at 1:250 (Zymed Laboratories Inc., catalog number 71-8700).

### Western Blot Analysis

Samples were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes. Samples were blocked 1 h with 5% nonfat dry milk, and primary antibody was applied for 1 h at room temperature or overnight at 4 °C. The blots were washed four times with TBS containing 0.1% Triton X-100 and incubated with peroxidase-conjugated secondary antibody (goat anti-rabbit or goat anti-mouse, catalog number 1:15,000, both from Jackson ImmunoResearch, West Grove, PA) for 1 h at room temperature followed by four more washes as above.



**FIGURE 1. FBA proteins differ in their ability to bind high mannose glycoproteins.** *A*, FLAG-tagged FBA proteins expressed in COS-7 cells were immunoprecipitated from nondenatured lysates with anti-FLAG conjugated beads. Bound proteins were eluted, separated by SDS-PAGE, and probed with ConA, a lectin that binds high mannose glycans. *Left lanes* of each panel contain 10% of the lysate used for IP. *Upper panel* was probed with ConA and *lower panel* with anti-FLAG antibody. FBXO2 shows robust binding, FBXO6 and FBXO27 modest binding, and FBXO44 and FBXO17 no binding of ConA-reactive (*i.e.* high mannose glycan) glycoproteins. *B*, null binding mutants of GST-FBXO fusion proteins abrogate binding to ConA-reactive glycoproteins. Purified GST-FBXO fusion proteins were incubated with COS-7 cell lysate for 30 min. After pull-down with glutathione-Sepharose, bound proteins were eluted with Laemmli buffer and separated by SDS-PAGE. *Upper panel* was probed with ConA. *Lower panels* show pulled down GST and GST fusions stained with Direct Blue. GST-FBA proteins effectively bound ConA-reactive glycoproteins, and this binding was lost when two key hydrophobic residues in the G domain were mutated (null mutants).

## RESULTS

**Divergent Binding of High Mannose Glycans**—Previous studies have shown that FBXO2 and FBXO6 bind glycoproteins containing *N*-linked high mannose glycans (3, 23, 26, 29, 33, 34). This raises the following important question. Do all five FBA family members bind similar substrate proteins and thus, like FBXO2 and FBXO6, have the capacity to participate in GERAD? To begin addressing this question, we first tested the ability of all five full-length FBA proteins to co-immunoprecipitate high mannose containing glycoproteins.

As anticipated, proteins co-precipitating with FBXO2 stained robustly with ConA, a lectin that specifically recognizes high mannose glycans (Fig. 1*A*). Thus, many if not all proteins bound by FBXO2 are high mannose glycoproteins. As shown in Fig. 1*A*, FBXO2 is also expressed at higher levels than other FBA proteins, consistent with our previous report (27). Both FBXO6 and FBXO27 displayed much more modest co-precipitation of ConA-staining proteins. In contrast, FBXO44 and FBXO17 dis-

TABLE 2

## Summary of the diverse properties reported for the FBA family in this study

Binding of FBA proteins to high containing glycoproteins was determined by ConA blotting of proteins co-immunoprecipitated with FBA family members (+++ = strong, + = weak, and - = no high mannose glycan binding). Families of glycans showing significant binding to FBA proteins on glycan arrays are also indicated. Proteins bound in pull-down assays are as follows: B, RNase B; L, lactoferrin; H, heparin sulfate; D, denatured glycoprotein. Tissue distribution was determined by Western blot analysis of mouse tissues with FBA protein-specific antibodies (H, heart; B, brain; L, liver; M, muscle; K, kidney; P, pancreas; A, adipose; letters in parentheses indicate tissues with additional immunoreactive proteins of higher than expected molecular weight for the FBA protein). P<sup>1</sup> and A<sup>1</sup> indicate FBXO2 detected in pancreas and adipose on overexposed Western blots.

FBXO	ConA	Glycan array	Pull-down assay	FBA protein tissue distribution
2	+++	High mannose glycans	B, BD, L, LD	B, P <sup>1</sup> , A <sup>1</sup>
6	+	High mannose glycans ≈ glycoprotein > sulfated glycan	LD > L	(H, B), L, K (P)
44	-	None	None	B, L, P, A
17	-	Glycoproteins > sulfated glycans	L, LD, H, HD, >> B, BD	B
27	+	Glycoproteins	None	H, B, M

played little if any binding to high mannose glycoproteins, validating earlier work performed with full-length FBXO44 and a truncated form of FBXO17 (19). Similar results were also obtained when immunoprecipitations were performed in the presence of either the proteasome inhibitor lactacystin or benzoyloxycarbonyl-VAD-fluoromethyl ketone, an inhibitor of the cytosolic glycosidase PNGase F (data not shown) (35). Thus, the absence of robust high mannose glycoprotein binding by some FBA family members is not because of rapid proteasomal degradation of substrates or the removal of ConA-binding sites by PNGase F.

To further characterize the binding of FBXO2, FBXO6, and FBXO27 to high mannose-containing glycoproteins, we created GST fusion proteins of these three FBA family members. After large scale purification from a bacterial expression system, aliquots of these recombinant proteins were incubated with COS-7 cell lysates. The GST-FBA fusion proteins were then precipitated with glutathione-Sepharose beads and the eluents separated on SDS-polyacrylamide gels. As shown in Fig. 1B, proteins co-precipitating with these fusion proteins again stained robustly with ConA, demonstrating that FBXO2, FBXO6, and FBXO27 do not require eukaryotic post-translational modification to bind substrate. The prominent 37-kDa band found in the GST-FBXO27 pull-down is not consistently observed, and the continued presence of this band in the null mutant suggests that this binding may be nonspecific.

To verify the specificity of binding, we used site-directed mutagenesis on each FBA protein to replace two hydrophobic amino acids in the G domain shown to form the sugar binding pocket (23) (supplemental Fig. 1, A and B). These predicted "Null" mutants of FBXO2 and FBXO6 demonstrated a loss of binding to ConA-detectable proteins (Fig. 1B). FBXO27-Null showed reduced binding to ConA-detectable proteins; this residual binding suggests that other amino acids in its G domain may contribute to glycan binding (23). The loss of binding by Null mutants indicates that the binding of FBXO2, FBXO6, and FBXO27 to ConA-detectable proteins is specific and mediated primarily by these two hydrophobic residues in the G domain.

N-Linked glycosylation is not the sole means of glycosylation in the cell. Another important type, O-linked glycosylation, occurs on serine or threonine residues. Although no lectins specifically recognize O-linked glycans, two antibodies recognize a subset of O-linked glycoproteins containing O-GlcNAc. Using these antibodies, we did not detect immunoreactive bands co-precipitating with any of the five FBA proteins (data

not shown). This result suggests that the FBA family does not recognize O-GlcNAc glycans.

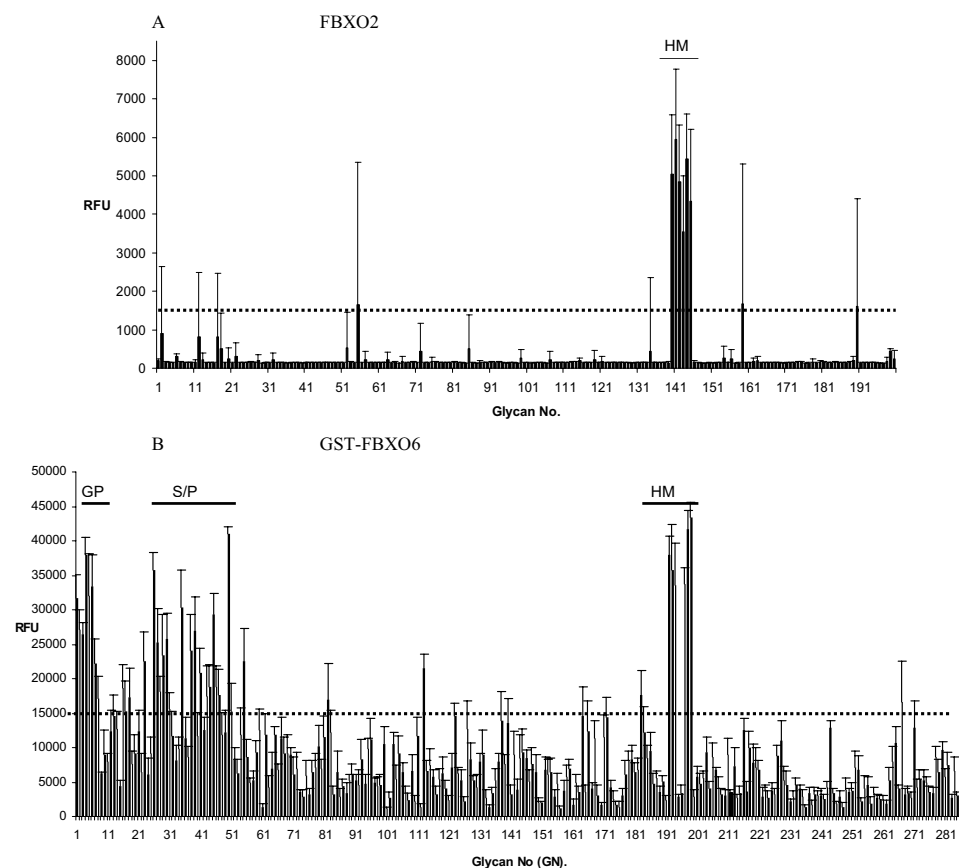
In summary, our ConA results suggest that FBA proteins differ in their affinity for glycoproteins decorated with high mannose glycans (Table 2), and that this affinity is largely determined by two hydrophobic amino acids in the G domain. The FBA proteins broadly can be separated into the following three classes: robust binding (FBXO2), moderate binding (FBXO6 and FBXO27), and little or no binding (FBXO44 and FBXO17) to high mannose glycans.

*Divergent Binding to Glycans*—Although the above results show that some FBA proteins co-precipitate high mannose glycoproteins, they do not demonstrate that these proteins have a specific and direct affinity for high mannose glycans, nor do they answer the question whether any FBA proteins can bind other glycans. A powerful way to assess affinity for a wide range of glycans is the glycan array developed by the NIH-sponsored Consortium for Functional Glycomics. Carbohydrates on the array are chemically synthesized without a protein backbone, allowing the affinity of FBA proteins for individual glycan units to be assessed independent of protein context. Binding affinities are reported as RFUs, and can be used to determine the relative binding affinity of a given FBXO protein for different glycans on the array. Individual cutoff values for significant binding were set as explained under "Materials and Methods." Glycan array experiments were performed using recombinant N-terminal GST-FBA fusion proteins except in the case of FBXO2, where GST was cleaved prior to final purification. Protein expression, confirmed by Direct Blue staining of purified fractions (supplemental Fig. 2A), revealed an overall purity of 50% full-length fusion proteins.

As shown in Fig. 2, supplemental Fig. 2, B and C, and Table 3, the five FBA proteins displayed diverse glycan binding. In contrast, GST as a negative control showed no affinity for any glycans on the array (supplemental Fig. 2B).

FBXO2 reproducibly and specifically bound high mannose glycans with high affinity (high RFU value; Fig. 2A). This result is consistent with our ConA results and previously published data (3, 19, 27, 29). FBXO2 bound avidly to Man5-chitobiose through Man9-chitobiose (glycan numbers (GNs) 140–145) but did not bind Man3, Man4, or Man5 alone (GNs 135–139) (Fig. 2A), demonstrating the importance of the chitobiose core. We also observed weaker binding to mannose 6-phosphate (GN 18). FBXO2 did not bind sulfated glycans (GNs 10–17, 19–22, 25–26) except for weak binding to 3'-sulfated neolactosamine (GN 12) and 4'-sulfated lactosamine (GN 17). Negli-

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**FIGURE 2. FBA proteins display diverse binding to glycans on arrays.** A–D, 600  $\mu$ g of recombinant FBA proteins were applied to glycan arrays, and binding affinity was assessed by measuring RFU. Bars represent mean RFU values, and error bars represent standard deviation ( $n = 4$ ). Dotted line indicates threshold for significant binding. A, FBXO2 specifically binds high mannose (HM) glycans. B, FBXO6 binds high mannose, sulfated/phosphorylated (S/P) glycans and a diverse set of glycoproteins (GP). C, FBXO17 binds select glycoproteins and individual sulfated/phosphorylated glycans. D, FBXO27 binds only select glycoproteins. FBXO44 failed to bind any glycans (supplemental Fig. 2C). All data used in generating graphs are available in the supplemental Table 1, A–E.

gible binding of FBXO2 was observed to the six glycoproteins included in the array (GNs 1–6). These glycoproteins possess a wide range of complex carbohydrates, including single and multiple, biantennary, and triantennary oligosaccharides (Table 3). Overall, the results demonstrate that FBXO2 specifically and directly binds a narrow set of glycans: of the  $\sim$ 200 sugars tested, high affinity binding was observed only for those glycans with a high mannose carbohydrate attached to a chitobiose core.

Using a newer version of the array, we found that FBXO6 binds a much broader range of glycans than FBXO2 (Fig. 2B). Like FBXO2, and consistent with our ConA results, FBXO6 bound high mannose glycans with relatively high affinity (GNs 194 and 197–199) but did not bind conformations of tri- or penta-mannose lacking the chitobiose core (GNs 189–191 and 195–196). In contrast to FBXO2, however, FBXO6 also bound with high affinity to all six glycoproteins (GNs 1–6) and to di-sulfated lactose and lactosamine families of glycans (GNs 26–30, 35, 38–39, and 45). FBXO6 also showed more modest binding to mono-sulfated sugars (GNs 31–34, 36–37, 42–43, 46–47, and 139–140) but negligible binding to the core lactose and lactosamine glycans (GNs 152–155) of these sulfated sugars, suggesting that FBXO6 binding is enhanced by sugar sulfa-

tion. Finally, mannose 6-phosphate was also bound by FBXO6 (GN 41).

In contrast to FBXO2 and FBXO6, FBXO17 did not bind high mannose glycans (GNs 192–194 and 197–199) (Fig. 2C). In many other respects, however, FBXO17 displayed similar binding properties to that of FBXO6. For example, it bound with high affinity to glycoproteins (GNs 1–6) and modest affinity to polysulfated glycans (GNs 26, 28, 29, 30, 34, 35, 38, 39, and 45). Unlike FBXO6, however, FBXO17 showed markedly reduced affinity for 6'-mono-sulfated glycans and negligible affinity for 4'-mono-sulfated glycans.

FBXO27 did not bind appreciably to any family of glycans on the array, including high mannose glycans (GNs 194 and 197–199), although it did bind a single isolated sulfated protein (GN 26) (Fig. 2D). The failure of FBXO27 to bind any other related sulfated glycans (GNs 27–30, 35, 38–39, and 45) suggests that this binding to a single sulfated protein may be nonspecific. FBXO27 did bind several glycoproteins containing a mixture of glycan moieties (GNs 1–6) (Table 3). The absence of binding to the high mannose glycans of the array differs from our earlier IP data for FBXO27

(Fig. 1), which showed weak co-precipitation of high mannose-containing glycoproteins. Thus, the IP results may reflect binding of FBXO27 to other glycans on co-precipitated proteins that coincidentally also contain high mannose groups.

Unique among FBA proteins, FBXO44 demonstrated no significant binding to any of the  $>$ 200 glycans on the array (supplemental Fig. 2C). Although we did not expect high mannose binding based upon our IP data (Fig. 1), we did not anticipate a complete absence of binding to any glycans in the array. Three separate preparations of recombinant FBXO44 protein from two different expression vectors gave the same negative results.

In summary, the array results reveal surprising diversity in the range of glycan binding by the FBA family of proteins (Table 3). FBXO2 and FBXO6 bound high mannose glycans. FBXO6, FBXO17, and to a lesser degree FBXO27 bound a set of glycoproteins with undefined glycan moieties containing complex oligosaccharides. FBXO6, and to a lesser degree FBXO17, also bound sulfated glycans, a class of potential target substrates not previously described for any ubiquitin ligases. The results suggest that FBXO2 and FBXO6 regulate high mannose glycoproteins; FBXO6 and FBXO17 regulate sulfated glycoproteins, whereas FBXO6, FBXO17, and FBXO27 may regulate certain complex glycoproteins. This diversity of glycan binding

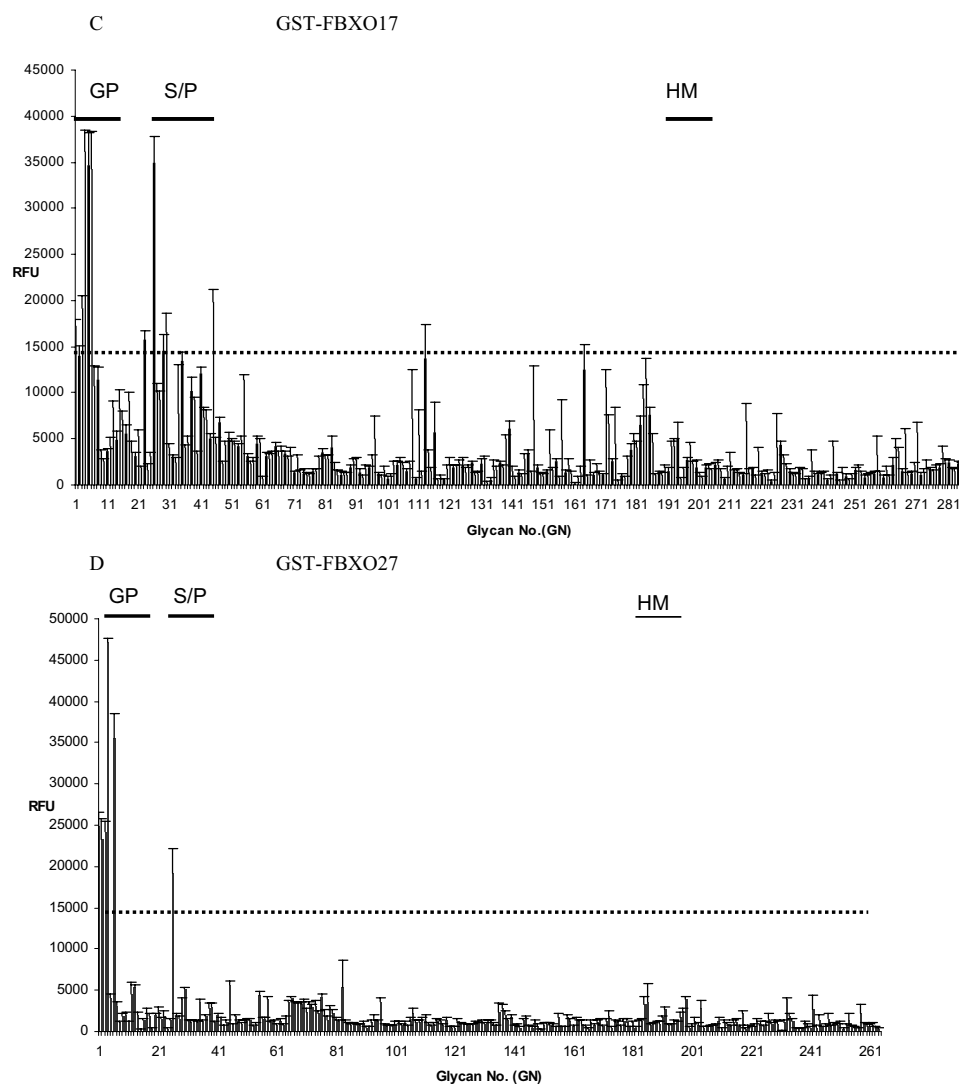


FIGURE 2—continued

expands the repertoire of potential substrates for this family of F-box proteins.

**Divergent Binding to Model Glycans and Glycoproteins**—The glycan array results demonstrated that members of the FBA protein family specifically and directly bind glycans. Although informative, glycan arrays are limited by the fact that each spot contains a single glycan homogeneously attached to an artificial matrix. *In vivo*, *N*-linked glycosylation is rarely complete and homogeneous; many glycoproteins contain multiple potential *N*-linked glycosylation sites that are partially occupied by a variety of oligosaccharides. To model FBA protein binding to actual glycoproteins, we selected potential candidate proteins that contain completely occupied *N*-linked sites with a fairly uniform set of oligosaccharides. Model glycoproteins selected for testing also possessed at least one glycan group to which one or more FBA proteins displayed significant binding on the glycan array.

To model high mannose glycan-containing glycoproteins, we chose bovine RNase B because it contains a single high mannose glycan (36). Because certain FBA proteins also bound some of the six arrayed glycoproteins containing heterogene-

ous, complex oligosaccharides (see Table 3), we also explored this binding, choosing two proteins: human  $\alpha$ 1-acid glycoprotein (AGP) and bovine lactoferrin. AGP, which on the array was bound with high affinity by three FBA proteins, contains a heterogeneous mixture of five complex glycans in multiple isoforms (37). Lactoferrin consists of two isoforms, each containing two high mannose glycans with variable additional complex glycans (38). To model sulfated glycans we chose heparin, a disaccharide repeat of GlcNAc and glucuronic acid (GlcUA) sulfated at the C-6 position and N position of GlcNAc, and at the C-2 position of IdoUA, the epimerized form of GlcA (39). Finally, to exclude the possibility that FBA protein binding to sulfated glycans on the array merely reflected nonspecific interactions with charged sulfates, we tested binding to a sulfated glycoprotein with a different glycan core, chondroitin sulfate. Chondroitin sulfate is a glycosaminoglycan with disaccharide repeat of GalNAc and GlcUA, sulfated at the C-4 and C-6 positions of GalNAc and at the C-2 position of IdoUA, again the epimerized form of GlcA (40).

We tested each individual FBA protein against this battery of test glycoproteins (Fig. 3A). These

model glycoproteins were attached to Affi-Gel beads and incubated with GST-FBA fusion proteins, and Affi-Gel bound fractions were eluted. Only binding by the uppermost GST-FBA band is reported as it represents full-length fusion protein. Because the natural substrates for FBA proteins may be misfolded glycoproteins in which the chitobiose core is more accessible (19), we also simulated the unfolded state by denaturing aliquots of immobilized test glycoproteins and assessing FBA protein binding.

FBXO2, as expected, bound both high mannose-containing model glycoproteins, RNase B and lactoferrin. Denatured and native forms of RNase B were bound equally well, whereas denatured lactoferrin bound slightly more FBXO2 than native lactoferrin. This result suggests that glycoprotein denaturation may enhance FBXO2 binding by fully exposing the chitobiose core (19). FBXO2 did not bind test proteins containing sulfated glycans, underscoring its narrow binding profile for high mannose glycans.

Unexpectedly, FBXO6 displayed very low affinity for all test glycoproteins, binding only modestly to lactoferrin and negligibly to RNase B. This lack of binding suggests that the affinity of

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**TABLE 3**

**Identity of glycans/glycoproteins bound by FBA family on glycan arrays**

Two FBA proteins bound six substrates (FBXO2 and FBXO27), and two FBA proteins bound 15 substrates (FBXO6 and FBXO17). Substrates fall under three distinct motifs: high mannose glycans, sulfated glycans, and glycoproteins. Other motifs present on the array, such as terminal sialic acids or Lewis structures, were not bound by the FBXO proteins. FBXO44 and GST (not shown) did not bind any substrates on arrays. Substrates are listed from highest (RLU value) to lowest significant binding. Ceruloplasmin is a serum copper-binding protein containing three complex glycans/molecule, two biantennary glycans, and one triantennary glycan (68, 69). Transferrin is a serum iron-binding protein containing four complex chains, three biantennary glycans, and one triantennary glycan (70, 71). Fibrinogen is a serum precursor of fibrin containing three pairs of  $\alpha$ ,  $\beta$ , and  $\gamma$  chains with a single complex biantennary glycan on both of its  $\beta$  and  $\gamma$  chains (72). AGP is an acute phase reactant serum protein containing a heterogeneous mixture of five complex glycans (37). Although they do not contain high mannose sugars, isoforms of AGP can be separated based on their retention on a ConA column (AGP-A does not bind the column and AGP-B is delayed on the column).

Binding Affinity	FBXO2	FBXO6	FBXO17	FBXO27	FBXO44
Higher ↑ Lower	Man5-9mix-N	Man5-9mix-N	Ceruloplasmin	Ceruloplasmin	
	Man8-GlcNAc $\beta$ 1-4GlcNAc $\beta$ -N	Man5-GlcNAcb1-4GlcNAcb-N	[3OSO3][6OSO3]Galb1-4[6OSO3]GlcNAcb	Transferrin	
	Man5-GlcNAcb1-4GlcNAcb-N	Man3-GlcNAcb1-4GlcNAcb-G	Fibrinogen	$\alpha$ 1-acid glycoprotein (AGP)	
	Man6-GlcNAc $\beta$ 1-4GlcNAc $\beta$ -N	Ceruloplasmin	Transferrin	AGP-B	
	Man9-GlcNAc $\beta$ 1-4GlcNAc $\beta$ -N	Man7-GlcNAcb1-4GlcNAcb-N	[6OSO3]Galb1-4[6OSO3]Glc	AGP-A	
	Man7-GlcNAcb1-4GlcNAcb-N	Man8-GlcNAcb1-4GlcNAcb-N	AGP-B	[3OSO3][6OSO3]Gal1-4[6OSO3]GlcNAc	
		[3OSO3][6OSO3]Galb1-4[6OSO3]GlcNAcb	$\alpha$ 1-acid glycoprotein (AGP)		
		Man6-GlcNAcGlcNAc-N	[3OSO3]Gal $\beta$ 1-4(6OSO3)Glc $\beta$		
		Man9-GlcNAcGlcNAc-N	b-GlcN(Gc)-		
		Transferrin	[3OSO3]Gal1-4(6OSO3)Glc $\beta$		
		Fibrinogen	AGP-A		
		$\alpha$ 1-acid glycoprotein (AGP)	Gala1-6Glc		
		[3OSO3]Galb1-4[6OSO3]GlcNAcb	[3OSO3]Galb1-4[6OSO3]GlcNAcb		
		[6OSO3]Galb1-4[6OSO3]Glc	GlcNAc $\beta$ 1-3Gal $\beta$ 1-4GlcNAc $\beta$		
		AGP-A	6-H2PO3Mana		

FBXO6 for a single high mannose glycan on a protein may be low. Lectins generally bind ligands with low affinity, and increasing the number of binding sites on a substrate (multivalency) can increase binding avidity by 1–3 orders of magnitude (41). Hence, FBXO6 may bind lactoferrin via its multiple high mannose sites but lack the ability to bind the single high mannose site present on RNase B. Alternatively, because FBXO6 bound both complex and high mannose glycans on the array (Fig. 2B), FBXO6 may bind lactoferrin through its complex glycans. When lactoferrin was denatured to fully expose the chitobiose core, FBXO6 binding was enhanced, highlighting the importance of the chitobiose core to binding. Although FBXO6 bound di- and monosulfated glycans on the glycan array, it failed to bind the primarily tri-sulfated heparin and chondroitin sulfate, suggesting that polysulfation could inhibit FBXO6 binding.

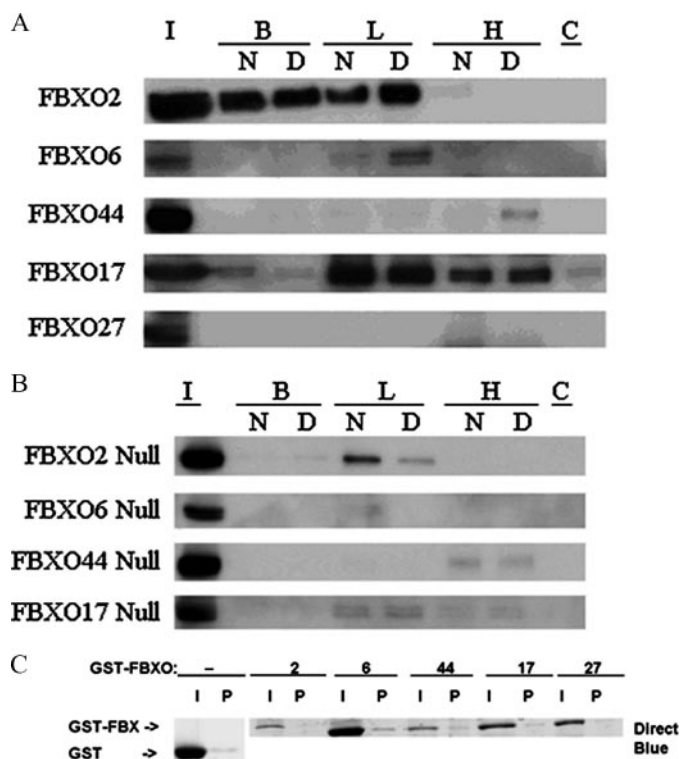
FBXO17 bound heparin strongly and chondroitin sulfate weakly, suggesting that FBXO17 binds sulfated glycans. The

preferential binding of FBXO17 to heparin may be due to differences in sulfation or in the underlying core sugars. FBXO17 bound strongly to lactoferrin but minimally to RNase B. Because FBXO17 did not bind high mannose glycans on the glycan array, its binding to lactoferrin likely reflects binding to the complex glycan moieties on the protein.

Consistent with the glycan array results, FBXO27 and FBXO44 did not bind test glycoproteins in our pulldown experiments. Weak binding of FBXO44 to denatured heparin was observed, but as discussed below, this binding may be nonspecific. These results suggest that these two FBA proteins may have a very limited set of target glycoproteins.

In these assays we focused on the binding of full-length recombinant GST-FBA proteins, avoiding any potential contributions from truncated fusion protein products. The results, which are generally consistent with our glycan array findings, again highlight the apparent diversity of glycan binding by this family of proteins.

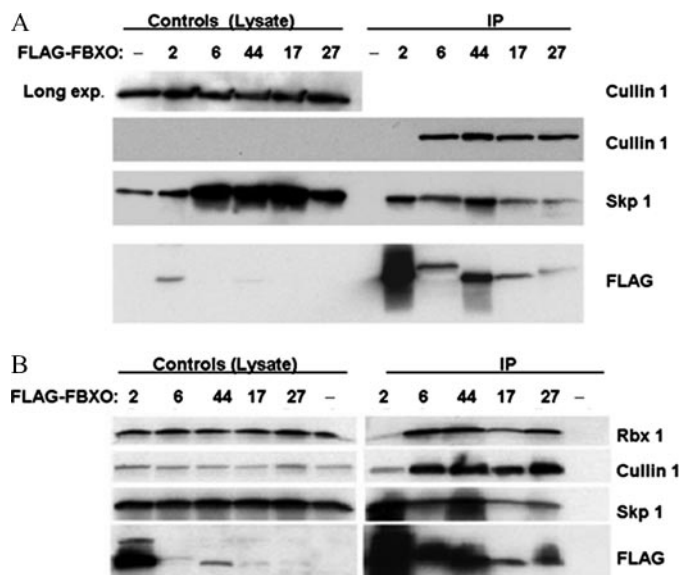




**FIGURE 3. FBA protein binding to candidate glycoproteins and glycans.** *A* and *B*, immobilized glycan and glycoprotein pull-down assays (*I* = 50% input, *B* = RNase B, *L* = lactoferrin, *H* = heparin, *C* = chondroitin, *N* = native glycoproteins/glycans, *D* = glycoproteins/glycans denatured with guanidine HCl). *A*, FBA proteins show diverse binding to model glycans/glycoproteins immobilized on beads. *B*, null mutants, with double alanine substitutions in the FBA hydrophobic pocket, show markedly diminished glycan/glycoprotein binding. *C*, 100  $\mu$ g of recombinant GST-FBA proteins or GST alone were incubated with AGP-conjugated beads for 30 min and pelleted with mild centrifugation. GST or GST fusion proteins were eluted in Laemmli buffer. (*I*, 50% input; *P*, pellet eluted fraction.) None of the FBXO protein bound significantly to AGP.

*A Hydrophobic Pocket in the G Domain Is Necessary for Glycoprotein Binding by FBA Proteins*—The structure of FBXO2 suggests that it binds the chitobiose core of glycans via a hydrophobic pocket formed by two consecutive amino acids containing aromatic rings, tyrosine and tryptophan (Tyr-278 and Trp-279 in the human FBXO2 sequence) (supplemental Fig. 1A) (23, 33). Because of the high degree of amino acid identity among the G domains of the FBA family members (supplemental Fig. 4), we hypothesized that all five bind glycans in a similar manner requiring this hydrophobic pocket. To test this hypothesis, we mutated these two critical amino acids to alanine in the four family members that were shown to bind glycoproteins (FBXO2, FBXO6, FBXO17, and FBXO44) (supplemental Fig. 1B). As can be seen in Fig. 3B, these double alanine substitutions markedly reduced binding in the pull-down assays. Thus the hydrophobic pocket formed by these adjacent amino acids appears to be essential for core glycan binding by all FBA family members. The nearly complete elimination of glycan and glycoprotein binding by alanine substitutions in just two amino acids in the G domain suggests that the G domains assume a highly conserved structure that binds core glycans in a similar fashion for all FBA proteins.

Finally we tested binding of all FBA proteins to AGP because FBXO6, FBXO17, and FBXO27 all bound AGP on the arrays.



**FIGURE 4. FBA family members differentially bind components of the SCF complex.** *A* and *B*, COS-7 cells were transfected with plasmids encoding the indicated FLAG-tagged FBA proteins or empty vector (*A* was first probed with ConA in Fig. 1A and then stripped and probed as described below) or were also co-transfected with plasmids encoding Rbx1, Cullin1, and Skp1 (*B*). After affinity purification of FLAG-tagged FBA proteins, immunoprecipitated proteins were probed on immunoblots with antibodies to RBX1, Cul1, Skp1, and FLAG as indicated. *Controls* represent 10% of the lysate used in IP. *A*, all FBA proteins bound Skp1, and all except FBXO2 bound Cul1. (Cul1 levels are low in COS-7 cells but are present in overexposed control lysates, *Long exp.*). *B*, whereas all components of the SCF complex co-IP with each FBA protein, FBXO2 binds much less Cul1 and Rbx1 than it does Skp1.

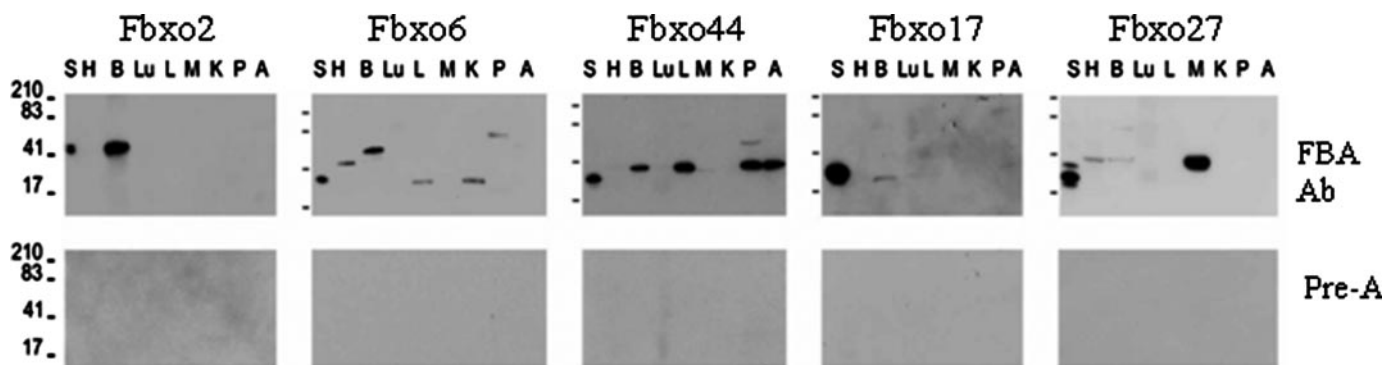
Unexpectedly, however, none of the FBA proteins were pulled down significantly by immobilized AGP (Fig. 3C). The lack of binding may be due to differences in glycoprotein attachment or presentation between the Affi-Gel beads and the array matrix. This negative result highlights the importance of confirming glycan array data by additional methods, including pull-down assays.

*Divergent Binding of FBA Proteins to Components of the SCF Complex*—As the substrate recognition subunits of multiprotein ubiquitin ligase complexes, F-box proteins have no intrinsic catalytic activity of their own. For F-box proteins, the canonical ubiquitin ligase is the assembled SCF complex (for Skp1, Cul1, F-box and Rbx1). The F-box domain interacts directly and primarily with Skp1 but also forms two hydrogen bonds with Cul1 (42). Cul1 is a large bridging protein that interacts with Skp1 through its N-terminal region and with Rbx1 through its C terminus. When an SCF complex binds a substrate protein, a ubiquitin-conjugating enzyme associates with the complex via Rbx1 and ubiquitinates the substrate protein.

To examine the ability of all five FBA proteins to associate with SCF complexes, we first performed immunoprecipitations after transfecting COS-7 cells with constructs encoding FLAG-tagged FBA proteins. All FBA proteins co-immunoprecipitated endogenous Skp1 (Fig. 4A). Although FBXO6, -44, -17, and -27 also co-immunoprecipitated Cullin1, FBXO2 failed to do so.

Very little Cullin1 was detected in our COS-7 cells (Fig. 4A), although a band was observed upon longer exposure in the *Control Long exp. lanes*. To ensure that the lack of FBXO2 binding to Cullin1 was not due to reduced levels of SCF components

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**FIGURE 5. Differential expression of FBA proteins in mouse tissues.** Lysates (50  $\mu$ g) from indicated mouse tissues were separated by SDS-PAGE and probed with each FBA protein-specific antibody (*top panels*) or pre-adsorbed antibody (*Pre-A, bottom panels*). At least one FBA protein is expressed in every tissue examined, and many tissues express several FBA proteins *H*, heart; *B*, brain; *Lu*, lung; *L*, liver; *K*, kidney; *M*, muscle; *P*, pancreas; *A*, adipose tissue; *S*, positive control (lysates from COS-7 cells transfected to express the indicated target FBA protein).

in COS-7 cells, we co-transfected COS-7 cells with plasmids expressing FLAG-tagged FBA proteins and the other three proteins of the SCF complex and then assessed complex formation by co-IP. All FBA family members co-immunoprecipitated components of the SCF complex (Fig. 4*B*), suggesting that they can assemble into an F-box ubiquitin ligase complex. However, not all FBA proteins co-precipitated SCF components identically. Although the amount of co-precipitated Skp1 correlated directly with the input for each FBA protein, FBXO2 bound far less Cul1 and Rbx1 than the other four FBA proteins. These results are consistent with recent work suggesting that FBXO2 exists primarily as a heterodimer with Skp1 in some tissues, such as the inner ear organ of Corti (30, 43, 44). This variation in Cul1 binding likely results from differences in the F-box amino acid sequence (supplemental Fig. 4) and the linker sequence between the F-box and G domain (45).

**Expression of the FBA Gene Family**—The emergence of the FBA family in higher metazoans (supplemental Fig. 5), together with the evidence presented above, suggests an expansion of functional diversity during evolution. Levels of high mannose, complex, and sulfated glycoproteins vary in a tissue-specific manner. Accordingly, FBA family members might display tissue-specific distributions reflecting differences in glycoprotein distribution. To address this possibility, we analyzed the expression of the entire FBA protein family by Western blot.

For the five FBA proteins, antibodies have only been reported against mouse Fbxo2 (Fbxo and FBXO refer to mouse and human FBA proteins, respectively) (29, 46). Rabbit polyclonal antibodies were thus generated against peptides specific for each mouse FBA protein (supplemental Fig. 3) and used to examine the tissue distribution for each FBA protein in adult mouse tissues (Fig. 5).

A strong immunoreactive protein of the correct molecular weight for Fbxo2 was expressed only in brain. On overexposed blots a faint signal was also detected in pancreas and adipose tissue (data not shown), suggesting weak Fbxo2 expression in these tissues (19, 27, 46).

An immunoreactive protein of the expected molecular weight for Fbxo6 was expressed in liver and kidney. The heart, brain, and pancreas contained immunoreactive bands of higher molecular weight. These bands could reflect post-translational modifications, higher molecular weight isoforms as noted for

other F-box proteins (47), or antibody cross-reactivity to unrelated proteins.

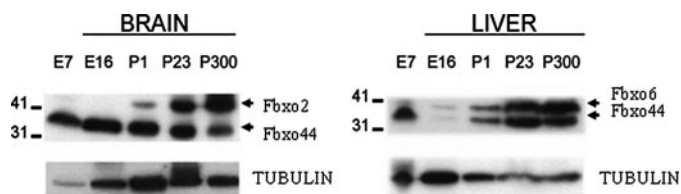
Fbxo44 was expressed in diverse tissues, including brain, liver, pancreas, and adipose tissue, with weaker signals seen in other tissues after longer exposures. The major identified protein migrated  $\sim$ 4–8 kDa larger than full-length Fbxo44 expressed in transfected COS-7 cells. However, when recombinant Fbxo44 was expressed in a murine hepatic cell line, it displayed the same apparent molecular weight as seen in tissue lysates (data not shown). This cell-specific difference in molecular weight may reflect a post-translational modification.

Like Fbxo2, Fbxo17 was primarily expressed in brain, where a single immunoreactive protein of the correct predicted molecular weight was detected. Much weaker Fbxo17 expression was also observed in lung.

The final FBA protein, Fbxo27, was expressed in muscle, heart, and brain. Endogenous Fbxo27 electrophoresed as a slightly larger protein than the truncated mouse Fbxo27 protein used as an antigenic marker.

Our expression analyses of the five FBA proteins yielded results similar, but not identical, to previously published mRNA expression analyses for the FBA genes (19, 21). The differences may reflect cross-reactivity of the nucleic acid probes or antibodies, weak antibody detection at low protein expression levels, or tissue-specific differences in post-transcriptional regulation, as reported for many other ubiquitin ligases (48–53).

In summary, the expression patterns for the FBA proteins differ substantially. Contrary to earlier mRNA studies, at least one FBA protein is recognized by our polyclonal antibodies in every tissue we examined, and many tissues express several FBA proteins. This divergence in tissue distribution may reflect the need for different FBA proteins to regulate the cellular glycome in a tissue-specific manner. For instance, major glycoprotein-producing tissues include the brain, liver, and pancreas, all of which express multiple FBA family members. In contrast, production of sulfated, galactose-terminated glycoproteins is particularly robust in the brain (54–56). Fbxo6 and Fbxo17, the only two FBA proteins that bind sulfated, galactose-terminated glycoproteins based on our analysis (Tables 2 and 3), are both expressed in brain.



**FIGURE 6. Divergent FBA protein expression during mouse development.** Lysates (25  $\mu$ g) from whole embryo at embryonic day 7 (E7), mouse brain or liver at embryonic day 16 (E16), or postnatal days 1, 23, or 300 were separated on SDS-polyacrylamide gels and probed with the indicated antibodies. Fbzo2 is predominantly expressed in adult brain, whereas Fbzo44 is maximally expressed in brain during embryonic development and later declines. In liver, Fbzo44 and Fbzo6 are coordinately expressed throughout development.

*Developmental Expression of the Fbzo2/Fbzo44/Fbzo6 Gene Cluster*—Because many glycoproteins and genes involved in glycoprotein synthesis are developmentally regulated, we wondered whether the expression of FBA family members likewise is developmentally regulated. To assess developmental expression, we chose brain and liver as target organs because each tissue expresses at least two FBA family members and supports robust production of glycoproteins. Developmental expression was examined for the three genes comprising the gene cluster on mouse chromosome 4 (*Fbzo2/Fbzo6/Fbzo44*), which is syntenic with the chromosome 1 cluster in humans. For this analysis, we used entire mouse embryo extracts at embryonic day 7 and brain or liver tissue isolated from embryonic day 16 through various postnatal ages.

The results reveal marked differences in the developmental expression of the three genes comprising this cluster (Fig. 6). *Fbzo44* was expressed abundantly, *Fbzo6* weakly, and *Fbzo2* not at all in lysates from whole embryos. In brain, Fbzo2 protein was expressed predominantly in adult tissue and was absent from embryonic tissue, consistent with earlier Northern blot results (19). In contrast, *Fbzo44* was maximally expressed in brain during embryonic development but declined thereafter. In the developing liver, however, *Fbzo44* and *Fbzo6* were coordinately expressed, increasing in parallel during development (Fig. 6). The differing patterns of developmental and tissue-specific expression for the three FBA genes comprising this gene cluster parallel the similar, divergent patterns observed for glycosyltransferases involved in glycoprotein synthesis (57–60).

## DISCUSSION

Among F-box ubiquitin ligase subunits, the FBA family displays a unique ability to bind glycoproteins. Our results reveal marked functional differences among the FBA proteins, beginning with differing affinity for high mannose-containing glycoproteins. Subsequent glycan array analysis demonstrated wide divergence in the glycans bound by individual FBA proteins; some FBA proteins only bound high mannose glycans whereas others bound a wider range, including complex, sulfated glycans. Guided by the array results, we further examined the ability of FBA proteins to bind model glycans in the context of native or denatured glycoproteins. This approach, coupled with site-directed mutagenesis, revealed that glycan binding requires the conserved hydrophobic pocket within the FBA domain. We also uncovered unexpected variability in the ability

of FBA proteins to assemble into a full SCF complex, the canonical complex for F-box protein ubiquitin ligases. Finally, studies with newly generated antibodies specific for each FBA protein revealed differing expression patterns during development and in adult tissues. Overall, we observed wide diversity in glycan binding, SCF complex formation, and tissue distribution for this small family of proteins. This diversity is reminiscent of that seen with glycotransferases and glycoprotein degradation enzymes, which regulate tissue and cellular glycomes (57–60).

Our results lead us to conclude that only some members of the FBA family (FBXO2 and FBXO6) could function in GERAD by recognizing the signature high mannose glycan moiety present on retrotranslocated ER proteins. We suggest instead that collectively the FBA proteins recognize a broader array of glycoproteins. In contrast to the narrow specificity of FBXO2 for high mannose glycans, FBXO6 is a more promiscuous glycan binder that also recognizes sulfated glycans and glycoproteins. The failure of FBXO44 to bind glycans on the glycan array or the immobilized glycoprotein pulldown assay suggests it may not bind glycans. However, FBXO44 does contain the highly conserved G domain; moreover, phylogenetic analysis places it in the FBXO2 clade, and uniquely it does undergo tissue-specific post-translational modification. Hence, although we believe FBXO44 may bind glycans, and suspect our failure to observe this is either because of a failure to discover the appropriate glycoprotein substrate or because of a post-translational modification of FBXO44, we cannot formally exclude the possibility that FBXO44 alone among the FBA family does not bind glycoproteins. FBXO17 has a similar binding profile to FBXO6, except that it fails to bind high mannose glycoproteins.

Because sulfation and conversion to complex glycans occur in the Golgi, the affinity of FBXO6 and FBXO17 for these classes of glycoproteins implies that they may regulate secreted or membrane glycoproteins within or beyond the Golgi in the secretory pathway. It is intriguing to speculate how glycoproteins processed by the Golgi might become available to interact with cytoplasmic FBA proteins. Perhaps misfolded, complex (sulfated) glycoproteins are exported directly from the Golgi into the cytoplasm through an unidentified quality control process. Alternatively, misfolded proteins from the Golgi might return to the ER for release into the cytoplasm via conventional ER-associated degradation. Finally, because lysosomes process most complex and sulfated glycoproteins, FBXO6 and FBXO17 could perform a scavenger function and degrade proteins that escape the lysosome or are left in residual vesicular bodies. Further work is needed to examine these hypotheses, identify substrate proteins, and define the residues within the FBA domain critical for binding sulfated glycans.

Although all FBA family proteins were shown to bind core components of the SCF ubiquitin ligase complex, the degree of binding varied. Interestingly, FBXO2 showed much less binding of Cul1/Rbx1 than the rest of the FBA family members. An *in silico* explanation for this observation is that, among FBA proteins, FBXO2 lacks the glutamate residue required for hydrogen bonding to tyrosine in Cul1. In FBXO2 this glutamate is replaced by a proline, likely reducing affinity of FBXO2 for Cul1 (42). Recent work has also shown that the linker region between the F box and G domain in FBXO2 may suppress Cul1

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binding (45). Replacing the linker sequence in FBXO2 with that found in FBXO6 restores some Cul1 binding (45), suggesting that there may be multiple regions of contact between Cul1 and these FBA proteins. Thus, although all members of the FBA protein family can form SCF complexes, FBXO2 may also participate in heterodimeric complexes with Skp1, as we and others have found in the organ of Corti of the inner ear (30, 61, 62).

The variable yet widespread distribution of FBA proteins in post-mitotic tissues highlights their potential importance in regulating the cellular glycome. At the same time, however, their divergent tissue distribution suggests a degree of cell-specific substrate recognition for individual FBA proteins. It may be significant that in three tissues dedicated to heavy glycoprotein synthesis and secretion (brain, liver, and pancreas), we observed expression of multiple FBA family members.

In some tissues for certain FBA genes, there are discrepancies in expression at the RNA level (19, 21) and at the protein level reported here. A similar discrepancy in expression patterns has been reported for other ubiquitin ligases (52), and tissue-specific distributions have been reported for different isoforms of lysosomal enzymes (63–65). These differences in expression could reflect tissue-specific auto-ubiquitination of ubiquitin-protein isopeptide ligases (50, 51) or the expression of splice variants. Approximately 20% of all gene products are expressed as splice variants (66), and tissue-specific isoforms have been reported for other ubiquitin ligases (47). Consistent with this possibility, multiple FBA protein splice variants exist in the NCBI data base (67). Because our antibodies were raised against peptides, they may not recognize particular FBA splice variants. The functional characterization of these potential variants is an interesting subject for further study.

In summary, our analysis of the FBA family of F-box proteins demonstrates it to be a novel and functionally divergent group of lectin-like ubiquitin ligase subunits. One subset of this family recognizes high mannose glycans, whereas another subset preferentially recognizes sulfated glycans. Despite their close similarity at the sequence level, the F-box proteins differ markedly in spatial and temporal expression. This functional diversity suggests that although one or more FBA proteins may participate in GERAD, the family as a whole assumes a broader role in glycoprotein homeostasis. It will be important to define the substrate specificity *in vivo* for each member of this family of ubiquitin ligases to understand the specific role each protein plays in glycoprotein quality control.

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