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Loss of Core Fucosylation of Low-Density Lipoprotein Receptor–Related Protein-1 Impairs Its Function, Leading to the Upregulation of Serum Levels of Insulin-Like Growth Factor–Binding Protein 3 in Fut8$^{-/-}$ Mice

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α1,6-Fucosyltransferase (Fut8) catalyzes the transfer of a fucose residue from GDP-fucose to the innermost *N*-acetylglucosamine residue of *N*-glycans. Here we report that the loss of core fucosylation impairs the function of low-density lipoprotein (LDL) receptor–related protein-1 (LRP-1), a multifunctional scavenger and signaling receptor, resulting in a reduction in the endocytosis of insulin like growth factor (IGF)–binding protein-3 (IGFBP-3) in the cells derived from Fut8-null (Fut8$^{-/-}$) mice. The reduced endocytosis was restored by the re-introduction of Fut8. Serum levels of IGFBP-3 were markedly upregulated in Fut8$^{-/-}$ mice. These data clearly indicate that core fucosylation is crucial for the scavenging activity of LRP-1 *in vivo*.

Key words: core fucosylation, endocytosis, Fut8, IGFBP-3, LRP-1

It is generally accepted that *N*-glycans play an important role in the folding, stability, and sorting of glycoproteins (*1–3*). The branching pattern of *N*-glycans, which is determined by the action of glycosyltransferases, affects both the physicochemical properties and the function of the protein. The GDP-L-Fuc:*N*-acytyl-β-D-glucosaminide α1,6-fucosyltransferase (Fut8, EC 2.4.1.152) catalyzes the transfer of a fucose residue from GDP-fucose to the 6 position of the innermost *N*-acetylglucosamine residue of *N*-glycans (*4–6*). The reaction products, α1,6-fucosylated oligosaccharides (core fucose), are widely distributed in mammalian tissues. To determine the physiological roles of Fut8, we developed Fut8$^{-/-}$ mice, which show growth retardation, semi-lethality and emphysematous changes in the lung (–). We found that TGF-β1 receptor signaling is dysregulated in Fut8$^{-/-}$ mice.

Low density lipoprotein (LDL)–related protein 1 (LRP-1), a large glycoprotein receptor, is capable of binding and endocytosing a number of ligands and is generally considered to be a multifunctional scavenger and signaling receptor (*8*, *9*). It is synthesized as a single-chain, 600-kDa precursor that is subsequently processed into a 515-kDa alpha chain and an 85-kDa beta chain by furin (*10*) (see Scheme 1). The alpha chain of LRP contains multiple clusters of cysteine-rich LDL receptor class A repeats, which function in ligand recognition; and the beta chain contains multiple epidermal growth factor repeats, a transmembrane domain, and a cytoplasmic domain. LRP-1 recognizes lipoproteins (*11*), proteases such as tissue-type plasminogen activator (*12*), matrix metalloproteinase-9 (*13*), proteinase inhibitor complexes (*14*), bacterial toxins (*15*) and growth factors (*16*). LRP-1 also plays important roles in modulating signaling events, either as a coreceptor or by activating cellular signaling cascades through receptor ligation (*17*, *18*). In this study, we demonstrate that the insulin-like growth factor (IGF)–binding protein-3 (IGFBP-3), the most abundant of the six IGF-binding proteins, is endocytosed *via* LRP-1, and that the core fucosylation of the *N*-glycan of LRP-1 is crucial for IGFBP-3 endocytosing activity. Alteration in the function of LRP-1 as a result of the loss of core fucosylation might lead to the elevated serum concentration of IGFBP-3 in Fut8$^{-/-}$ mice.

MATERIALS AND METHODS

Animals—Mice were kept in a specific pathogen–free mouse facility at the Institute of Experimental Animal Sciences, Osaka University Medical School. All procedures of animal experiments were performed in accordance with the Osaka University Medical School guidelines. The establishment of Fut8-null mice has been described previously (*7*).

Reagents—The mouse monoclonal anti-LRP beta chain 5A6 was obtained from Progen (Germany) and the rabbit 488 anti LRP-1 antibody was generously provided by Dr. Strickland (University of Maryland, MD). The normal goat IgG and a goat anti–mouse IGFBP-3 antibody were purchased from R&D Systems, Inc. (Minneapolis, MN). Recombinant mouse IGFBP-3 was obtained from Techne Co. (Minneapolis MN). Biotinylated Aleuria aurantia lectin

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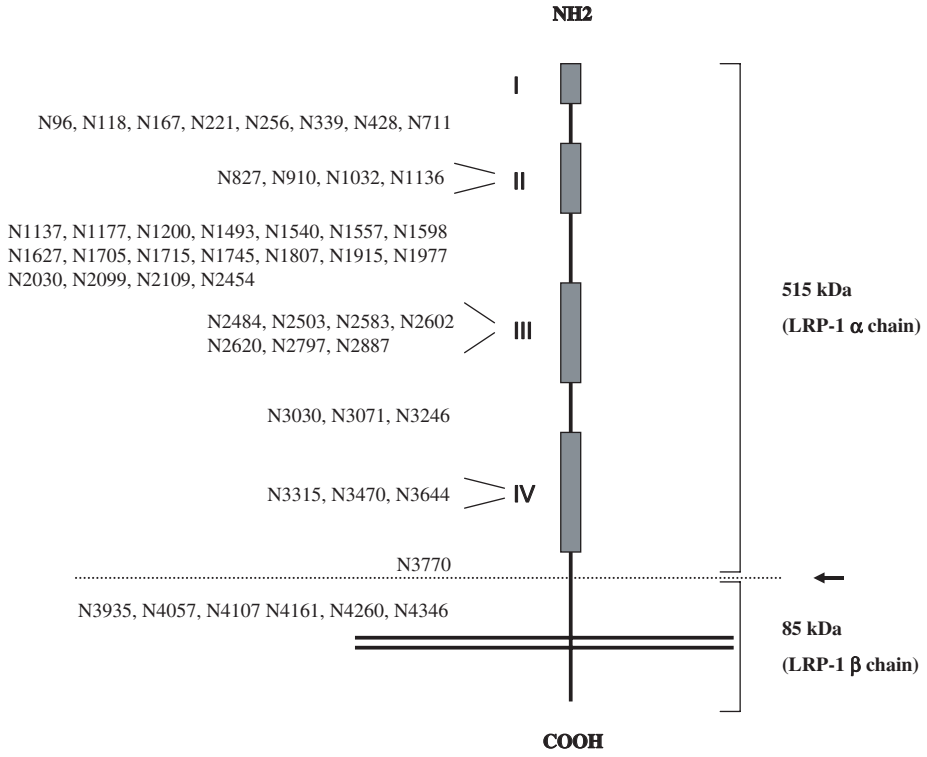

Scheme 1. **Schematic diagram of mouse LRP-1 with putative *N*-glycosylation sites.** The four putative ligand-binding domains are labeled with numerals I, II, III and IV. The site of furin cleavage is indicated with an arrow. LPR-1 alpha and beta chains have 44 and 6 putative *N*-glycosylation sites respectively. Amino acids of mouse LPR-1 are numbered without a signal sequences like those of human LPR-1.

(AAL), Concanavalin A (Con-A) and Sambucus sieboldiana agglutinin (SSA) were obtained from Seikagaku Corp. (Japan). Streptavidine-alexa was purchased from Molecular Probes Co. (Netherlands).

Western Blotting—Samples were subjected to SDS–polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred to a nitrocellulose membrane and, after blocking with 5% skim milk, the blots were reacted with the primary antibodies. After incubation with the peroxidase-conjugated secondary antibody, the membranes were processed with ECL reagents (Amersham) and exposed to an X-ray film.

RT-PCR—Total RNAs from kidneys, lungs and livers were isolated using the TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. First-strand cDNA synthesis was performed using a reverse transcription reaction with a random primer. PCR was then performed using the transcription product in a 25-μl reaction mixture. The 251-bp mouse IGFBP3 product was amplified using the primers 5′-CGTCTA-CACGGAGCGCTGTG-3′, and 5′-GATGGAACTTGGAAT-CGGTCA-3′. β-Actin was amplified as control using the primers 5′-TTACCAACTGGGACGACATG-3′ and 5′-AGG-AGCCAGAGCAGTAATCT-3′. PCR product was electrophoresed on 2% agarose gel.

Total IGF-1 Concentration—Serum total IGF-1 concentrations were determined using an Active Mouse/Rat IGF-1 enzyme immunoassay kit from Diagnostics Systems Laboratories (Webster, TX). To measure total serum IGF-1 levels, IGF-1 was dissociated from IGFBPs prior to the enzyme immunoassay using the acid ethanol cryoprecipitation method according to Breier BH *et al.* (*19*).

Cell lines, Culture and Transfection—Wild type (MEF-1) and LRP-null (PEA-13) mouse embryo fibroblasts were obtained from the American Type Culture Collection (Rockville, MD) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 10 units penicillin/ml, and 10 μg/ml of streptomycin. Kidney epithelial cells were prepared from a 2-week-old Fut8$^{-/-}$ mouse kidney using the Percoll gradient method according to Richardson *et al.* (*20*) and maintained in DMEM containing 4.5 g/liter glucose with 10% FBS, 10 units penicillin/ml, and 10 μg/ml of streptomycin. The cells were immortalized with the SV40 gene and named KK cells. For restoring Fut8, pCXN2/human Fut8 was cotransfected with pcDNA 3.1hygro into KK cells using the LipofectAMINE reagent (Life Technologies, Inc. Grand Island, NY) following the manufacturer's instructions. Selection was performed in a medium that contained 300 μg/ml of hygromycin (Calbiochem) and, after a 2-week incubation, stable transfectants were isolated and positive clones were selected by Western blotting and named KF cells.

Preparation of Receptor-Associated Protein (RAP)—Human GST-RAP and GST in the pGEX expression vector were kindly provided by Dr. Bu (Washington University, St. Louis), expressed in *E. coli* and prepared as described previously (*21*).

Radioiodination of Proteins—Protein labeling with ^{125}I was performed using IODO-GEN Pre-Coated Iodination

tubes (Pierce Co.) according to the manufacturer's protocol. Labeled protein was separated from free ^{125}I on PD-10 column (Pierce Co.).

Internalization of IGFBP-3—To monitor IGFBP-3 internalization, a previously described protocol was followed (*22*). Namely, 1.5×10^5 cells were plated in a 24-well plate and incubated with 6 nM ^{125}I-labeled IGFBP3 in DMEM–0.1% BSA in the presence of RAP-GST or GST at 37°C for the indicated times. After incubation, the cells were rapidly rinsed with ice-cold DMEM–0.1% BSA three times to remove unbound ligands. The cells were then incubated for 5 min with acid strip buffer (0.5 M sodium chloride, 0.2 M acetic acid, pH 2.8) at 4°C. The acid wash was combined with a second short rinse with the same acidic solution to determine the amount of surface-bound ^{125}I-ligand. Finally, the cells were solubilized in 1 M sodium hydroxide for quantitation of the internalized ^{125}I-ligand. The rate of internalization was calculated using the amount of internalized IGFBP-3/surface bound IGFBP-3 (I/S).

Preparation of Membrane Samples from Lungs—For the membrane sample preparation, lung tissues were homogenized on ice in 500 μl of phosphate-buffered saline (PBS) containing a proteinase inhibitor cocktail (Roche, Germany), then centrifuged at 15,000 rpm for 15 min. Pellets were incubated for 30 min on ice in 300 μl of solubilization buffer containing 10 mM Tris-HCl (pH 8.0), 140 mM NaCl, 300 mM KCl, 4 mM NaN_3, 0.5% Triton X-100, 12 mM sodium deoxycholate, 1 mM EDTA, and proteinase inhibitor coctail, then sonicated four times for 10 s each time. The solubilized samples were centrifuged at 40,000 rpm for 1 h at 4°C, and the supernatant was used in the lectin blotting and Western blotting experiments.

Preparation of Cell Extracts—Cell cultures (60–80% confluent) were rinsed twice with ice-cold PBS, then harvested in lysis buffer [20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% (w/v) Nonidet P-40, 10% (w/v) glycerol, 5 mM sodium pyrophosphate, 10 mM NaF, 1 mM sodium orthovanadate, 10 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 5 μg/ml leupeptin, and 1 mM dithiothreitol]. Cell lysates were centrifuged at 15,000 × *g* for 10 min at 4°C. The supernatants were collected, and protein concentrations were determined using a CBB protein assay kit (Nacalai Tesque, Japan).

Lectin Cytochemistry—KK and KF cells were seeded in a 35 mm glass-bottomed dish (Matsunami Glass Ind. Ltd., Japan) and fixed in 4% paraformaldehyde in PBS for 10 min, then permealized with 0.2% Triton X-100 for 10 min at room temperature. The cells were incubated in a 5% BSA/PBS for 30 min, incubated with biotinylated AAL, Con-A, or SSA in 5% BSA solution for 1 h, then labeled with streptavidin-alexa for 1 h. Fluorescence images were observed by LSMS PASCAL confocal microscopy (Carl Zeiss, Germany).

Immunoprecipitation and Lectin Blotting—For the immunoprecipitation of LRP-1, the samples (500 μg) were incubated overnight with 4 μg of rabbit 488 anti–LRP-1 antibody at 4°C, then with 20 μl of Protein G–Sepharose 4 Fast Flow (Amersham Pharmacia Biotech) for 1 h at room temperature. The immunoprecipitated LRP-1 was electrophoresed on 8% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked with 3% (w/v) bovine serum albumin (BSA) in Tris-buffered saline containing 0.1% (v/v) Tween-20 (TBST), and then incubated with a 2 μg/ml biotinylated AAL in TBST for 30 min at room temperature. After washing three times with TBST, lectin-reactive proteins were detected by use of a Vectastain ABC kit (Vector Laboratories, Burlingame, CA) and ECL reagent.

Cell Surface Biotinylation—Cell surface biotinylation was performed as described previously (*23*). Briefly, cells were rinsed twice with PBS supplemented with 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$, then incubated with freshly prepared sulfosuccinimidobiotin (s-NHS-biotin; Pierce Co.) diluted in the same solution (1 mg/ml) for 30 min on ice. The reaction was quenched by adding 50 mM NH_4Cl. The resulting cell lysate was immunoprecipitated with the rabbit 488 anti–LRP-1 antibody as described as above, then subjected to 8% SDS-PAGE, and transferred to a nitrocellulose membrane. After blocking the membrane with 3% (w/v) bovine serum albumin (BSA) in Tris-buffered saline containing 0.1% (v/v) Tween 20 (TBST, pH 7.5), the biotinylated proteins were visualized using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA) and an ECL kit.

RESULTS

Upregulation of Serum Levels of IGFBP-3 in Fut8$^{-/-}$ Mice—Establishment of Fut8$^{-/-}$ mice prompted us to determine molecules that are affected by a loss in core fucosylation. We examined serum IGFBP-3, which has growth inhibitory effects, by controlling the function of IGF-1. As shown in Fig. 1A, serum levels of IGFBP-3 were markedly increased in Fut8$^{-/-}$ mice. The upper two bands are an IGFBP-3 doublet (*24*), and the lowest band is considered to be a proteolytic fragment (*25*). Since the mRNA levels were not significantly different in Fut8$^{-/-}$ mice (Fig. 1B), it was assumed that post-translational modifications such as impaired degradation processes were involved in the increase in protein levels. Since IGFBP-3 binds to and controls the function of IGF-1, total IGF-1 levels in Fut8$^{-/-}$ mice were also determined (Fig. 1C). As a result, total IGF-1 levels were found to be increased significantly in Fut8$^{-/-}$ mice ($P < 0.05$).

LRP-1 Is a Catabolic Receptor for IGFBP-3—Since LRP-1 is responsible for the endocytosis and degradation of various types of serum proteins, and IGFBP-3 has been reported to be a possible ligand of LRP-1, we examined the possibility that IGFBP-3 is internalized by cells via LRP-1. LRP-expressing MEF-1 cells and LRP-deficient PEA13 cells were cultured, and the internalization of IGFBP-3 was measured. As shown in Fig. 2, the rate of internalization of IGFBP-3 was much lower in PEA13 cells than in MEF-1 cells. The internalization of IGFBP-3 in MEF-1 cells was inhibited by RAP, indicating that the internalization occurred via LRP-1. These results strongly suggest that LRP-1 functions as a catabolic receptor of IGFBP-3, along with other proteins.

Core Fucosylation of LRP-1 Is Crucial for Endocytosis of IGFBP-3—Next, we examined the effect of the core fucosylation of LRP-1. As shown in Fig. 3, the results of the AAL lectin blot experiments confirmed that LRP-1 in the wild-type mice lung was modified by core fucose and that it was abolished in Fut8$^{-/-}$ mice. To examine the role of core fucosylation in the LRP-1–mediated internalization of IGFBP-3, we established an epithelial cell line from

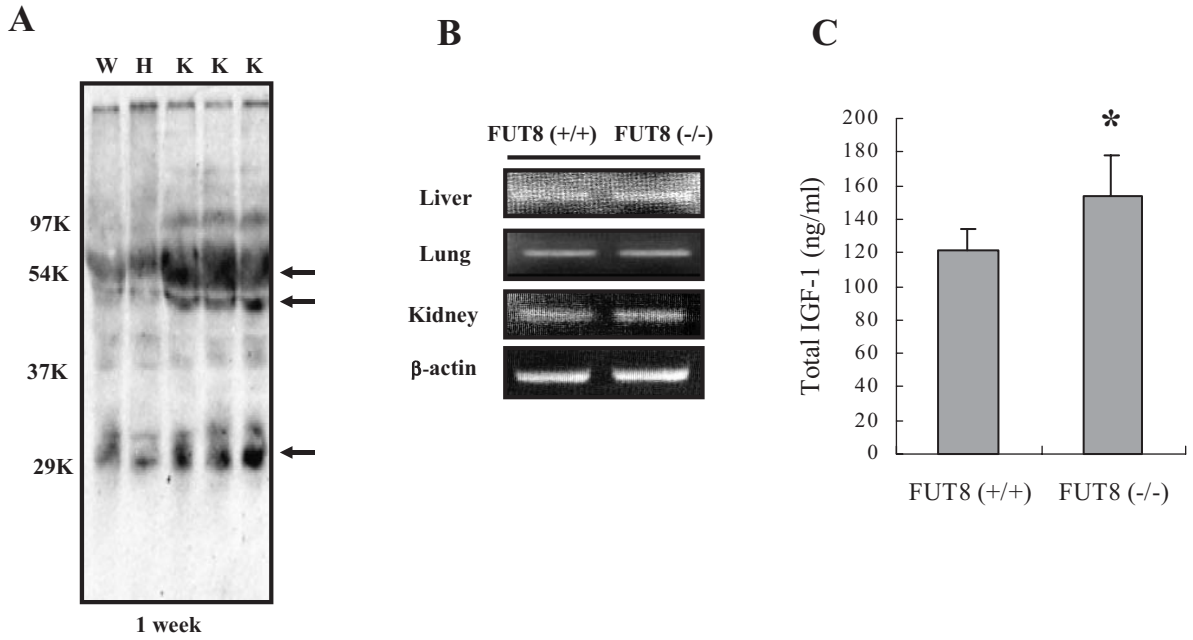

Fig. 1. **IGFBP-3 and IGF-1 is increased in Fut8$^{-/-}$ mice serum.** (A) Two microliters of serum from postnatal 6.5-day-old wild-type, hetero and Fut8$^{-/-}$ mice were subjected to 12% SDS-PAGE under reducing conditions and analyzed by Western blotting using a goat anti–IGFBP-3 antibody. Arrowheads indicate the IGFBP-3 doublet (upper two bands) and fragmentated IGFBP-3 (lowest band) (W: Wild-type, H: Hetero, K: Fut8$^{-/-}$). (B) Total RNA was prepared from kidney, lung and liver, and 1 μg of total RNA was used for RT-PCR for IGFBP-3. (C) Serum IGF-1 levels of wild-type (+/+) (n = 5) and Fut8$^{-/-}$ (–/–) (n = 6) mice of postnatal day 1 were determined as described in "MATERIALS AND METHODS" (+/+: Wild-type, +/–: Hetero, –/–: Fut8$^{-/-}$). *P < 0.05 compared with wild-type mice.

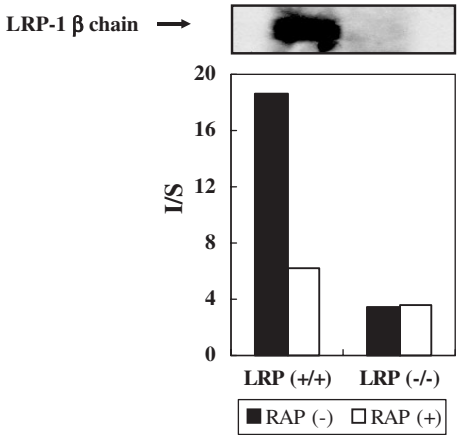

Fig. 2. **LRP-1 is catabolic receptor for IGFBP-3.** The internalization assay with ^{125}I-IGFBP-3 was performed using LRP-1 (+/+) MEF1 and LRP-1 (–/–) PEA13 cells. LRP-1 expression level of MEF1 and PEA13 cells was checked by Western blotting with an anti-LRP antibody 5A6 (upper pannel). Cells were incubated with 6 nM ^{125}I-IGFBP-3 in the presence of 500 nM GST-RAP or GST at 37°C for 2 h (lower panel). After washing with ice cold M-BSA, both the surface bound and internalized ^{125}I-IGFBP-3 were evaluated using a γ-counter. The rate of internalization was calculated by dividing the amount of internalized ^{125}I-IGFBP-3 (I) by the amount of surface-bound ^{125}I-IGFBP-3 (S):(I/S).

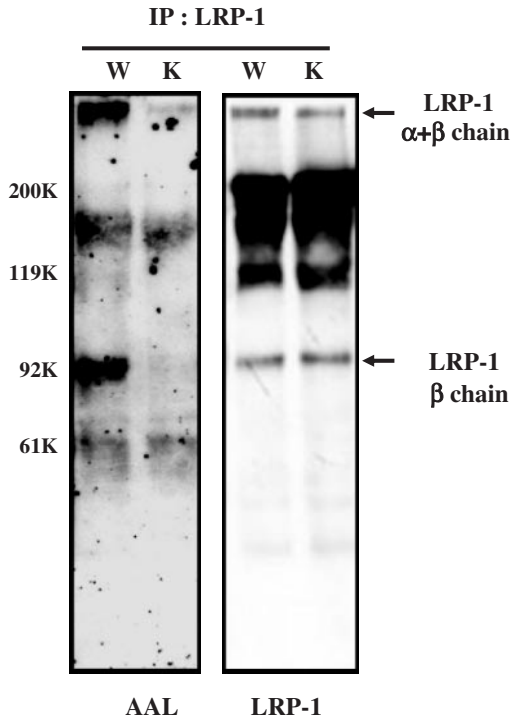

Fig. 3. **LRP-1 is fucosylated protein.** Membrane proteins prepared from wild-type and Fut8$^{-/-}$ mice lung (300 μg) were immunoprecipitated with rabbit 488 anti–LRP-1 antibody and subjected to 8% SDS-PAGE under non-reducing conditions. After transferring to nitrocellulose membranes, the blots were probed with AAL lectin (left panel) or anti mouse LRP-1 antibody 5A6, which recognizes only 85-kDa LRP-1 β chain (right panel) (W: wild-type, K: Fut8-null).

Fut8$^{-/-}$ mice (KK cells). We also restored Fut8 in the KK cells and denoted these cells as KF cells. The results of lectin cytochemistry and lectin blotting with AAL, Con-A, and SSA, which preferentially recognize core fucose, high mannose type of oligosaccharide and

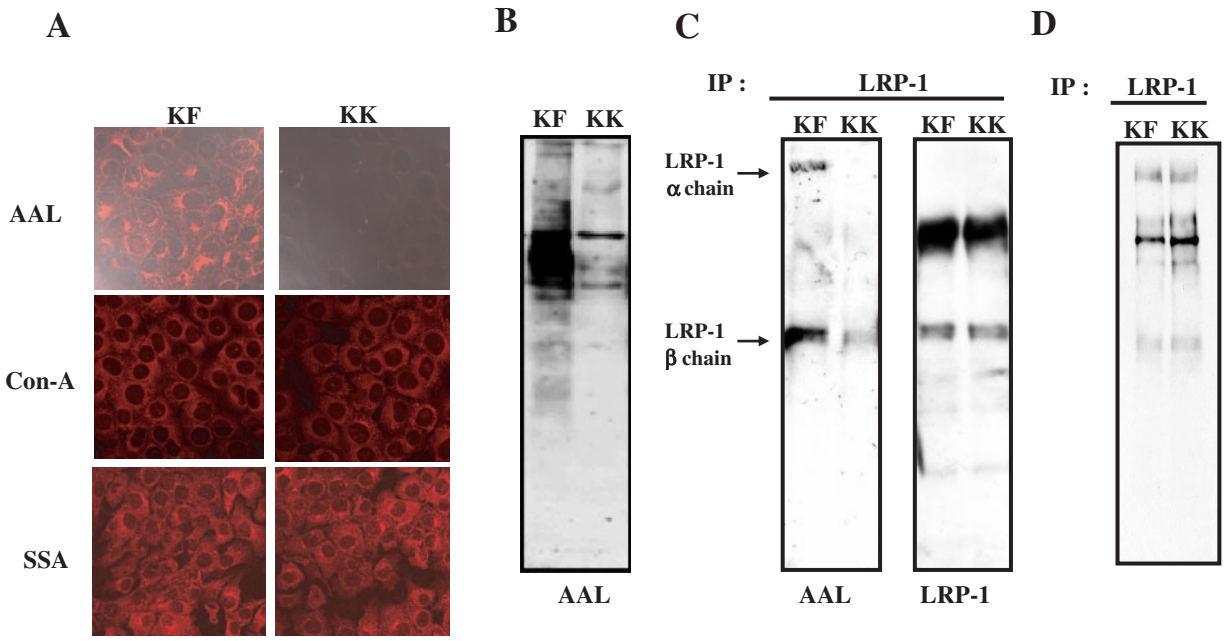

Fig. 4. **Establishment of kidney epithelial cells from Fut8$^{-/-}$ mice.** (A) Lectin cytochemistry assay using AAL, Con-A, and SSA lectin. (B) AAL-lectin blotting analysis using KF and KK cells. Ten micrograms of total proteins were subjected to 10% SDS-PAGE under reducing conditions. Lectin blotting was performed as described in "MATERIALS AND METHODS." (C) Total cell lysates from KF and KK cells were immunoprecipitated with rabbit 488 anti–LRP-1 antibody and subjected to 8% SDS-PAGE under non-reducing conditions. After transferring to nitrocellulose membranes, the blots were probed with AAL lectin (left panel) or anti–mouse LRP-1 antibody 5A6 (right panel). (D) Cell surface biotinylation and immunoprecipitation of LRP-1 from the KF and KK cells. Cells were biotinylated, and whole cell lysates were immunoprecipitated with rabbit 488 anti–LRP-1 antibody. The samples were subjected to 8% SDS-PAGE and transferred to a nitrocellulose membrane, and the biotinylated proteins were detected using a Vectastain ABC kit and an ECL kit.

α2,6-sialic acid, respectively, indicate that the KK and KF cells were both glycosylated and the levels of α2,6-sialylation were not significantly changed, but that the levels of fucosylation were reduced substantially in KK cells (Fig. 4, A and B). The fucosylation of LRP-1 was also significantly reduced in KK cells, which was confirmed by AAL lectin blotting after immunoprecipitation (Fig. 4C). However, the cell-surface expression levels of LRP-1 were not different between KK and KF cells (Fig. 4D). An internalization assay using ^{125}I-IGFBP-3 indicated that IGFBP-3 internalization levels were significantly decreased in KK cells compared to KF cells (Fig. 5). These results suggest that the core fucosylation of LRP-1 is crucial for internalization of IGFBP-3 *via* LRP-1.

DISCUSSION

The findings of the present study show that core-fucosylation is crucial for the function of LRP-1. We have found that serum levels of IGFBP-3 increased in Fut8$^{-/-}$ mice. Since mRNA levels were not changed, we assumed that this is due to a decreased internalization or the degradation of IGFBP-3. It was confirmed that IGFBP-3 is endocytosed *via* LRP-1, and therefore, we examined the function of core fucose deficient LRP-1. The data suggest that the IGFBP-3 endocytosing activity of LRP-1 is significantly decreased in Fut8$^{-/-}$ cells compared to cells that had been restored by the reintroduction of Fut8. Thus, we consider that the elevated serum

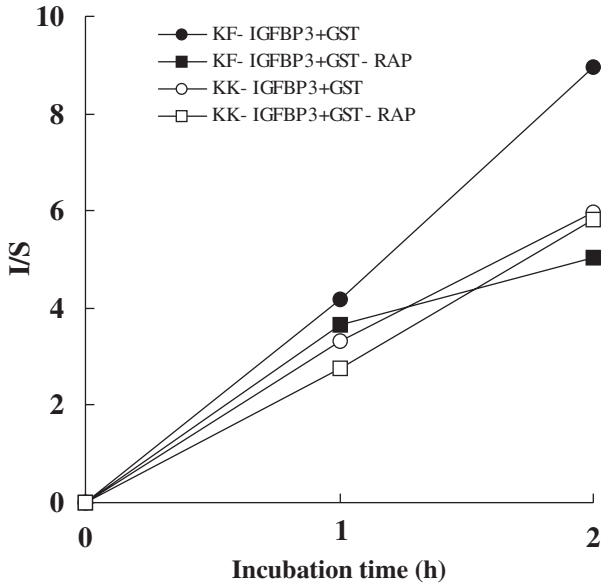

Fig. 5. **Fucosylation of LRP-1 is important in internalization of IGFBP3 Internalization assay of ^{125}I-IGFBP-3 using KF (solid circles, solid squares) and KK (open circles, open squares) cells.** Cells were incubated with 6 nM ^{125}I-IGFBP3 in the presence of 500 nM GST (solid and open circles) or GST-RAP (solid and open squres) at 37°C for the indicated times. Data are expressed as the ratio of internalized radioactivity to surface-bound radioactivity. Data represent the averages of three independent experiments. (I, internalized IGFBP-3; S, surface-bound IGFBP-3).

concentration of IGFBP-3 in Fut8$^{-/-}$ mice is due to a decrease in the endocytosis of IGFBP-3 *via* LRP-1.

Since IGFBP-3 is known to be one of the causes of growth retardation (*26*), and IGFBP-5 transgenic mice show neonatal mortality and whole-body growth inhibition (*27*), it is possible that the upregulation of IGFBP-3 is involved in semi-lethality and the severe growth retardation observed in Fut8$^{-/-}$ mice. We are currently attempting to define the relationship between the increased serum level of IGFBP-3 and growth retardation in Fut8$^{-/-}$ mice.

Many receptors are reported to be regulated by their *N*-glycans. The deletion of *N*-glycan on asparagine 420 from the epidermal growth factor receptor (EGFR) leads to spontaneous oligomerization and the constitutive activation of the receptor (*28*). The modification of the *N*-glycan of EGFR by bisecting GlcNAc leads to the upregulation of EGFR endocytosis (*29*). A mutation in the α2A/β1-adrenergic receptor at the asparagine in the *N*-glycan consensus sequence enhances heterodimerization (*30*).

LRP-1 is a very large glycoprotein and contains many putative *N*-glycosylation sites. It has been proposed that glycosylation is involved in the function of LRP-1; May *et al.* reported that the hypoglycosylation of LRP-1 is correlated with an increased shedding of the LRP ectodomain, resulting in the release of the LRP intracellular domain (*31*). McCormick *et al.* have shown that glycosylation is involved in the proper folding of LRP-1 (*32*). The findings herein demonstrate that core fucosylation is crucial for IGFBP-3 catabolism.

The antibody (5A6) used in Western blotting recognizes only the 85-kDa LRP-1 beta chain. Since upper and lower bands were detected by a LRP-1 5A6 antibody in Fig. 3, the upper band is considered to be a 600-kDa precursor form (alpha + beta chain) of LRP-1. On the other hand, in Fig. 4C, only the lower band was detected by Western blotting, indicating that the upper band in the AAL lectin blot represents the alpha chain. Thus, we consider that both alpha and beta chains of LRP-1 are core-fucosylated (Figs. 3 and 4C). In the previous study, we demonstrated that the profile of *N*-glycans in Fut8-null mice is not significantly changed other than loss of core fucosylation (*7*). The present data also indicated that the degrees of sialylation of *N*-glycans are not significantly altered by loss of core fucosylation (Fig. 4A).

LRP-1 is a multifunctional scavenging receptor and is also involved in the catabolism of various molecules. It is generally thought to be involved in lipid metabolism including cholesterol homeostasis thus, the approximately twofold increase of total cholesterol in the serum of Fut8$^{-/-}$ mice (Table 1) may support the hypothesis that the internalization activity of LRP-1 is decreased by the loss of core fucosylation.

IGFBP-3 is the most abundant of the six IGFBPs in plasma. It has been reported to act as a potent inhibitor of cell growth and to cause apoptosis (*33–35*). It is thought that IGFBP-3 exerts its function by binding to IGF-1 and modulating IGF signaling, and also by binding to its own receptor. IGF-independent effects are supported by many findings: for example, Blat et al. reported that IGFBP-3 had the ability to inhibit DNA synthesis in chick embryo fibroblasts stimulated by serum, fibroblast growth factor, or TGFβ, but not IGF (*36*). In addition, IGFBP-3 has antiproliferative effects on breast cells that are unresponsive to IGF-I (*37*), and on mouse fibroblasts that lack IGF-I receptors (*38*). It has been proposed that the IGFBP-3 receptor is identical to TGFβ receptor V (TβR-V), which mediates IGF-independent growth inhibition. Interestingly, in our present study, serum levels of IGF-I are upregulated along with IGFBP-3. We consider that this is a responsive increase, since a similar effect was also observed in IGFBP-3 transgenic mice (*39*). We confirmed that the IGF-I signaling pathway itself is not impaired in cells derived from Fut8$^{-/-}$ mice (data not shown).

In a previous study, we reported that the loss of core fucosylation of TGF-β receptor reduced its ligand binding activity, leading to the dysregulation of TGF-β signaling (*7*). Interestingly, it has been reported that IGFBP-3 binds TβRV and inhibits TGF-β binding and that the IGFBP-3 induced growth inhibition in wild-type, TβRI- and TβRII-deficient mink lung cells could be blocked by a TGF-β1 peptide antagonist (*40*). It is possible that the upregulation of serum levels of IGFBP-3 is involved in the dysregulation of TGF-β signaling in Fut8$^{-/-}$ mice.

Very recently, Huang et al. proposed that TβR-V, an IGFBP-3 receptor, is identical to LRP-1 and is important for the signal transduction of IGFBP-3 (*41*). It is possible that the internalization effect and signal transduction effect are distinct activities for LRP-1, as is suggested for PDGFbb/LRP-1 signaling. It is also possible that endocytosing-defective LRP-1 keeps the IGFBP-3 on the cell surface to exert continuous signaling. To clarify this issue, we are now determining the signaling activity of core fucose-deficient LRP-1.

Glycosylation of IGFBP-3 seems to affect its binding to the cell surface, but not to IGF-1 (*42*). Therefore, the loss of core fucosylation in IGFBP-3 may affect its binding to the cell surface. However, the IGFBP-3 in serum seems to be non-fucosylated, because it is mainly produced in the liver, where Fut8 expression is quite low. We have checked the glycosylation of IGFBP-3 using embryonic fibroblast cells. Immunoprecipitated IGFBP-3 from both Fut8 null-cells and control cells were detected with Con-A but not with AAL lectin, indicating that IGFBP-3 has high mannose type of sugar chain but not fucose (data not shown).

Table 1. **Biochemical analysis of serum from wild-type and Fut8-null mice.**

	Total cholesterol (mg/dl)*	BUN (mg/dl)*	Creatinine (mg/dl)	AMY (IU/liter)	AST (IU/liter)	ALT (IU/liter)	CPK (IU/liter)	Keton body (μmol/liter)
FUT8 (+/+)	22.7 (±7.2)	5.16 (±1.60)	0.133 (±0.05)	1,766.6 (±2,599.2)	69.5 (±34.58)	21.8 (±18.6)	58.33 (±29.89)	454.5 (±334.507)
FUT8 (–/–)	46.8 (±8.4)	11.5 (±2.38)	0.125 (±0.05)	479.5 (±70)	93 (±33.655)	16.75 (±6.23)	108.5 (±62.7)	753.25 (±567.7)

BUN, blood urea nitrogen; AMY, amylase; AST, glutamic-oxaloacetic transaminase; ALT, glutamic-pyruvic transaminase; CPK, creatine phosphokinase. * $P < 0.05$ compared with wild-type mice. Sera were analyzed using an autoanalyzer (Hitachi 747, Hitachi, Japan). Values shown are averages for four to six wild-type and Fut8-null mice, and differences were analyzed using the unpaired Student's *t* test.

Thus, it is unlikely that the loss of core fucosylation in IGFBP-3 affects its biological effects in our system, although the possibility cannot be totally ruled out.

About lectin blotting, although AAL lectin has strong binding affinity to core fucose, it also has weak binding affinity to α1-2, and α1-3 fucose (*43*). Therefore, it is possible that the two distinct AAL-positive bands in KK cell in Fig. 4B represent fucose residues other than core fucose. The difference between Fig. 4A and Fig. 4B might come from the difference of sensitivity.

We observed increased levels of BUN in Fut8-null mice serum, but no change in those of creatinine (Table 1), suggesting that catabolism of proteins was enhanced in Fut8-null mice.

It has been revealed that core fucosylation catalyzed by Fut8 plays an important role in various biological phenomena. Fut8 transgenic mice show steatosis in the liver and kidney due to a decrease in lysosomal acid lipase activity accompanied by its over-fucosylation (*44*). Core fucose-deficient IgG1 showed increased binding to FcγRIIIA, and a dramatically increased antibody-dependent cellular cytotoxicity activity, which is a key strategy in antibody therapy against tumors (*45*, *46*). In the present study, we have found that core fucosylation is required for the endocytosing activity of LRP-1. Since LRP-1 is involved in various biological events, it is possible that core fucosylation is important for these diverse phenomena. Investigations of the complex mechanisms by which core fucosylation regulates cellular physiology would aid in our understanding of the role of glycosylation.

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