題目：A novel missense mutation causing abnormal LMAN1 in a Japanese patient with combined deficiency of factor V and factor VIII

（新規遺伝子変異による血液凝固第 V・VIII 因子合併欠損症の分子病態解析）

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【緒言】

第 V・VIII 因子合併欠損症（F5F8D）は、先天性凝固因子欠損症のうち、常染色体劣性遺伝病である第 V 因子（FV）欠損症、伴性劣性遺伝病である第 VIII 因子（FVIII）欠損症（いわゆる血友病 A）とは全く異なる稀な常染色体劣性遺伝病である。F5F8D は FV、FVIII の抗原・活性が同等に正常血漿レベルの 5～30％に低下し、出血症状は中等度から軽度で FV や FVIII のそれぞれの単独欠乏症と似ている。

F5F8D の変異責任遺伝子として、現在、LMAN1 (lectin, mannose-binding 1) 遺伝子と MCFD2 (multiple coagulation factor deficiency 2) 遺伝子が挙げられる。LMAN1 は蛋白分泌過程における小胞体からゴルジ体への糖蛋白質の輸送に関与するカーゴレセプターであり、FV、FVIII を含む特定の糖蛋白質の糖鎖を認識しその細胞内輸送・分泌に関与すると考えられている。また、MCFD2 分子は、LMAN1 とカルシウム依存的に相互作用し機能しており、MCFD2 欠損においても F5F8D が発症することが報告されている。今回、我々は、F5F8D 患者の遺伝子解析、およびその分子病態について検討を行った。

【方法】

名古屋大学医学部倫理委員会の承認のもと、インフォームドコンセントを得た後、発端者およびその家族の末梢血白血球分画より定法により DNA を抽出した。*LMAN1* および *MCFD2* の遺伝子配列データベースを基に、それぞれの mRNA をコードする各エクソンならびに、そのイントロンとの境界領域を増幅させるプライマーを設定し PCR を行い、ダイレクトシークエンス法にてそれぞれの塩基配列を解析した。また、患者および健常人リンパ球の EB ウィルスによる不死化クローン株を樹立し、それぞれのリンパ球で LMAN1 蛋白の発現をウエスタンブロット解析および ELISA にて検索した。

LMAN1/MCFD2 複合体形成を確認するため、EB ウィルスによる不死化クローン株を用いて、Ca イオンまたは EDTA 存在下、抗 MCFD2 抗体にて IP (Immunoprecipitate)-ウエスタンブロットを行った。また、変異 LMAN1 分子の糖鎖認識能を解析する為、myc および His タグを付加した野生型および変異型 LMAN1 分子の発現ベクターをリコンビナント PCR 法にて作製した。作製したベクターを COS-1 細胞に導入後、His タグを標的にニッケルカラムにて精製した。精製物を透析後、マンノース結合アッセイを行い、myc 抗体にて IP 後、抗 LMAN1 抗体を用いてウエスタンブロットを行った。

【結果・考察】

シークエンスの結果、発端者の *LMAN1* のエクソン１に、c,200G＞C (p.Trp67Ser)の新規の一塩基変異をホモ接合体として、またその娘にはヘテロ接合体として同定した。一方、*MCFD2* には変異を認めなかった。変異を同定した LMAN1 分子の 67Trp は各種生物界で高く保存されており、重要なアミノ酸であることが示唆された。

EB ウィルスによる症例リンパ球の不死化クローン株を用いたウエスタンブロット

解析では、発端者、娘、健常者検体すべてにおいて LMAN1 蛋白を検出した。また、ELISA においても各検体間の LMAN1 蛋白発現量に有意な差を認めなかった。

LMAN1/MCFD2 複合体形成においては、抗 MCFD2 抗体による IP-ウエスタン解析により、健常者検体での LMAN1/MCFD2 複合体形成は Ca イオン依存的であり、発端者検体では Ca イオン存在下においても LMAN1/MCFD2 複合体形成不全が認められた。この結果より、LMAN1/MCFD2 複合体形成は Ca イオン依存的であり、分子モデル解析により LMAN1 蛋白の 67Trp が Ser に変異することにより、MCFD2 が結合する LMAN1 分子の糖鎖認識ドメイン(Carbohydrate Recognition Domain :CRD)を構成するβシート構造の一部に立体構造変化を生じ、LMAN1/MCFD2 複合体形成を障害することが推察された。

マンノース結合能実験では、野生型 67Trp−LMAN1 はマンノースとの結合能を有したが、変異型 67Ser−LMAN 1 は結合能を示さなかった。LMAN1 分子は 67Trp から Ser への置換により、上述の如く CRD の立体構造変化を生じ、マンノース型糖鎖との結合能が低下することが推察され、B ドメインに N 結合型糖鎖を多く含む凝固第 V・VIII 因子の細胞内蛋白輸送に共通して障害を引き起こすことが予想された。

【結語】

p.Trp67Ser 変異 *LMAN1* をホモ接合体にもつ発端者は、LMAN1/MCFD2 複合体形成不全ならびにマンノース型糖鎖結合能低下に伴い、凝固第 V・VIII 因子の細胞内輸送に障害を引き起こし、F5F8D を発症したものと推測された。

Summary

Combined deficiency of coagulation factor V (FV) and factor VIII (FVIII) (F5F8D) is an inherited bleeding disorder characterized by a reduction in plasma concentrations of FV and FVIII. F5F8D is genetically linked to mutations in either *LMAN1* or *MCFD2*. Here, we investigated the molecular basis of F5F8D in a Japanese patient, and identified a novel missense mutation (p.Trp67Ser, c.200G>C) in the *LMAN1*, but no mutation in the *MCFD2*. The amount of LMAN1 in Epstein-Barr virus-immortalized lymphoblasts from the patient was found to be almost the same as that in cells from a normal individual. Interestingly, an anti-MCFD2 antibody did not co-immunoprecipitate the mutant LMAN1 with MCFD2 in lymphoblasts from the patient, suggesting the affinity of MCFD2 for the mutant LMAN1 is weak or abolished by the binding of the anti-MCFD2 antibody. In addition, a Myc/6×His-tagged recombinant form of wild-type LMAN1 could bind to D-mannose, but that of the mutant could not. The p.Trp67Ser mutation was located in the carbohydrate recognition domain (CRD), which is thought to participate in the selective binding of LMAN1 to the D-mannose of glycoproteins as well as the EF-motif of MCFD2. Taken together, it was suggested that the p.Trp67Ser mutation might affect the molecular chaperone function of LMAN1, impairing affinity for D-mannose as well as for MCFD2, which may be

responsible for F5F8D in the patient. This is the first report of F5F8D caused by a qualitative defect of LMAN1 due to a missense mutation in *LMAN1*.

Introduction

Coagulation factor V (FV) and factor VIII (FVIII) are both essential in the blood coagulation cascade, as cofactors for the proteases factor X and factor IX, respectively. Combined deficiency of FV and FVIII (F5F8D) is an autosomal recessive bleeding disorder first described by Oeri et al. in 1954 (1), and a distinct clinical entity from chance co-inheritance of hemophilia A (FVIII deficiency) and parahemophilia (FV deficiency). F5F8D is extremely rare (1:2,000,000) in the general population (2), and characterized by a mild-to-moderate bleeding tendency manifested after surgical trauma, abortion, and delivery. Menorrhagia is also common, but hematuria and gastrointestinal bleeding are infrequent, and hemarthrosis is rare (3). Generally, patients with F5F8D show plasma levels of FV and FVIII in the range of 5% - 30% of normal (4).

Positional cloning has identified 2 genes, *LMAN1* (lectin, mannose-binding, 1; also known as ERGIC-53) and *MCFD2* (multiple coagulation factor deficiency gene 2), associated with F5F8D (4, 5). LMAN1 is a type-1 transmembrane protein that cycles between the endoplasmic reticulum (ER) and the ER-Golgi intermediate compartment (ERGIC) (6, 7). It contains a mannose-specific carbohydrate recognition domain (CRD) on the ER luminal side, and ER exit and retrieval motifs on the cytoplasmic side (8). MCFD2 has an EF-hand domain

that interacts with LMAN1 in a Ca^{2+}-dependent manner (5). The LMAN1-MCFD2 protein complex functions as a cargo receptor that facilitates the transport of FV and FVIII from the ER to the Golgi apparatus (5, 9).

Extensive genetic analyses of F5F8D patients have identified many causative mutations in *LMAN1* and *MCFD2*, which may account for nearly all cases, with about 70% of familial cases of F5F8D attributable to *LMAN1* mutations and about 30% to MCFD2 mutations (10). Most of these mutations, especially in *LMAN1*, were nonsense, frame shift, splicing defect or missense mutations, resulting in null alleles.

In this study, we examined the molecular basis of F5F8D in a Japanese patient, and identified a novel homozygous missense mutation in *LMAN1* that led to a functionally abnormal LMAN1 causing the F5F8D.

Materials & Methods

Patients and samples

The study was approved by the Ethics Committee of the Nagoya University School of Medicine. After obtaining informed consents, blood samples were collected from the patient and one of her children, but other family members did not participate in this study. Genomic DNA was isolated from peripheral blood leukocytes according to a standard procedure. We also established Epstein-Barr virus (EBV)-immortalized lymphoblast lines from the patient, her children and a control individual.

DNA sequencing of LMAN1 and MCFD2

All coding exons and intron-exon junctions of *LMAN1* and *MCFD2* were amplified from the genomic DNA by a polymerase chain reaction (PCR) as described previously (18). The PCR products were directly sequenced using a Big-Dye Terminator Cycle Sequencing kit and a 310 Genetic Analyzer (Applied Biosystems. Foster City, CA, USA).

Western blot analysis

EBV-immortalized lymphoblasts derived from the patient, her child and a healthy control were dissolved in lysis buffer (10mM Tris-HCl, pH7.4, 150mM NaCl, 1mM $MgCl_2$, 10mM $CaCl_2$, and 1% Triton X-100), the lysate were cleared by centrifugation at 1500x g, and protein concentrations were determined using a Bio-Rad Protein Assay Kit (Bio-Rad, Richmond, CA). Equal amounts of cell lysate (1.5μg) were separated by 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and transferred to PVDF membranes. LMAN1 was detected by immunostaining with a chicken anti-LMAN1 IgY antibody (GenWay Biotech, Inc., San Diego, CA) and a rabbit anti-chicken IgY conjugated with horseradish peroxidase (GenWay Biotech, Inc.), using an ECL PLUS Western blotting detection system (Amersham Biosciences, Piscataway, NJ).

Enzyme-linked immunosorbent assay for the measurement of LMAN1

We also measured antigen levels of LMAN1 in equal amounts of lysate (15μg) with an enzyme-linked immunosorbent assay (ELISA) as described previously (19). Briefly, a polyclonal chicken anti-LMAN1 antibody was used for capturing, while amplification and detection of the signals were achieved with a biotinylated polyclonal chicken anti-LMAN1

antibody and avidin peroxidase using an ECL Protein Biotinylation Module kit (Amersham Biosciences). We calculated the absorbance of the sample relative to that of a normal sample (mean ± S.D., n=3).

Immunoprecipitation (IP)-Western blot analysis

Equal amounts of lymphoblast lysate (250μg) in the presence of 10mM Ca^{2+} or 10mM EDTA in the lysis buffer were immunoprecipitated with a goat anti-MCFD2 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) using protein G PLUS-Agarose (Santa Cruz Biotechnology, Inc.). The precipitated samples were then separated by 10% SDS-PAGE and transferred to PVDF membrane~~s~~. The signals were detected as described above for LMAN1.

Construction of expression vectors for wild-type and mutant LAMN1s bearing an N-terminal c-myc epitope tag and a C-terminal 6×His tag

A full-length human LMAN1 cDNA was amplified by PCR from a human liver cDNA library (Clonetech, Mountain View, CA), and cloned into the pCI vector (Promega, Madison, WI). To allow for the simplified detection and purification of the recombinant LMAN1, we engineered a myc/6×His-tagged LMAN1 expression vector as described previously (11). First,

a c-myc epitope was introduced at the N-terminus of mature LMAN1 by recombinant PCR using mutagenesis primers, 5'-CAGATCCTCTTCTGAGATGAGTTTTTGTTC GCCCCGGACGAAGCGAC (forward: mutated nucleotides are underlined) and 5'-CTCATCTCAGAAGAGGATCTGGACGGCGTGGGAGGAGA (reverse), as described elsewhere (20). Second, a 6×His tag was introduced at the C-terminus, replacing the targeting signal (KKFF) of LMAN1, by substitution with a PCR fragment using a mutagenesis reverse primer, 5'-CCTCAATGGTGATGGTGATGATGGGCAGCTGCTTCTTGCT. Subsequently, we introduced a c.200G>C mutation into the myc/6×His-tagged LMAN1 by substitution with a PCR product using a forward mutagenesis primer, 5'-GCACCTGGTGCAGAGCGACGGGACCGTGCCCTTCTCGGCC. The presence of the desired mutations and the absence of a second mutation in the expression vectors were confirmed by DNA sequencing.

Transient expression of recombinant LMAN1 in COS1 cells

COS-1 cells were grown in DMEM supplemented with 10% fetal calf serum. The cells were cultured in 100-mm dishes until about 50% confluent, and then transiently transfected with 20μg of the expression plasmid vectors using the calcium phosphate method as described

previously (21). After 48 h of incubation, the cells were harvested and dissolved in the lysis buffer as described above. The cell lysates were then centrifuged at 1500x g for 10 min and cleared supernatants were used for subsequent mannose-binding experiments.

Mannose-binding assay for recombinant LMAN1

The cleared cell lysates containing wild-type or mutant Myc/6×His LMAN1 were incubated with Ni-NTA agarose (Invitrogen, Carlsbad, CA) under constant agitation for 1h at 4°C. After washing with binding buffer (10mM Tris-HCl, pH7.4, 150mM NaCl, 1mM $MgCl_2$, 10mM $CaCl_2$, and 0.15% Triton X-100), bound proteins were eluted with 0.25M imidazole and dialyzed against the binding buffer. The protein concentrations in the dialyzed samples were quantified using the Bio-Rad Protein Assay Kit. The samples (4μg each of total protein) were incubated with 200μl of mannose agarose (EY Laboratories, San Mateo, CA) under constant agitation overnight at 4°C. After washing, bound recombinant LMAN1 was eluted with 200μl of 0.2M D-mannnose (Sigma-Aldrich, Inc. St Louis, MO) in the binding buffer, and then immunoprecipitated with anti-c-myc antibody (Santa Cruz Biotechnology, Inc.) coupled to Protein G PLUS-Agarose. Subsequently, the precipitates and the pre-mannnose agarose samples (0.12μg of total protein for the wild-type and 0.4 μg for the mutant) were analyzed

by 10% SDS-PAGE and Western blotting using a polyclonal chicken anti-LMAN1 antibody as described above.

Results

Case report

The patient was a 32-year-old female suffering from bleeding after the 3rd delivery, having had similar bleeding episodes in her previous two deliveries. Her platelet count, FDP-E and liver function tests were normal, but both her PT (19.7s/control 12.0s) and APTT (72.5s/control 43.6s) were prolonged. She was diagnosed with F5F8D, because her FV and FVIII coagulant activities were 14% and 19%, respectively, and the activities of the other coagulation factors were normal (fibrinogen, 275mg/ml; FII, 78%; FVII, 96%; FX, 83%; FIX, 110%; FXI, 70%; FXII, 84%; PK, 70%; HMWK, 104%). In order to investigate the molecular basis of her F5F8D, she was referred to the Nagoya University hospital.

Mutational analysis in the F5F8D patient

We analyzed coding regions and intron-exon boundaries of *LMAN1* and *MCFD2* of the patient with F5F8D by PCR-mediated direct sequencing, and identified a novel missense mutation in *LMAN1*, but no mutation in *MCFD2*. The mutation, found in exon 1 of *LMAN1*, was a G-to-C transversion at nucleotide 200 (c.200G>C), leading to the substitution of TGG (Trp) at codon 67 with TCG (Ser) (p.Trp67Ser) (Fig. 1). The patient turned out to be

homozygous for the c.200G>C mutation, as confirmed by a *Apa*I PCR-restriction fragment length polymorphism (RFLP) analysis (data not shown). A large deletion of *LMAN1* in the other allele of the patient was excluded because of heterozygosity for a SNP (refSNP ID: rs11354119) in intron 6 of *LMAN1* (data not shown). Her child was also found to be heterozygous for this mutation by DNA sequencing as well as the *Apa*I- RFLP analysis.

Western blot analysis and ELISA for LMAN1 protein

To determine expression levels of LMAN1 *in vivo*, we prepared Epstein-Barr virus (EBV)-immortalized lymphoblast lines derived from the patient, her child and a healthy volunteer, and measured LMAN1 antigens by Western blot analysis as well as by enzyme-linked immunosorbent assay (ELISA). LMAN1 proteins of the expected molecular size (53kDa) were detected in all samples by Western blotting (Fig. 2A), and no difference in relative absorbance was found among the samples by ELISA for LMAN1 (Fig. 2B). These results indicated that the amounts of mutant LMAN1 expressed in lymphoblasts from the patient were same as those of wild-type LMAN1 in normal control lymphoblasts.

Immunoprecipitation (IP)-Western blot analysis.

To examine the relation between LMAN1 and MCFD2 in the EBV-immortalized lymphoblasts, we performed immunoprecipitation (IP)-Western blotting using anti-MCFD2 and anti-LMAN1 antibodies, with 10mM Ca^{2+} or 10mM EDTA present in the lysis buffer. In lymphoblasts from the normal individual, we observed that the anti-MCFD2 antibody co-immunoprecipitated LMAN1 with MCFD2 in the presence of 10mM Ca^{2+}, but not 10mM EDTA (Fig. 3A). In lymphoblasts from the patient, however, the anti-MCFD2 antibody did not co-immunoprecipitate mutant LMAN1 at all, even in the presence of 10mM Ca^{2+}. In lymphoblasts from the child carrying the heterozygous mutation, the signal for LMAN1 co-immunoprecipitated with the anti-MCFD2 antibody was slightly weaker than that in lymphoblasts from the normal control in the presence of 10mM Ca^{2+}. These results indicated that the mutant 67Ser-LMAN1 associated with MCFD2 to a much lesser extent than wild-type LMAN1 in lymphoblasts.

Mannose binding assay for recombinant Myc/6×His-LMAN1

To test the ability to bind to mannose agarose, we prepared wild-type and mutant recombinant LMAN1 bearing myc/6×His tags. The recombinant myc/6×His-LMAN1 eluted

from mannose agarose with 0.2M D-mannose were immunoprecipitated with an anti-c-myc antibody, and detected by Western blotting with an anti-LMAN1 antibody. We demonstrated that the wild-type myc/6×His LMAN1 bound to mannose agarose, whereas the mutant myc/6×His LMAN1 did not (Fig. 4). We also observed that the wild-type recombinant LMAN1 bound to the mannose in a Ca^{2+}-dependent manner as reported previously (11) (data not shown).

Discussion

F5F8D is a very rare congenital bleeding disorder and recent extensive genetic analyses of F5F8D patients have identified many causative mutations in the *LMAN1* and *MCFD* genes. We here investigated the molecular basis of F5F8D in a Japanese patient, and identified a novel missense mutation (p.Trp67Ser, c.200G>C) in the *LMAN1*, but no mutation in the *MCFD2*.

To date, at least 32 *LMAN1* mutations have been reported throughout all 13 exons of the gene, most of which are either nonsense or frameshift mutations leading to the complete absence of a functional LMAN1 protein (12-14). So far, only two missense mutations have been reported in *LMAN1*, p.Met1Thr and p.Cys475Arg, which resulted in little or no LMAN1 protein in the cells (13, 15). In this report, we showed that lymphoblasts from a patient with a homozygous p.Trp67Ser mutation possessed a variant LMAN1 protein in the same amount as LMAN1 in lymphoblasts from a normal individual. These results implied that the variant 67Ser-LMAN1 might be functionally abnormal as a cargo receptor in a complex with MCFD2 for trafficking FV and FVIII in this F5F8D patient.

LMAN1 is thought to form a Ca^{2+}-dependent complex with MCFD2 in the ER lumen at a stoichiometry of 1:1. Direct molecular interaction between LMAN1 and MCFD2 can occur *in*

vivo, because these two proteins can be co-immunoprecipitated in a complex from cells (5, 9). We examined the interaction between LMAN1 and MCFD2 in lymphoblasts by IP-Western blot analysis using anti-MCFD2 antibody, and found that the variant 67Ser-LMAN1 of the patient impaired Ca^{2+}-dependent association with MCFD2. The CRD of LMAN1 would contain the MCFD2-binding site, because the LMAN1-MCFD2 complex was reconstituted *in vitro* using the purified CRD of LMAN1 and MCFD2 (16). In addition, the alignment of LMAN1 and related sequences from different organisms within the animal kingdom revealed a high degree of sequence identity including 67Trp (Fig. 5), which located in the CRD of LMAN1 is one of the tryptophans forming a hydrophobic ladder running through the hydrophobic core of the protein (17). In the crystal structure study of the CRD, Velloso et al. showed that 67Trp locates in the first strand (β2) of the major β-sheet consisting an edge of CRD, and concluded that the major β-sheet (β2, β5, β14, β7-β10) curved giving rise to a concave surface, which is the putative ligand binding site (Fig. 6) (17). Therefore, it is likely that the p.Trp67Ser missense mutation would affect the conformation of the CRD in LMAN1 leading to a change in affinity for MCFD2.

It was reported that the recombinant CRD of LMAN1 could bind to sugars in a Ca^{2+} -dependent manner, and this was enhanced by its interaction with MCFD2 (16). In the present

study, we demonstrated that the recombinant wild-type LMAN1 could bind to mannose in the presence of Ca^{2+} ions *in vitro*, even in the absence of MCFD2. In contrast, the mutant 67Ser-LMAN1 lost its sugar-binding ability, suggesting that the p.Trp67Ser missense mutation in the CRD would abolish the binding to mannose, possibly due to a conformational change of the CRD in LMAN1.

It was likely that the p.Trp67Ser mutation in LMAN1 abolished its sugar-binding ability and impaired its interaction with MCFD2, resulting in the reduced secretion of factors V and VIII in this F5F8D patient. To our knowledge, this is the first report of F5F8D caused by a functionally abnormal LMAN1 due to a missense mutation in *LMAN1*.

Acknowledgements

We would like to thank Dr H. Saito (Nagoya Central Hospital), and Dr T. Matsushita (Department of Hematology-Oncology, Nagoya University Graduate School of Medicine) for helpful suggestions and for reading the manuscript. We are most grateful to Dr A. Takagi and Dr T. Murate (Department of Medical Technology, Nagoya University School of Health Sciences), Dr M. Sano (Maeda Hospital), Y. Fujimori, A. Suzuki and Y. Miyawaki (Department of Pathophysiological Laboratory Sciences, Nagoya University Graduate School of Medicine) for their helpful discussions. We also thank C. Wakamatsu, S. Yamamoto and S. Suzuki for their expert technical assistance, and members of the Kojima laboratory for their helpful discussions.

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Figure legends

Fig. 1. Sequence analysis of *LMAN1*.

Nucleotides and predicted amino acids sequences surrounding the mutation of exon 1 in the *LMAN1* gene. Arrows indicate the site of mutation. The mutation was a G-to-C transversion at nucleotide 200, leading to a substitution of TGG (Trp) at codon 67 with TCG (Ser).

Pt: patient, Ch: child, N: normal individual.

Fig. 2. Quantification of LMAN1 proteins in lymphoblasts.

A: Western Blot analysis of LMAN1 in lymphoblasts from the patient, her child and a normal individual. LMAN1 protein of the expected size, 53kDa, was detected in all samples.

B: LMAN1 levels in lymphoblasts determined by ELISA. We calculated the absorbance of the sample relative to that of a normal sample (mean ± S.D., n=3). Pt: patient, Ch: child, N: normal individual.

Fig. 3. IP-Western blot analysis of LMAN1/MCFD2 complex

Samples immunoprecipitated with a goat anti-MCFD2 antibody from lymphoblast lysates, with 10mM Ca^{2+} or 10mM EDTA present in the lysis buffer, were analyzed by Western blotting for LMAN1 using a chicken anti-LMAN1 antibody. Pt: patient, Ch: child, N: normal individual.

Fig. 4. Mannnose-binding assay.

A wild-type (67Trp) or mutant (67Ser) recombinant LMAN1 bearing myc/6×His tags were expressed in COS1 cells and purified by Ni-NTA agarose. The samples were then incubated with mannose agarose, eluted with 0.2M D-mannnose, immunoprecipitated with an anti-c-myc antibody, and detected by Western blotting with an anti-LMAN1 antibody.

pre: samples pre-treated with mannose agarose, elute: samples eluted from mannose agarose.

Fig. 5. Comparison of amino acid sequences of human, monkey, rat, mouse, fruit fly, C. elegans and Laccaria bicolor LMAN1.

Open boxes denote amino acids that do not differ between human LMAN1 and LMAN1 from other species. 67Trp (W in bold in the shaded box) is conserved in all species.

Fig. 6. Structural model of Carbohydrate Recognition Domain of LMAN1

Overall structure of LMAN1 monomer was maid by MacPyMOL according to the data form PDB: 1GV9 (17). 67Trp is one of the tryptophans (67Trp, 94 Trp, 126 Trp, and 144W) forming a hydrophobic ladder running through the hydrophobic core of the protein. 67Trp locates in the first strand (β2) of the major β-sheet consisting an edge of CRD, and the major β-sheets (β2, β5, β14, β7–β10) are though to curve giving rise to a concave surface, which is the putative ligand binding site (17).

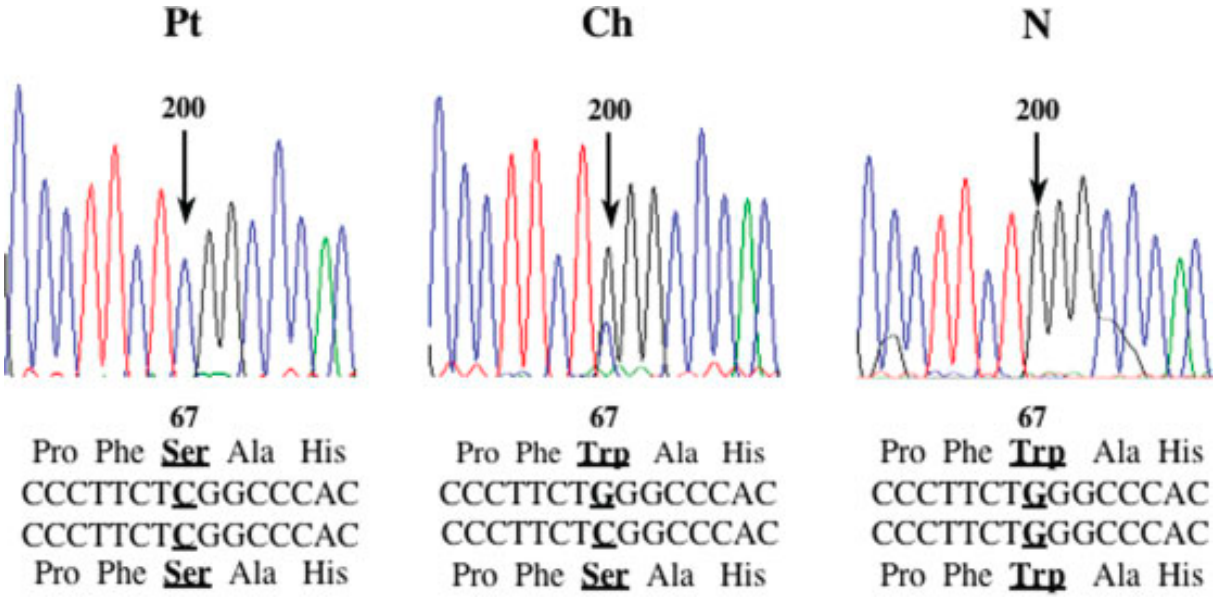
Pt
200
67
Pro Phe Ser Ala His
CCCTTCTCGGCCCAC
CCCTTCTCGGCCCAC
Pro Phe Ser Ala His
Ch
200
67
Pro Phe Trp Ala His
CCCTTCTGGGCCCAC
CCCTTCTCGGCCCAC
Pro Phe Ser Ala His
N
200
67
Pro Phe Trp Ala His
CCCTTCTGGGCCCAC
CCCTTCTGGGCCCAC
Pro Phe Trp Ala His

Fig. 1.

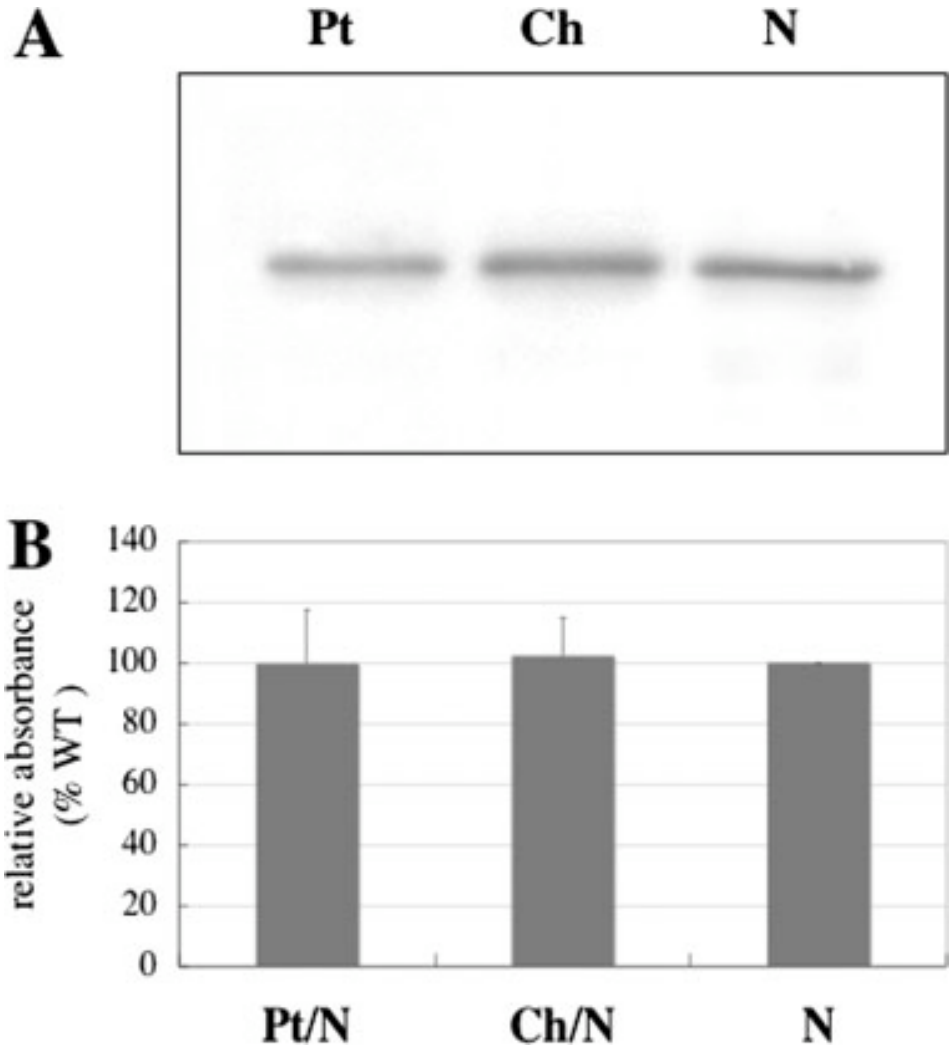
A
Pt
Ch
N
B
relative absorbance
(% WT)
140
120
100
80
60
40
20
0
Pt/N
Ch/N
N

Fig. 2.

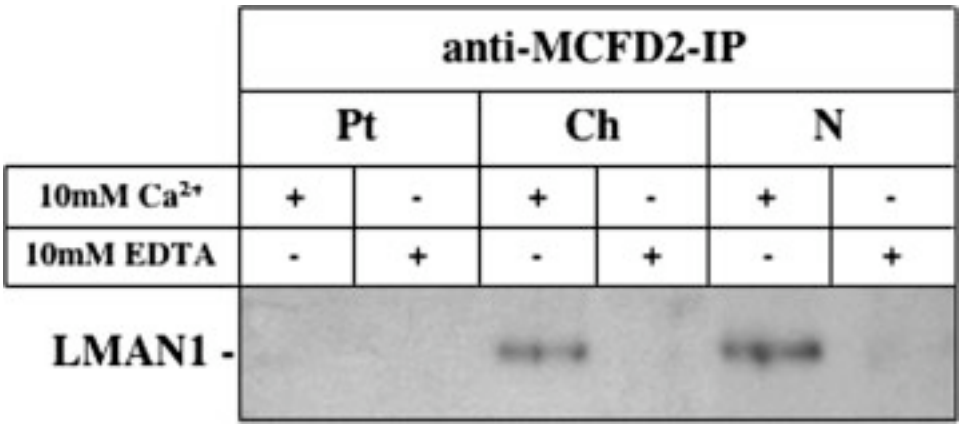

Fig. 3.

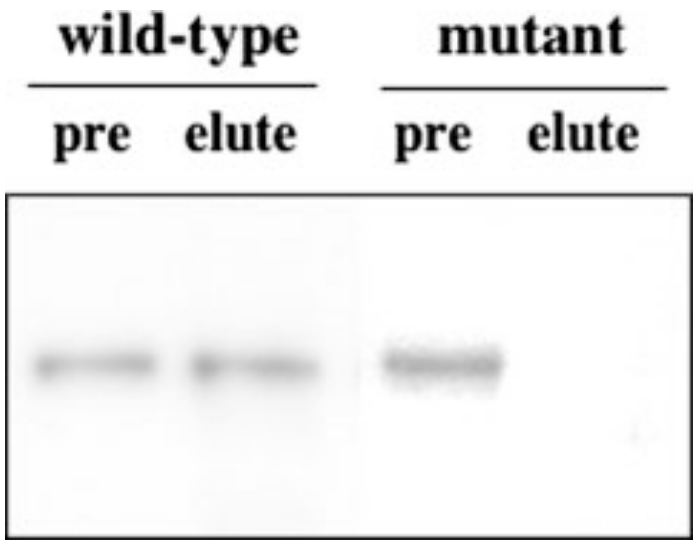

Fig. 4.

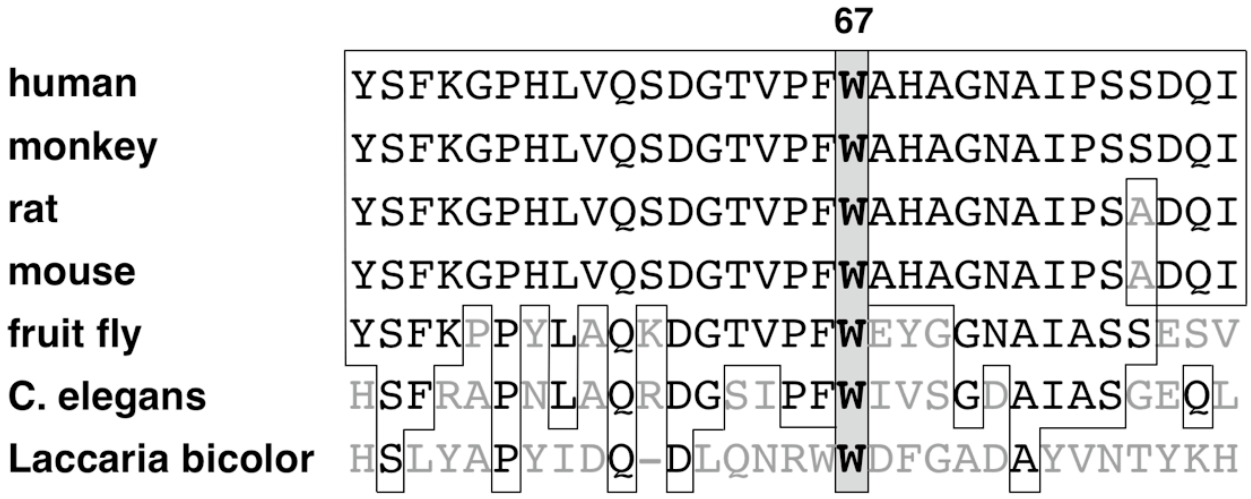
67
human YSFKGPHLVQSDGTVPFWAHAGNAIPSSDQI
monkey YSFKGPHLVQSDGTVPFWAHAGNAIPSSDQI
rat YSFKGPHLVQSDGTVPFWAHAGNAIPSADQI
mouse YSFKGPHLVQSDGTVPFWAHAGNAIPSADQI
fruit fly YSFKPPYLAQKDGTVPFWEYGGNAIASSESV
C. elegans HSFRAPNLAQRDGSIPFWIVSGDAIASGEQL
Laccaria bicolor HSLYAPYIDQ-DLQNRWWDFGADAYVNTYKH

Fig. 5.

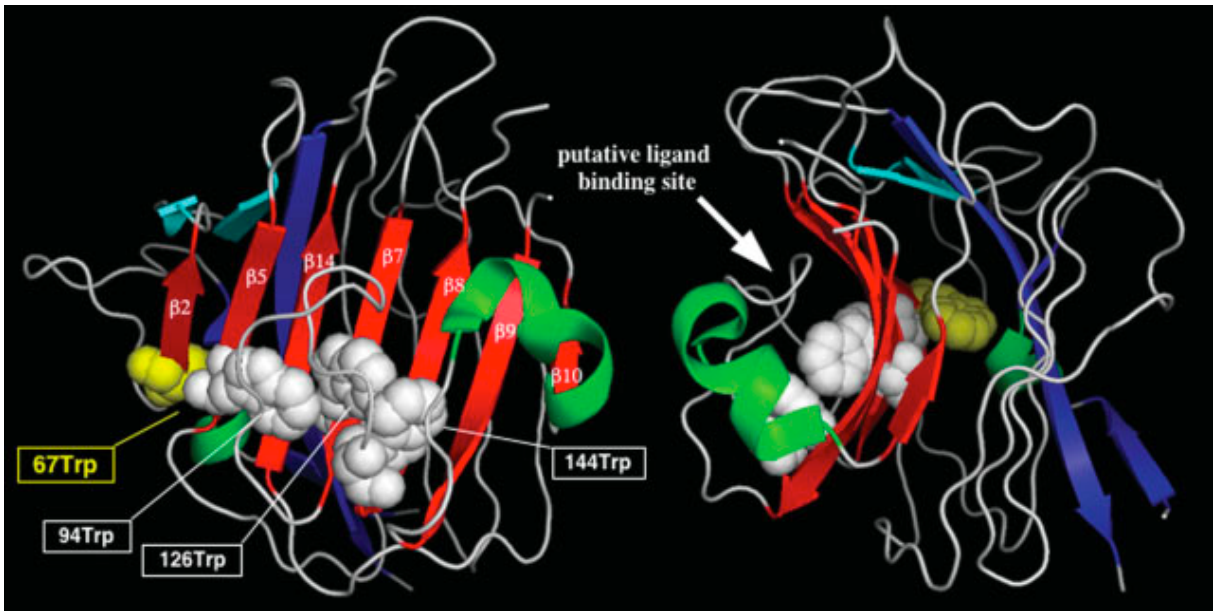
putative ligand
binding site
β2
β5
β14
β7
β8
β9
β10
67Trp
94Trp
126Trp
144Trp

Fig. 6.