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A carboxy-terminal truncation of human alpha-galactosidase A in a heterozygous female with Fabry disease and modification of the enzymatic activity by the carboxy-terminal domain. Increased, reduced, or absent enzyme activity depending on number of amino acid residues deleted.

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Research Article

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A Carboxy-terminal Truncation of Human α-Galactosidase A in a Heterozygous Female with Fabry Disease and Modification of the Enzymatic Activity by the Carboxy-terminal Domain

Increased, Reduced, or Absent Enzyme Activity Depending on Number of Amino Acid Residues Deleted

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Abstract

Fabry disease is an X-linked disorder of glycosphingolipid metabolism caused by a deficiency of α-galactosidase A (α-Gal A). We identified a novel mutation of α-Gal A gene in a family with Fabry disease, which converted a tyrosine at codon 365 to a stop and resulted in a truncation of the carboxy (C) terminus by 65 amino acid (AA) residues. In a heterozygote of this family, although the mutant and normal alleles were equally transcribed in cultured fibroblasts, lymphocyte α-Gal A activity was ~ 30% of the normal control and severe clinical symptoms were apparent. COS-1 cells transfected with this mutant cDNA showed a complete loss of its enzymatic activity. Furthermore, those cotransfected with mutant and wild-type cDNAs showed a lower α-Gal A activity than those with wild type alone (~ 30% of wild type alone), which suggested the dominant negative effect of this mutation and implied the importance of the C terminus for its activity. Thus, we generated mutant cDNAs with various deletion of the C terminus, and analyzed. Unexpectedly, α-Gal A activity was enhanced by up to sixfold compared with wild-type when from 2 to 10 AA residues were deleted. In contrast, deletion of 12 or more AA acid residues resulted in a complete loss of enzyme activity. Our data suggest that the C-terminal region of α-Gal A plays an important role in the regulation of its enzyme activity. (*J. Clin. Invest.* 1996. 98:1809–1817.) Key words: X-linked disorder • de novo mutation • one base insertion • site-directed mutagenesis • electroporation

Introduction

Human α-galactosidase A (α-Gal A;[1] EC 3.2.1.22) is a lysosomal enzyme that catalyzes the hydrolysis of α-galactosidic linkages of glycoconjugates (1). An X-linked inborn deficiency of this hydrolase, which is the cause of Fabry disease, leads to the accumulation of neutral glycosphingolipids, predominantly ceramide trihexoside primarily in the plasma and in the lysosomes of the vascular endothelium. Affected hemizygous males show various clinical symptoms, such as acroparesthesias, angiokeratoma, hypohidrosis, corneal and lenticular opacities, and progressive vascular disease of the kidney, heart, and brain, leading to death in early adulthood (1).

The gene structure of α-Gal A has been determined (2–8) and subsequent studies on unrelated families with Fabry disease have shown a variety of molecular pathology. The 14-kb genomic sequences contain 7 exons encoding the 429 amino acid (AA) α-Gal A polypeptide, including an NH_2-terminal 31-residue signal peptide. More than 70 mutations in the coding region of the α-Gal A gene have been reported (9–21). Most of these result in a classical phenotype with no α-Gal A activity. However, a few mutations with single amino acid substitutions which are located in the carboxy (C) -terminal region lead to an atypical variant of Fabry disease in males, with manifestations limited to the heart (11, 13, 15, 21). It has been suggested that this variant type might be more common than previously believed, occurring in male patients with unexplained left ventricular hypertrophy (21). Several mutations of the α-Gal A gene with different premature stop codons have been described, and all hemizygous patients with these mutations, including E398X which presumably lacks only 32 AA residues of the C terminus, are reported to manifest a classical phenotype (18, 20). Whereas, it was described that 26 or 28 AA residues might be proteolytically removed from the C terminus of α-Gal A to generate a final peptide (5, 6). These suggested that the C-terminal region might be one of active sites or it had an influence on the conformation of the active site.

In the present study, we have identified a novel mutation in a Japanese family with Fabry disease, which resulted in a C-terminal truncation of 65 AA residues. Affected males displayed the classical phenotype of the disease. The proband, who is a heterozygous female, retained relatively high residual α-Gal A activity in lymphocytes compared with the atypical variant males. However, she manifested severe clinical symptoms without limitation to the heart, suggesting some specific effects of the C-terminal truncation on enzyme function. We examined the nature of this mutant α-Gal A by the transfection of its cDNA into COS-1 cells. And to further understand the role of the C-terminal region, we analyzed the activities of artificial α-Gal A mutants obtained by site-directed mutagenesis, which lack various numbers of AA residues at the C terminus.

Methods

Subjects. The proband, a 52-yr-old Japanese female(II-3) with typical manifestations of Fabry disease except for angiokeratoma, and

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1. *Abbreviations used in this paper:* AA, amino acid; α-Gal A, α-galactosidase A; C-, carboxy; RFLP, restriction fragment length polymorphism; RT-PCR, reverse transcriptase–polymerase chain reaction; WT, wild type.

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her kindred were studied (see Fig. 1). Informed consent was obtained from each subject.

Enzyme sources, enzyme assay, and protein determination. Lymphocytes were separated from heparinized blood with Lymphoprep (Daiichi Pure Chemicals, Tokyo, Japan). COS-1 cells transfected with wild type or various mutant α-Gal A cDNAs were collected from culture dishes and lysed in PBS by three cycles of freezing and thawing. The lysates were assayed for α-Gal A activity with 4-methylumbelliferyl α-galactoside as substrate and *N*-acetylgalactosamine (Sigma Chemical Co., St. Louis, MO) as α-galactosidase B inhibitor, according to the method of Beutler et al and Mayes et al (22, 23). Protein concentration was determined by the method of Bradford (24) with Coomassie Protein Assay Reagent (Pierce Chemical Co., Rockford, IL).

Identification of the α-Gal A mutation. Genomic DNA was isolated from peripheral blood lymphocytes (25). Each of the seven exons of the α-Gal A gene was amplified by the PCR (26), using seven primer sets designed according to the human α-Gal A gene sequences (7) (Table I). PCR amplification with 500 ng of genomic DNA was carried out using 100 pmol of each primer, 2.5 mmol/liter of dNTPs and 2.5 U of Ampli Taq DNA polymerase (Perkin Elmer/Cetus, Norwalk, CT) in 100 μl of reaction mixture containing PCR buffer (10 mmol/liter Tris pH 8.3, 50 mmol/liter KCl, 1.5 mmol/liter $MgCl_2$, 0.001% gelatin) using a thermal cycle programmer (model 300; Astec, Fukuoka, Japan) with denaturation at 94°C for 1 min, annealing at 58°C for 1 min, and extension at 74°C for 2 min for 30 cycles. PCR products were purified using Suprec® (Takara Shuzo Co., Ltd., Kyoto, Japan). All coding regions and exon intron junctions of the amplified DNA fragments were directly sequenced according to the snap cooling method (27), using 0.5–1 μg of amplified fragments as the templates, and/or sequenced after subcloning into pUC 19 by the standard method (28), using Sequenase version 2.0 DNA sequencing kits (United States Biochemical Company, Cleveland, OH). In cases of sequence after subcloning, multiple clones were sequenced.

Reverse transcription polymerase chain reaction (RT-PCR) -direct sequencing analysis of α-Gal A mRNA. To analyze α-Gal A gene expression in the heterozygous subject(II-3, Fig. 1), we performed direct sequencing of the RT-PCR (29) products from skin fibroblasts isolated from the forearms, and cultured in MEM supplements with 10% FBS in an atmosphere of 5% CO_2 and 95% air in 10-cm culture dishes. Total RNA was isolated from the cells using Isogen® (Nippon Gene Co., Ltd., Tokyo, Japan). After treatment with DNAase (RNase free) (Sigma) at 37°C for 60 min in DNAase buffer (0.1 mmol/liter sodium acetate, 5 mmol/liter $MnCl_2$), 3 μg of total RNA was reverse transcribed into cDNA with 50 pmol of oligo$(dT)_{17}$, 20 mmol/liter of dNTPs, and 50 U of RNase inhibitor (Promega, Madison, WI) using 12.5 U of Avian Moloney virus-reverse transcriptase (Takara Shuzo Co., Ltd., Kyoto, Japan) in reverse transcriptase buffer (50 mmol/liter Tris pH 8.3, 50 mmol/liter KCl, 8 mmol/liter $MgCl_2$, 5 mmol/liter DTT) at 42°C for 120 min. The sample was then heated to 98°C for 10 min, and immediately chilled on ice.

For PCR amplification of α-Gal A cDNA, a sense primer 5′-ATTGTTGATGTTGCTGGACCAG-3′ (nt 753-774 [nucleotide numbered for sequences of full length α-Gal A cDNA (8)]) and an antisense primer 5′-GTCTTTTAATGACATCTGCATT-3′ (nt 1260–1281) were designed to encompass exon 5 to 7 in order to eliminate possible amplification of genomic DNA. PCR amplification was carried out using one-tenth of the total reverse transcription mixture as described above. The PCR products were directly sequenced as described above.

Detection of the Y365X mutation in the pedigree. For rapid screening of the mutation identified in the proband, which creates a novel MseI restriction site at nt 1095, genomic DNA from the family members were examined by PCR-RFLP (polymerase chain reaction-restriction fragment length polymorphism) analysis. A primer pair (a sense primer, 5′-GAGACAACTTTGAAGTGAAGTGTGG-3′ [nt 1001–1020] and an antisense primer, 5′-AATGACATCTGCATTGTATTTTC-3′ [nt 1274–1252]) was used for amplification, under the same PCR conditions as above using 500 ng of genomic DNA as the template. Amplified DNA fragments were then digested in a 15-μl reaction mixture containing 10 μl of the PCR product, 1.5 μl of 10 × NEB2 buffer, and 10 U of the restriction enzyme MseI (New England Biolab, Beverly, MA). The fragments were then fractionated in a 5% acrylamide gel (8 cm × 6 cm × 0.5 cm, acrylamide: *N*, *N*′-methylene-bis-acrylamide = 19 : 1) and photographed under ultraviolet light after staining with ethidium bromide.

Cloning the mutant α-Gal A cDNA and construction of the expression plasmid. Two overlapping α-Gal A cDNA fragments, one 851-bp fragment covering nt 1–851, and another 998-bp fragment covering nt 299–1296, both with an additional EcoRI site and HindIII site at the 5′ and 3′ end, respectively, were synthesized by the RT-PCR method using 3 μg of total RNA prepared from cultured fibroblasts either from an affected or an unaffected hemizygote. Each amplified fragment was isolated and cleaved with EcoRI and HindIII, and ligated into the EcoRI-HindIII site of pUC 19. The sequence of each fragment was confirmed as described above. The plasmid containing the 851-bp fragment was cut with EcoRI and BglII, and the resultant 520-bp fragment was inserted into the EcoRI-BglII site of expression plasmid pCMV. The plasmid containing the 998-bp fragment was cut with BglII and HindIII, and the resultant 788-bp fragment was ligated to the BglII-HindIII site of pCMV, into which had previously been

Table I. Oligonucleotides Used for α-Gal A Gene Amplification

No.	Sequence	Orientation	Genomic Coordinates
1	5′-AATAAGTCATCGGTGATTGGTCCGC-3′	Sense	1090 to 1114
2	5′-AAGGGAAGGGAGTACCCAATATCTG-3′	Antisense	1378 to 1402
3	5′-CCCAAGGTGCCTAATAAATGGGAGG-3’	Sense	5016 to 5040
4	5′-GCTTACAGTCCTCTGAATGAACAAG-3′	Antisense	5289 to 5313
5	5′-GGTGACTCTTTTCCTCCCTCTCATT-3′	Sense	7240 to 7264
6	5′-CCTTTGTGGCTAAATCTCTGGAATG-3′	Antisense	7456 to 7480
7	5′-GCCCCAGCTGGAAATTCATTTCTTT-3′	Sense	8265 to 8289
8	5′-ACAGTTCTATTGGATTCTGGGCTCAC-3′	Antisense	8421 to 8445
9	5′-GAGAAGGCTACAAGTGCCTCCTTTA-3’	Sense	10041 to 10065
10	5′-ATAGGAAACAAGCCTACCGCAGGGT-3′	Antisense	10321 to 10345
11	5′-GGTTTCTCCATATGGGTCATCTAGG-3′	Sense	10429 to 10453
12	5′-GGCCCAAGACAAAGTTGGTATTGGG-3′	Antisense	10745 to 10769
13	5′-CAGGGCCACTTATCACTAGTTGCTA-3′	Sense	10926 to 10950
14	5′-GGTGGACAGGAAGTAGTAGTTGGCA-3′	Antisense	11283 to 11307

The nucleotide coordinates for genomic sequences are those numbered for the sequences of the 12-kb gene (7).

Table II. Antisense Primers Used in Deletional Mutagenesis of Carboxy-terminal Amino Acid Residues

Mutation	Sequence	cDNA coordinates
pCMV-AGΔ2	5′-TTAAGCT**TCA**GTCTTTTAATGAC-3′	1266 to 1288
pCMV-AGΔ4	5′-GTAAGC**TTA**TAATGACATCTGC-3′	1260 to 1281
pCMV-AGΔ5	5′-TAAGCTTT**TTA**TGACATCTGCATTGT-3′	1255 to 1280
pCMV-AGΔ6	5′-TAAAGCTTTTAA**TTA**CATCTGCAT-3′	1258 to 1281
pCMV-AGΔ7	5′-TAAAGCTTTTAATGA**CTA**CTGCAT-3′	1258 to 1281
pCMV-AGΔ8	5′-TAAAGCTTTTAATGACAT**CTA**CATTGT-3′	1255 to 1281
pCMV-AGΔ9	5′-TTAAGCTTATCTG**CTA**TGTATTTTC-3′	1249 to 1273
pCMV-AGΔ10	5′-CATAAGCTTT**TCA**ATTTTCTAGCTGAAGC-3′	1239 to 1266
pCMV-AGΔ11	5′-CATAAGCTTTGT**CTA**TTCTAGCTGAAGC-3′	1239 to 1266
pCMV-AGΔ12	5′-GCAAGCTT**TTA**TAGCTGAAGCAAAAC-3′	1234 to 1259
pCMV-AGΔ17	5′-CTGAAGC**TTA**AACAGTGCCTGTGGG-3′	1219 to 1243

Oligonucleotide sequences are those of the antisense strand of the carboxy-terminal region of α-Gal A, cDNA as numbered for sequences of full-length cDNA (8). Deviations from the wild-type sequence are underlined. Stop codons are indicated in boldface. HindIII sites are attached to the 5′-end.

inserted the 520-bp-EcoRI-BglII-fragment. Thus, each 1.3-kb fragment, which contained the entire coding region of the mutant or wild type cDNA, was inserted into the cloning site of EcoRI-HindIII of the expression plasmid pCMV. The plasmids were designated pCMV-AGK (for α-Gal A-Kumamoto) or pCMV-AGW (for α-Gal A-wild type), respectively.

Construction of mutant α-Gal A cDNAs. 11 sets of primer pairs, with a sense primer (5′-CAAGGGTACCAGCTTAGACAGG-3′ (nt 979–1000) with a KpnI site at nt 985) and antisense primers, which were modified to create terminating codons at various C-terminal sites and with an additional HindIII site (Table II), were used together to generate PCR fragments using 5 ng of pCMV-AGW as the template. Each fragment generated with each primer pair (sized 258 ∼ 303-bp) was digested with KpnI and HindIII, and inserted into the corresponding KpnI-HindIII site of pCMV-AGW. The resulting plasmids which encode α-Gal A with deletions of C-terminal 2, 4, 5, 6, 7, 8, 9, 10, 11, 12, and 17 AA residues, were designated pCMV-AGΔ2, pCMV-AGΔ4, pCMV-AGΔ5, pCMV-AGΔ6, pCMV-AGΔ7, pCMV-AGΔ8, pCMV-AGΔ9, pCMV-AGΔ10, pCMV-AGΔ11, pCMV-AGΔ12, and pCMV-AGΔ17, respectively. To eliminate any possible PCR error, the entire coding regions of the cDNAs used for the study were sequenced before transfection.

Expression of α-Gal A mutants in COS-1 cells. COS-1 cells were grown at 37°C in Dulbecco's modified Eagle's medium supplemented with 10% FBS in an atmosphere of 5% CO_2 and 95% air to a density of 50% in 10-cm culture dishes. Cells were collected, washed three times with PBS, and separated into aliquots of 5×10^6 cells in 0.7 ml of modified PBS containing 30.8 mM NaCl, 120.7 mM KCl, 8.1 mM Na_2HPO_4, 1.46 mM KH_2PO_4, and 10 mM $MgCl_2$, and used for transfection. Transfections were performed with 10 μg of each plasmid for each experiment using the electroporation method according to the manufacturer's instructions (Gene Pulser Transfection Apparatus, Bio-Rad Laboratories, Richmond, CA). The condition of electroporation was set at either 300 V and 500 μF with a time constant of 14.2±0.5 ms, or 280 V and 250 μF with a time constant of 8.1±0.35 ms. Independent transfections were performed three or four times for each plasmid. After transfection, each sample was separated into two 10-cm culture dishes and grown as described above. The medium was aspirated 12 h after transfection, and after a wash with PBS new medium was added. Cells were harvested from one of two dishes per transfection after 72 h, lysed by three cycles of freezing and thawing in PBS, and α-Gal A activity measured as described above. Cells from the second dish were harvested for RNA preparation. Total RNA was isolated from the cells using Isogen® for Northern blot analysis.

Analysis of α-Gal A mRNA. Northern blot analysis was performed by standard procedures (30). 3 to 10 μg of total RNA were size-fractionated by agarose gel electrophoresis, blotted onto a nitrocellulose membrane, and hybridized for 12 h with cDNA probes labeled by the random primer method. Two cDNA probes were used for hybridization. The first probe was a 1.3-kb EcoRI-HindIII fragment of α-Gal A cDNA prepared from the expression plasmid pCMV-AGW. After hybridization, membranes were washed twice with 0.1X SSC with 0.1% SDS at 60°C for 30 min and exposed to Imaging-Plate® (Fuji Film Co.,Ltd., Tokyo, Japan) and/or x-ray film for an appropriate time. Membranes were then washed with 5X SSC, 5X Denhardt's, 50% formamide, with 0.1% SDS at 70°C for 2 h and rehybridized with the second probe, a ∼ 400-bp fragment of β-actin cDNA (Nippon Gene Co., Ltd.) as an internal control for normalization of the transfection efficiency. Radioactivity was measured using a Bio Image-Analyzer BA100® (Fuji Film Co., Ltd.).

Statistical analysis. Statistical analysis was performed using the nonpaired Student's *t* test. Results are given as mean±SD.

Results

Clinical data. A 52-yr-old female (Fig. 1, II-3) visited a hospital complaining of chest pain and occasional breathlessness since the age of 40, accompanied by periodic crises of severe pain in the extremities, and hypohidrosis since the age of 11 (Table III). On suspicion of hypertrophic cardiomyopathy, myocardial biopsy was performed, which revealed marked vacuolar degeneration. She was admitted to our hospital for further examination. Upon physical examination, she had mild hypertension. Cardiac auscultation revealed a moderate systolic murmur. Slit-lamp examination revealed corneal opacities and tortuous retinal vessels. Electrocardiographic abnormality, and mild cardiomegaly on x-ray film were observed. Angiokeratoma was not present. Hematogram, blood chemistry, and other routine laboratory examinations revealed no abnormality except for proteinuria of 0.4-0.8 g per d. Two of the patient's three sons (Fig. 1, III-2 and III-3) showed almost the same clinical symptoms, and both had angiokeratoma. In addition, the second son (III-2) had had an episode of slight bleeding in the midbrain at 28 yr of age, and the third son (III-3) had been undergoing hemodialysis since the age of 26 because of uremia (Table III). The proband's mother (I-2) and first son (III-1) were clinically normal and the father (I-1) , though not examined because of death due to an unknown cause at 65 yr of age, was considered to be normal according to information from family members. Assays of α-Gal A in lymphocytes re-

Table III. Clinical Characteristics of Affected Individuals

	Heterozygote II-3	Hemizygote 1 III-2	Hemizygote 2 III-3
Age	58	34	29
Acroparesthesia	Since childhood	Since childhood	Since childhood
Hypohidrosis	Moderate	Moderate	Severe
Corneal dystrophy	+	−	n.d.
Angiokeratoma	−	Since adolescence	Since adolescence
Hypertension	+	−	+
Cardiomyopathy	+*	+	+
Proteinuria	Mild	Mild	Severe
Creatinine clearance (ml/min)	60.8	51.2	< 5‡
Brain vascular disease	−	+§	−
Lymphocyte α-Gal A activity (nmol/h per mg protein)	24.5	0.7	0.9

n.d., not determined. *myocardial vacuolar degeneration was detected by biopsy; ‡receiving hemodialysis since the age 26; §developed small bleeding in the midbrain at the age of 28.

vealed partial deficiency of enzymatic activity in the proband (24.5 nmol/h/mg protein), and severe deficiency in two affected sons (0.7 and 0.9 nmol/h/mg protein, respectively).

Identification of the α-Gal A mutation. Direct nucleotide sequencing of the PCR products from genomic DNA of the affected individuals (proband and two sons) revealed an adenine nucleotide insertion at nt 1095 in exon 7 of the coding sequence, which resulted in a tyrosine-to–stop codon substitution at amino acid residue 365 (Y365X), causing a C-terminal truncation by 65 AA residues (Fig. 2). Because of the presence of the insertion in one allele and the absence in the other, overlapped bands were observed after nt 1096 on sequence analysis of the proband.

RT-PCR-direct sequencing analysis of α-Gal A mRNA. Transcription of the Y365X mutation into mRNA was confirmed by the RT-PCR method in the hemizygous males (III-2 and III-3, data not shown). In RT-PCR-direct sequence analysis of cultured fibroblasts from the heterozygous female (II-3), overlapped bands were observed after nt 1096 (Fig. 3). Corresponding bands derived from mRNA transcribed from the wild type (WT) or the mutant alleles showed almost the same densities as those observed in genomic PCR direct sequencing shown in Fig. 2. Assuming that the density of each band in direct sequence analysis corresponded to the amount of each template (i.e., RT-PCR product), and that the amount of each RT-PCR product was consistent with the amount of mRNA transcribed from each allele, this finding suggested that there was no difference between the amounts of mRNAs transcribed from the WT and the mutant allele (compare bands 1 and 2, 3 and 4, 5 and 6, 7 and 8, 9 and 10, and 11 and 12, respectively, in Fig. 3).

Detection of the Y365X mutation in the pedigree. In order to identify the Y365X mutation in the members of the family, we performed PCR-RFLP analysis. Two bands of 208 and 66-bp were observed for the proband's mother and the unaffected son, three bands of 115, 94, and 66-bp for two sons, and four bands of 208, 115, 94, and 66-bp for the proband (Fig. 4). Therefore, two affected sons (III-2 and III-3, respectively, in

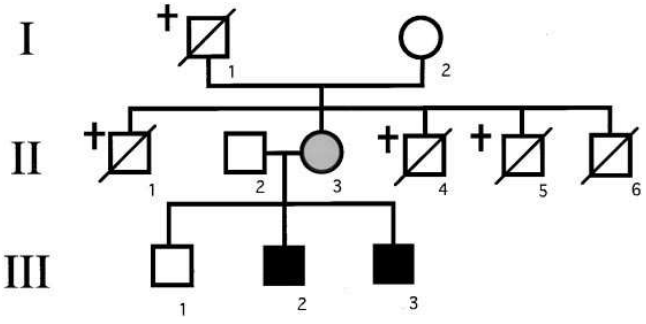


Figure 1. Family pedigree. Males are denoted by squares, females by circles. Clinical status is indicated as follows: closed symbols, typical clinical manifestations of Fabry disease; shaded symbol, the proband; open symbols, unaffected individuals; oblique line, not examined; †, deceased; (the proband's father [*I-1*], deceased at age 65, was reported to have no clinical symptoms of Fabry disease).

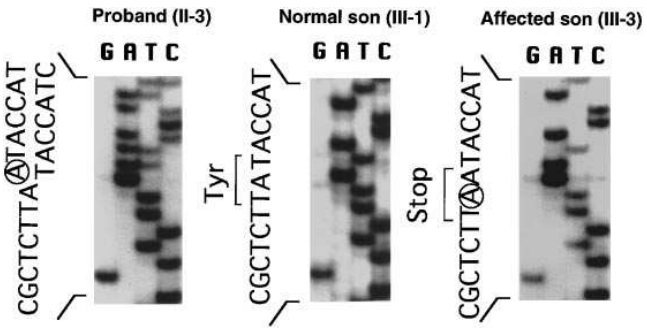


Figure 2. Sequence analysis of the α-Gal A gene of family members. PCR products from the genomic DNAs of subjects II-3, III-2, and III-3 in Fig. 1 were directly sequenced as described in Methods. The nucleotide inserted at position 1095 in exon 7 is indicated by the circle. This insertion results in substitution of Tyr^{365} by a stop codon.

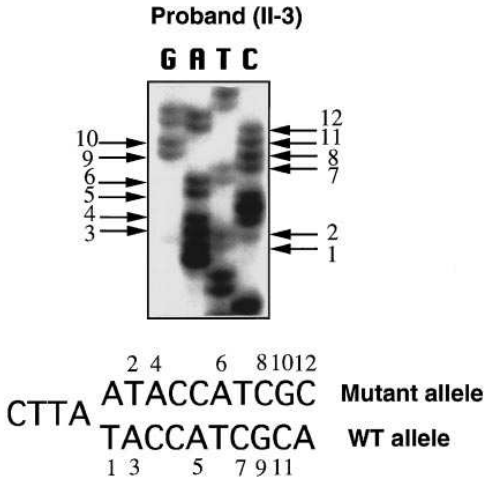


Figure 3. Analysis of expression of wild type and mutant genes in a heterozygote. The amplified product from the proband's cDNA (*II-3*), which was obtained from the RNA preparation from cultured fibroblasts by reverse transcription, was directly sequenced as described in Methods. Bands 1, 3, 5, 7, 9, and 11 are considered to be those extended from the transcript of wild-type mRNA in the cultured fibroblasts, while bands 2, 4, 6, 8, 10, and 12 are from that of the mutant mRNA. The ratios of the density of each band derived from WT to the corresponding band from the mutant, 1:2, 3:4, 5:6, 7:8, 9:10, and 11:12, respectively, were determined as described in Methods.

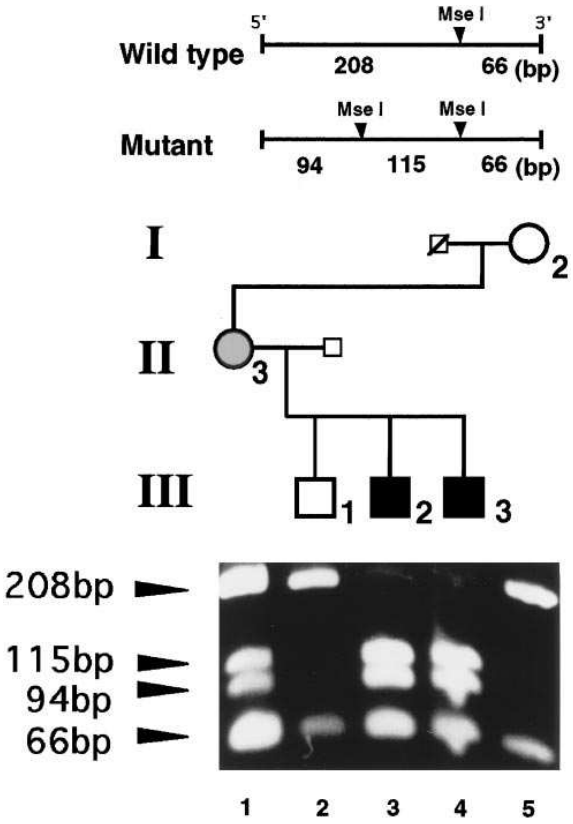


Figure 4. Detection of the Y365X mutation in the pedigree. Genomic DNA from individuals (symbols in Fig. 1) was amplified by PCR to obtain a 270-bp fragment including the codon 365. Amplified DNA fragments were then digested with the restriction enzyme Mse I, fractionated on a 5% polyacrylamide gel, stained with ethidium bromide, and photographed under ultraviolet light. Four bands derived from normal and mutant fragments were obtained from the proband (lane *1*). Three bands from the mutant fragment were obtained from the two affected sons (lanes *3* and *4*), and only two bands from the normal fragment were observed in an unaffected son and the proband's mother (lanes *2* and *5*).

Fig. 1) were hemizygous, and the proband (II-3) was heterozygous for the mutation. Although the proband's mother (I-2) had been expected to be an asymptomatic heterozygote by pedigree analysis (Fig. 1), both she and the unaffected son (III-1) were shown to be normal. The proband's mother's genomic DNA was directly sequenced and it was confirmed that there was no mutation at codon 365 (data not shown). This implied that this was a de novo mutation, which had occurred in the germ line of either of the proband's parents, although our data do not exclude the possibility that the proband's father was an asymptomatic or a mildly symptomatic hemizygote.

Cloning the mutant α-Gal A cDNA and expression in COS-1 cells. The expression plasmids, pCMV-AGW and pCMV-AGK, which contain the entire coding region of the wild type and mutant cDNAs of α-Gal A, respectively, were constructed as described above (Fig. 5 *A*). Expression of the transfected α-Gal A cDNA was examined by Northern blot analysis. Both the cells without transfection and with a mock transfection (transfected with vector alone) showed a very faint band of α-Gal A mRNA, which was considered to be an endogenous product of COS-1 cells. Cells transfected with pCMV-AGW or with pCMV-AGK showed distinct α-Gal A bands. There was no significant difference in the amounts of α-Gal A mRNA observed in cells transfected with pCMV-AGW or with pCMV-AGK (Fig. 5 *B*). However, COS-1 cells transfected with pCMV-AGW showed a 16-fold increase of α-Gal A activity when compared with those with a mock transfection, while those transfected with pCMV-AGK showed no significant increase (Fig. 5 *C*). These results suggest that transcription of Y365X cDNA is normal, but that α-Gal A activity is completely absent in this mutant.

Since it is known that α-Gal A forms a homodimer in lysosomes (1), Y365X mutant could have the dominant negative effect in the heterozygous genotype. To examine this possibility, 10 μg of plasmid DNA containing a 1: 1 mixture of mock plasmid and pCMV-AGW or a 1: 1 mixture of pCMV-AGK and pCMV-AGW were cotransfected to COS-1 cells. The experiments were performed in triplicate for each cotransfection. The Northern blots of α-Gal A mRNA are shown in correspondence with β-actin mRNA (Fig. 6 *A*). After subtraction of the α-Gal A activity of mock transfected cells and normalization with the amount of α-Gal A mRNA (with the wild type/mutant co-transfection, it was assumed that half the total α-Gal A mRNA was derived from WT α-Gal A cDNA, and the rest was from the Y365X mutant), there was a significant decrease ($\sim$ 40%) in the α-Gal A activity of cells cotransfected with WT/mutant mixture compared with the wild type/mock plasmid mixture (Fig. 6 *B*, $P < 0.05$, $n = 3$). The α-Gal A activity of cells cotransfected with pCMV-AGK and pCMV-AGW, when corrected for the total α-Gal A mRNA (including both Y365X mutant and WT), was $\sim$ 30% of that of cells cotransfected with mock plasmid and pCMV-AGW. This suggested that the mutant α-Gal A had a dominant negative effect.

In vitro expression of the C-terminal truncated mutants. To further elucidate the function of the C-terminal region of

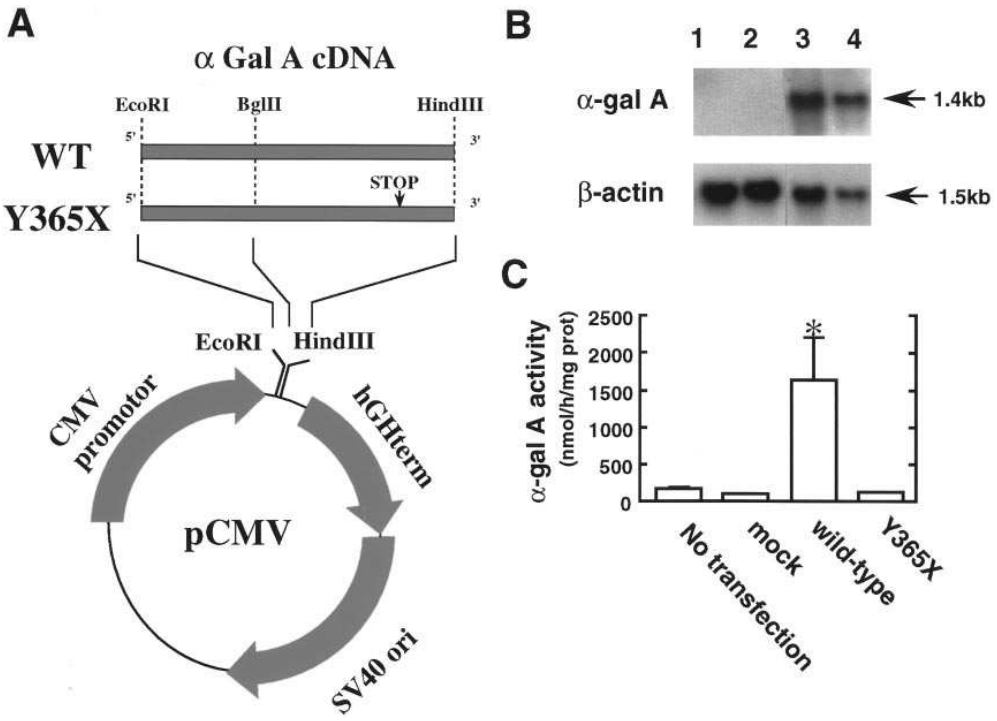
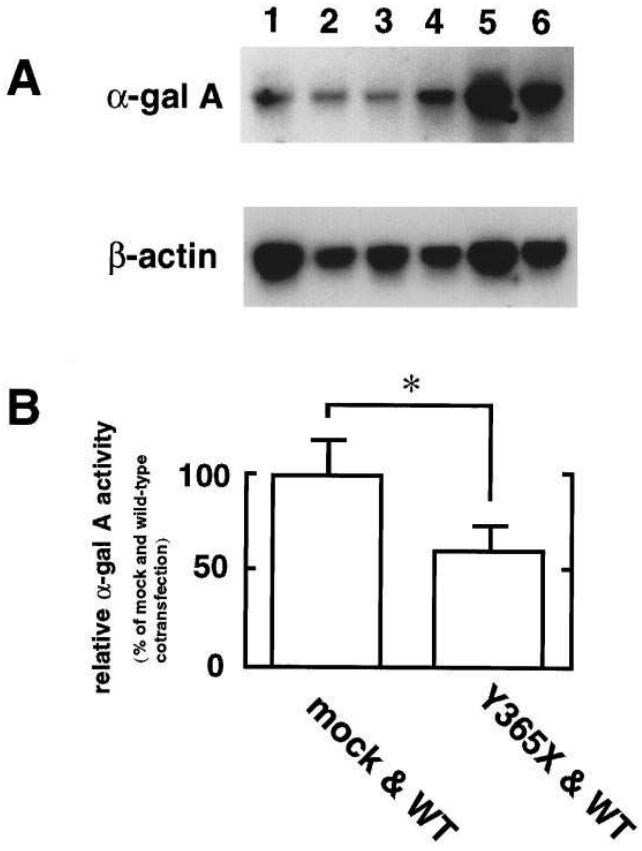


Figure 5. Expression of the Y365X mutant α-Gal A in COS-1 cells. (*A*) The construction of expression plasmids. Normal (WT) or mutant (Y365X) α-Gal A cDNA were inserted into the EcoRI-HindIII cloning site of pCMV. (*B*) Northern blot analysis of COS-1 cells transfected with α-Gal A cDNA. 10 μg of plasmids were transfected to COS-1 cells by the electroporation method. 3 d after transfection, RNA was extracted from the cells transfected with each plasmid. 10 μg of total RNA was size-fractionated by the agarose gel electrophoresis and hybridized with α-Gal A cDNA probe (*upper panel*), and with β-actin cDNA probe as an internal control (*lower panel*). Lane *1*; no transfection, lane *2*; mock transfection (the vector without cDNA insertion), lane *3*; pCMV-AGW (WT cDNA), lane *4*; pCMV-AGK (Y365X). (*C*) α-Gal A enzyme assay. α-Gal A activities of the cell lysates were assayed by standard methods. $*P < 0.05$ vs. mock transfection ($n = 3$).



α-Gal A, we constructed mutant α-Gal A cDNAs which resulted in various C-terminal truncations of the α-Gal A protein and studied their activities in COS-1 cells. Mutant plasmids, pCMV-AGΔ2 (Δ2), pCMV-AGΔ4 (Δ4), pCMV-AGΔ5 (Δ5), pCMV-AGΔ6 (Δ6), pCMV-AGΔ7 (Δ7), pCMV-AGΔ8 (Δ8), pCMV-AGΔ9 (Δ9), pCMV-AGΔ10 (Δ10), pCMV-AGΔ11 (Δ11), pCMV-AGΔ12 (Δ12), and pCMV-AGΔ17 (Δ17), which were considered to express α-Gal A mutants lacking C-terminal 2, 4, 5, 6, 7, 8, 9, 10, 11, 12, and 17 AA residues, respectively, were transfected to COS-1 cells (C-terminal sequences are shown in Fig. 7 *A*). The efficiency of each trans-

Figure 6. Effect of cotransfection with Y365X mutant and WT α-Gal A cDNA. 5 μg of pCMV-AGW (WT plasmid) was transfected together with either 5 μg of pCMV-AGK (Y365X mutant) or 5 μg of vector to COS-1 cells, and analyzed by Northern blot and enzyme assay. (*A*) Northern blot analysis. Northern blot was performed as in Fig. 4. 10 μg of total RNA prepared from the transfected cells were used for hybridization. Radioactivities of the bands were measured using Bio Image-Analyzer BA100 (Fuji Film Co., Ltd.). Lanes *1*, *2*, and *3* each represent an independent cotransfection with mock and wild-type plasmid, lanes *4*, *5*, and *6* each represent an independent cotransfection with Y365X and WT. (*B*) α-Gal A enzyme assay. The α-Gal A activities of the cell lysates were assayed as in Fig. 4. Obtained data in each experiment was subtracted with that of mock-transfection, and normalized by the ratio of the WT α-Gal A mRNA to the β-actin mRNA in Northern blot. Values represent mean±SD (as percent control of the activity in cells cotransfected with mock and WT plasmid) for $n = 3$. $*P < 0.05$.

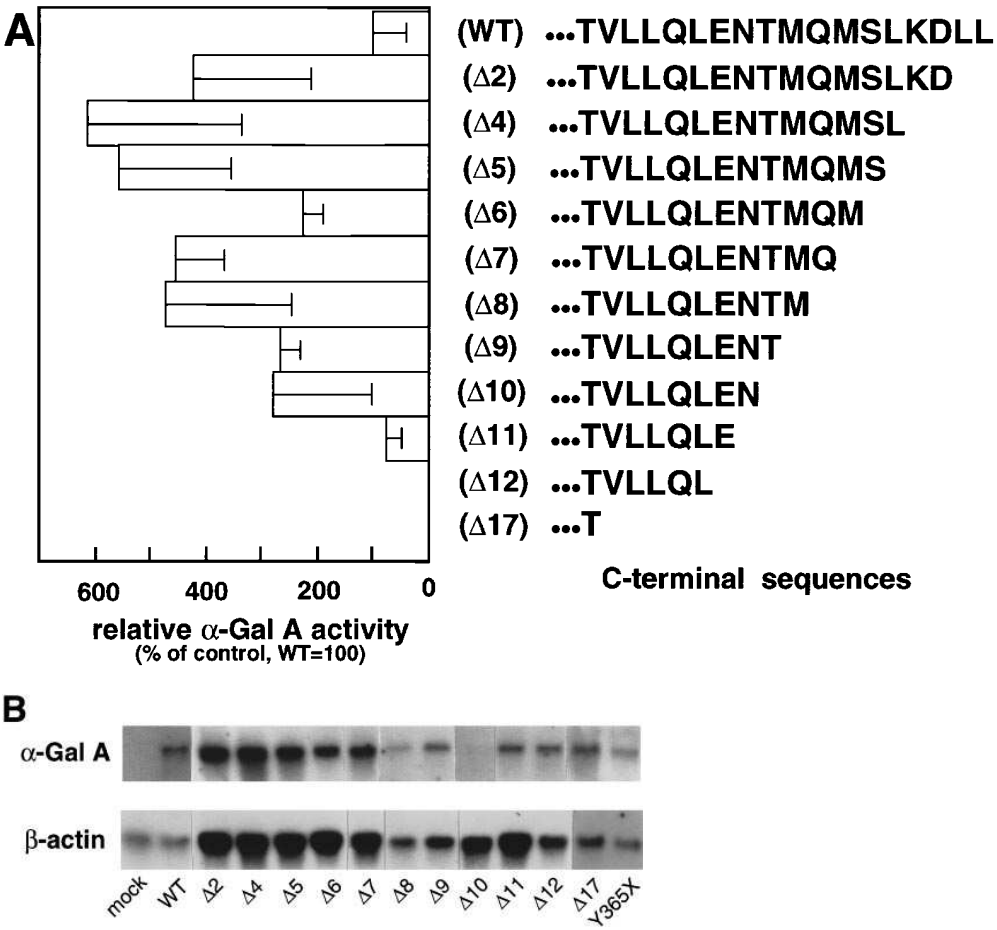


Figure 7. Enzyme activity of artificial α-Gal A mutants with C-terminal deletions with various numbers of amino acid residues. (*A*) Mutant cDNAs with premature stop codons yielding the deletions described above, were obtained by site-directed mutagenesis, and inserted into the EcoRI-HindIII cloning site for the transient expression. Amino acid sequence of the C-terminal residues of wild-type α-Gal A and mutants are shown in the right. α-Gal A activities obtained from cell lysates in transfections with these plasmids are shown in the left. α-Gal A activity was adjusted by subtraction of activity seen after mock-transfection, and normalization by the ratio of the α-Gal A mRNA to the β-actin mRNA in the Northern blot. Values are shown as % control (WT = 100), representing mean for $n = 2$ or 3. (*B*) Northern blots were performed as in Fig. 4. A representative autoradiogram is shown.

fection was estimated by the ratio of the radioactivity of the α-Gal A mRNA band to that of the β-actin band in Northern blot, as described in the Methods (a representative autoradiogram of the Northern blot is shown in Fig. 7 *B*). Unexpectedly, transfections with Δ2, Δ4, Δ5, Δ6, Δ7, Δ8, Δ9, and Δ10 plasmids markedly increased α-Gal A activity in comparison with mock transfection. Moreover, even when compared with WT, transfections with these C-terminal truncated mutants showed significant increases in enzyme activity. The α-Gal A activities observed after normalization and mock subtraction were ~ 420, 620, 560, 230, 460, 480, 270, and 280% of that of WT α-Gal A with Δ2, Δ4, Δ5, Δ6, Δ7, Δ8, Δ9, and Δ10, respectively (Fig. 7 *A*). In the transfection experiments with Δ10, α-Gal A mRNA bands in the Northern blot were faint compared with β-actin, suggesting either an abnormality in transcription or an instability of this mRNA (Fig. 7 *B*). Transfection with Δ11 resulted in mild reduction of activity compared with WT, and those with Δ12 and Δ17 plasmids resulted in complete depletion of α-Gal A activity (Fig. 7 *A*).

Discussion

Fabry disease is an X-linked recessive disorder caused by a deficiency of α-Gal A, which affects about 1 in 40,000 people (1). After the isolation and characterization of the cDNA and genomic sequences encoding human α-Gal A, a variety of molecular lesions causing Fabry disease has been demonstrated, as reviewed by Eng and Desnick (31). In the present study, we identified a novel mutation of the human α-Gal A gene (Y365X), which resulted in a C-terminal truncation by 65 AA residues. Two hemizygous siblings of the pedigree showed a classical form of Fabry disease, and an affected female showed relatively severe clinical symptoms. By the expression study of Y365X mutant α-Gal A, we could demonstrate that this mutation led to a complete depletion of enzyme activity.

Interestingly, in the proband who was a heterozygous female, clinical symptoms were more severe than those seen in the usual heterozygote including cardiac involvement and hypohidrosis, of which each estimated incidence based on review of over 122 heterozygous females has been reported to be less than 1%, respectively (1). A few heterozygotes have previously been reported in whom the expression of the disease was comparable to that observed in severely affected hemizygous males (32–34). In contrast, obligate heterozygotes without any clinical manifestations and with normal levels of leukocyte α-Gal A have been reported (34, 35). Such markedly variable expression is expected in females heterozygous for X-linked diseases due to random X inactivation (36). At the cellular level, carriers of Fabry disease have been shown to have two distinct clonal populations of cells, one with normal and the other with defective α-Gal A activity, providing evidence of random X inactivation (37). However, verifications of this theory in most cases have not easily been done. In this study, in order to analyze the expression of α-Gal A gene, we introduced a novel method of RT-PCR direct sequencing. This enabled us to estimate the ratio of the expression of the normal

allele to that of the mutant allele to be 1:1 in cultured fibroblasts from the heterozygous female of the family. Lymphocytes α-Gal A activity was lower than that expected, to be ~ 30% of the normal control. This suggested that, apart from the random X chromosomal inactivation, there might be some interaction between the normal and mutant α-Gal A proteins such as heterodimerization, since this enzyme is known to exist as a homodimer (7). To investigate this, we cotransfected equal amounts of WT and mutant cDNAs to COS-1 cells for transient expression. The resultant α-Gal A activity, corrected by the total α-Gal A mRNA (including both Y365X mutant and WT), was ~ 30% of that of cells transfected with wild type cDNA alone (i.e., cotransfection with mock plasmid). This could be due to an interaction between the normal and Y365X mutant α-Gal A proteins. To understand this dominant negative effect of Y365X, further analysis determining the functional domains of the α-Gal A protein is necessary.

Several mutations of the α-Gal A gene with different C-terminal truncations have previously been reported, and all hemizygous patients with these mutations are reported to manifest a classical phenotype. Whereas a few mutations with single amino acid substitutions which are located in the C-terminal region are known to lead to an atypical variant in males, with manifestations limited to the heart. These findings and our present study of Y365X mutant suggested that the C-terminal region might carry an important functional role for α-Gal A enzymatic activity. Therefore, we further examined the role of the C-terminal domain by generating artificial mutant α-Gal As which lacked various number of AA residues at the C terminus, and analyzed their enzymatic activity. One striking result was the demonstration that deletions up to 10 AA residues in the extreme C terminus of α-Gal A led to a significant increase in its enzymatic activity. Deletion of the first two residues, the Leu-Leu sequence, from the C-terminal end of α-Gal A resulted in a 4.2-fold increase in enzyme activity in COS-1 cells compared with WT. Removal of 4, 5, 6, 7, 8, 9, and 10 AA induced 6.2, 5.6, 2.3, 4.6, 4.8, 2.7, and 2.8-fold increases, respectively. In transfection with Δ10, the α-Gal A mRNA band in the Northern blot was always faint compared with other mutants, suggesting either an abnormality in transcription or an instability of the mRNA. The mutant which lacks C-terminal 11 AA residues (Δ11) showed a 23% reduction in enzyme activity compared with WT. Removal of only one additional AA (Δ12), and also deletion of 17 C-terminal residues (Δ17) resulted in complete depletion of the enzyme activity in COS-1 cells. Thus, three of the mutant α-Gal A enzymes with a C-terminal truncation (Δ12, Δ17, and Δ65) retained no enzyme activity.

Our data show that the mutant α-Gal A activity is increased compared with wild type when from 2 to 10 AA residues in the C-terminal region are deleted, mildly reduced when 11 AA residues are deleted, and completely absent when 12 or more AA residues are deleted. These data suggest that there might be some functional domain in the C-terminal sequence of the last 10 AA residues which inhibit enzyme activity of α-Gal A. In order to specify the functional domains, we searched databases for sequences similar to the C terminus of α-Gal A. However, any similar or homologous sequence could not be identified, including in the sequence of α-galactosidase B (α-*N*-acetylgalactosaminidase) which has remarkable amino acid homology and is considered to have evolutionary relatedness with α-Gal A (38). As regards other cytoplasmic proteins such as endoplasmic reticulum resident proteins or peroxisomal proteins, the role of the C-terminal domain has been described. In endoplasmic reticulum resident proteins such as grp78, disulfide isomerase, and glucose-6-phosphatase, the tetra peptide Lys-Asp-Glu-Leu (39) or the Leu-Leu motif (40–42) in the C-terminal domain have been identified as the specific signals for retention in endoplasmic reticulum. A conserved tripeptide Ser-Lys-Leu in the extreme C terminus of peroxisomal proteins such as rat acyl-CoA oxidase has been identified as a peroxisomal targeting sequence (43, 44). These findings of other cytoplasmic proteins and our data suggest the possibility that some specific signals in the C terminus of α-Gal A regulate its enzymatic function, and importance of the role of C-terminal domain.

Therapeutic approaches using enzyme replacement are under investigation for lysosomal storage diseases, including Fabry disease. Recent success in the therapy of Gaucher's disease using mannose-terminated (macrophage-targeted) human glucocerebrosidase has demonstrated that this approach is valid and that improvement of the enzyme delivery system is important (45, 46). For Fabry disease, α-Gal A replacement using α_2-macroglobulin as a transport vehicle has been suggested (47). As described above, removal of several residues from the C-terminal sequence of WT α-Gal A resulted in a remarkable increase of its enzyme activity, which suggests the possibility of using these mutant enzymes in replacement therapy, as has been done in insulin therapy using a C-terminal mutant (48), and in the future, in gene therapy.

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