Volume 259, number 2, 353-356 FEB 07999 January 1990

A case of Fabry's disease in a patient with no α-galactosidase A activity caused by a single amino acid substitution of Pro-40 by Ser

Tsuyoshi Koide*, Masahiro Ishiura*, Kunimitu Iwai°, Michitoshi Inoue+, Yasufumi Kaneda†, Yoshio Okada† and Tsuyoshi Uchida*†

*Division of Cell Fusion, Department of Cell Biology, National Institute for Basic Biology, Myodaijicho, Okazaki, Aichi 444, °The First Department of Medicine, Osaka University Medical School, Nakanoshima, Kitaku, Osaka 530, +Department of Medical Information Science, Osaka University Medical School, Nakanoshima, Kitaku, Osaka 530 and †Institute for Molecular and Cellular Biology, Osaka University, Yamada-oka, Suita, Osaka 565, Japan

We analyzed a male patient with Fabry's disease who had no activity of the lysosomal hydrolase α-galactosidase A (α-GalA) and female members of his family. We cloned a cDNA that encoded the mutant α-GalA, determined its nucleotide sequence, and found two nucleotide differences between the mutant and the wild-type cDNAs. Although one difference was silent, the other difference, a C-to-T transition at nucleotide number 118, resulted in an amino acid substitution of Pro-40 by Ser. A transient expression assay demonstrated that this missense mutation was the cause of the deficiency of α-GalA activity in the patient. In vitro mutagenesis experiments demonstrated that Pro-40 is critical for the appearance of α-GalA activity.

Fabry's disease; α-Galactosidase A; cDNA; Nucleotide sequence; Missense mutation; (Human)

1. INTRODUCTION

FD is an X-linked, recessive, genetic disease which is caused by a deficiency in the activity of the lysosomal hydrolase α-GalA (EC 3.2.1.22) [1]. The absence of this enzymatic activity causes the accumulation of glycosphingolipids, such as globotriaosylceramide and galabiosylceramide, primarily in the lysosomes of the vascular endothelia and in plasma [1]. α-GalA is synthesized as a 50 kDa precursor glycopeptide and processed to a 46 kDa glycopeptide, and the mature enzyme is a homodimer of the 46 kDa subunits [1]. Recently, the nucleotide sequences of a cDNA and a genomic gene encoding human α-GalA were determined [2,3]. However, the functional domains of α-GalA protein that are required for its maturation, including glycosylation and modification of the sugar residues, transport into lysosomes, dimerization, stabilization, and the enzymatic activity per se are poorly understood. The accumulation of missense mutations in α-GalA protein by an analysis of many patients with FD should be useful for characterization of the functional domains. Only one missense mutation that results in the deficiency of α-GalA activity has been reported thus far [4].

We report that a single amino acid substitution of Pro-40 by Ser resulted in the deficiency of α-GalA activity in a male patient with FD and we describe results of in vitro mutagenesis experiments that demonstrate that the Pro-40 residue is critical for the appearance of the enzymatic activity.

Correspondence address: M. Ishiura, National Institute for Basic Biology, Myodaijicho, Okazaki, Aichi 444, Japan

This paper is dedicated to the late Professor Tsuyoshi Uchida, who initiated and organized this project

Abbreviations: α-GalA, α-galactosidase A; α-MEM, alpha modification of Eagle's minimum essential medium; EF2, elongation factor 2; FCS, fetal calf serum; FD, Fabry's disease; PCR, polymerase chain reaction; Pro-40, Pro at the 40th amino acid residue of an α-GalA precursor protein which has a signal sequence of 31 amino acids at its amino terminus; SV40, Simian virus 40

These sequence data will appear in the EMBL/GenBank/DDBJ Nucleotide Sequence Databases under the accession number X16889

2. MATERIALS AND METHODS

2.1. *Cell cultures*

Skin fibroblasts were isolated from the forearms of a patient with FD (FIS cells) and a normal male volunteer (NSF cells). They were transformed with the gene for the large T-antigen of SV40 and maintained in α-MEM supplemented with 10% FCS.

2.2. *Preparation and analysis of RNA, construction of cDNA library, and nucleotide sequencing*

Preparation and analysis by Northern blotting of poly(A)$^+$ RNAs were carried out as described previously [5]. A λgt11 cDNA library was constructed from the poly(A)$^+$ RNA of FIS cells by a standard procedure [5]. We isolated a full-length cDNA that encoded α-GalA from the library by plaque hybridization, using two ^{32}P-labeled 30-mer oligonucleotides, RAG1 (5'-GAGTACACAATGCTTCTGCCAGTCCTATTC-3') and RAG2 (5'-AAGGTTGCACATGAAGCGCTCCCAGTGCAG-3') and RAG2 (5'-AAGGTTGCACATGAAGCGCTCCCAGTGCAG-3'), as hybridization probes. The *Eco*RI insert of λAGF11 was subcloned into the *Eco*RI site of pUC118 (pAGF11-5). Nucleotide sequences were determined by the chain termination method [5].

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50

2.3. *Analysis of the α-GalA gene in genomic DNA by the PCR*

Genomic DNAs were prepared from fibroblasts or leukocytes by a standard method [5]. A specific 192-bp segment of DNA from the α-GalA genomic gene was amplified by the PCR [6], using a GeneAmp DNA amplification reagent kit (Perkin Elmer Cetus, Norwalk, USA), genomic DNAs derived from various individuals as templates, and PFS1 (5′-CAATGAAGCTGAGGAACCCA-3′) and PFS2 (5′-GATGCAGGAATCTGGCTCTT-3′) oligonucleotides as primers. The PFS3 oligonucleotide (5′-AGAACTGCATCTGGGCT-3′) was used as a sequencing primer.

2.4. *Site-directed mutagenesis and transient expression assay*

pMUP, pMUT and pMUG were constructed by site-directed mutagenesis [7], with single-stranded pAGF11-5 DNA as a template and, as primers, the three following oligonucleotides, which contained single or double point mutations (underlined), 5′-GATTGGCAAGGACGCCTACCATGGGCTGGC-3′, 5′-GATTGGCAAGGACGACTACCATGGGCTGGC-3′, and 5′-GATTGGCAAGGACGGGTACCATGGGCTGGC-3′. Each *Eco*RI insert of pMUP, pMUT, pMUG and pAGF11-5 was inserted into the *Eco*RI site of the pBactE expression vector, and the products were named pBa40P, pBa40T, pBa40G and pBa40S, respectively. pBactE is a derivative of pBactS′ (Furuichi et al., unpublished; a gift from Furuichi) which carries the 5′-flanking region and the first intron of the chick gene for β-actin [8], which contains the promoter and enhancer (see fig.3B). These plasmids were transiently introduced into FIS cells deficient in α-GalA activity by the calcium phosphate method [9]. α-GalA activity was determined by a standard method [10], using 4-methylumbelliferyl α-D-galactoside (Sigma, St. Louis, USA) as substrate.

3. RESULTS AND DISCUSSION

3.1. *Northern blotting analysis*

Poly(A)$^+$ RNAs from mutant FIS cells and wild-type NSF cells were analyzed by Northern blotting using an anti-sense probe (RAG1) that contained a sequence that is complementary to α-GalA mRNA (fig.1). A mouse EF2 gene probe was also used as a control. Only a single 1.3-kb band of α-GalA mRNA was observed whether the poly(A)$^+$ RNA isolated from NSF cells or that from FIS cells was analyzed. The intensities of both the band of α-GalA mRNA and the band of EF2 mRNA, as a control in the preparation of FIS poly(A)$^+$ RNA were slightly stronger than those obtained with the preparation of NSF poly(A)$^+$ RNA. However, the intensity of the band of α-GalA mRNA normalized by reference to the intensity of the band of EF2 mRNA was almost the same in the two preparations of poly(A)$^+$ RNA, suggesting that the level of α-GalA mRNA was almost the same in both FIS and NSF cells.

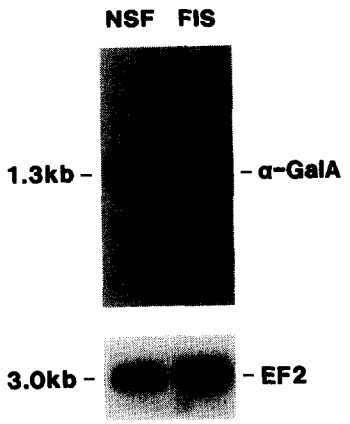

Fig.1. Analysis by Northern blotting of α-GalA mRNA. Poly(A)$^+$ RNAs (2 μg) prepared from wild-type NSF and mutant FIS cells were subjected to electrophoresis on an agarose gel, blotted onto a nylon membrane, and hybridized with the RAG1 probe (α-GalA gene probe, upper panel). The same membrane was also rehybridized with a control probe for the EF2 gene (lower panel).

3.2. *Sequence of the cDNA for the mutant α-GalA cloned from the patient*

We isolated the full-length cDNA λAGF11 that encoded the mutant α-GalA from FIS cells, determined its nucleotide sequence, and found two nucleotide differences between the FIS mutant cDNA and the reported sequence of wild-type cDNA [2]: A-to-G and C-to-T nucleotide substitutions at nucleotide numbers 24 and 118, respectively (fig.2). Although the former substitution was silent, the latter resulted in an amino acid substitution of Pro-40 by Ser. This missense mutation is located at the 9th amino acid residue of the mature α-GalA protein and on the first exon of the genomic gene. According to calculations by the Chou-Fasman program [11], the wild-type protein forms a β-sheet structure and an α-helix at the regions of the amino acid residues from positions 38 to 40 and 43 to 52, respectively (fig.3A,a), whereas the mutant protein forms a β-sheet structure at the region of the amino acid residues from positions 39 to 52 (fig.3A,b).

3.3. *Transient expression assay of α-GalA activity*

To determine whether the deficiency of α-GalA activity in the patient resulted from the amino acid substitution described above, we introduced two cDNAs that encoded the wild-type (pBa40P) and the mutant (pBa40S) α-GalA, respectively (fig.3B), into FIS cells and assayed the enzymatic activity of the transfected cells (table 1). Mutant FIS cells showed only 0.6% of the α-GalA activity observed in wild-type NSF

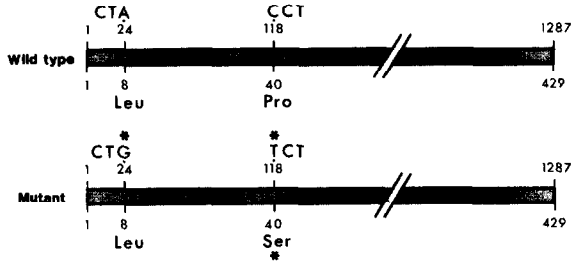

Fig.2. Differences in nucleotide sequences between FIS mutant and wild-type cDNAs that encode α-GalA. The triplet codons containing point mutations and the corresponding amino acid residues are shown. The numbers above and beneath the bars indicate the nucleotide numbers of α-GalA cDNA and the amino acid residue numbers of an α-GalA precursor protein, respectively. The asterisks indicate the nucleotide and amino acid substitutions.

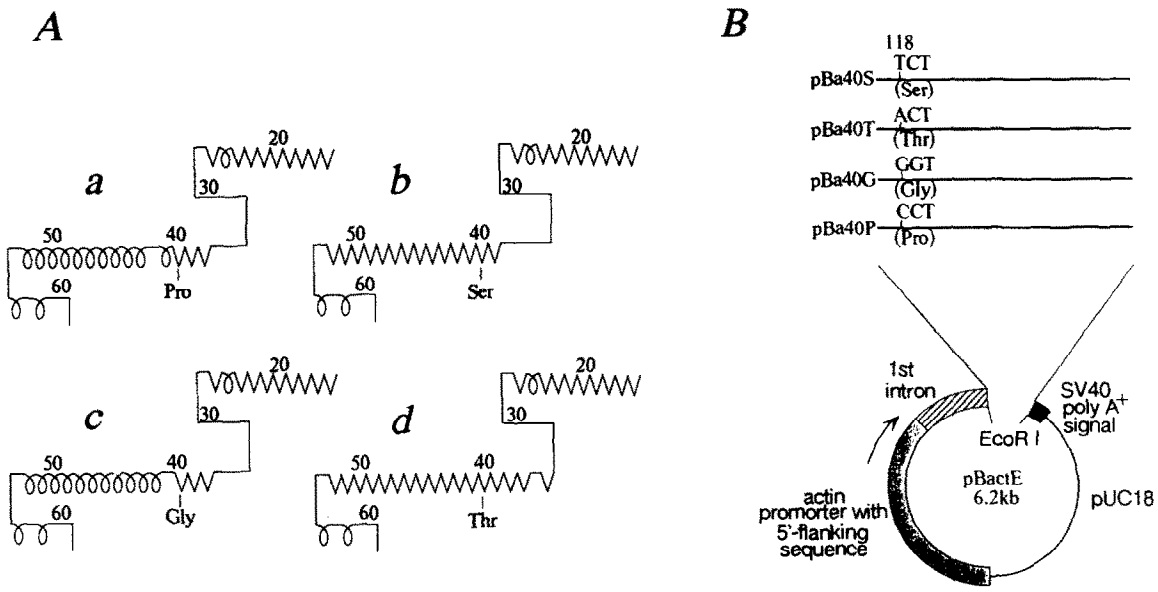

Fig.3. Secondary structures of α-GalA proteins expressed by pBactE expression plasmids that contain various α-GalA cDNAs. (A) Secondary structures of α-GalA proteins. The region between amino acid residues 16 to 60 is shown. The coils and notches indicate α-helices and β-sheet structures, respectively. a, wild-type protein with Pro-40; b, FIS mutant protein with Ser-40; c, mutant protein with Gly-40; d, mutant protein with Thr-40. The numbers indicate the positions of the amino acid residues. (B) Structures of pBactE plasmids that contain α-GalA cDNAs. Four different α-GalA proteins: the wild-type protein with Pro-40; a mutant protein with Ser-40 (FIS mutant); and two other mutant proteins with Thr-40 or Gly-40, were produced by the transient transfection of FIS mutant cells with pBa40P, pBa40S, pBa40T, and pBa40G, respectively. The arrow indicates the orientation of the β-actin promoter.

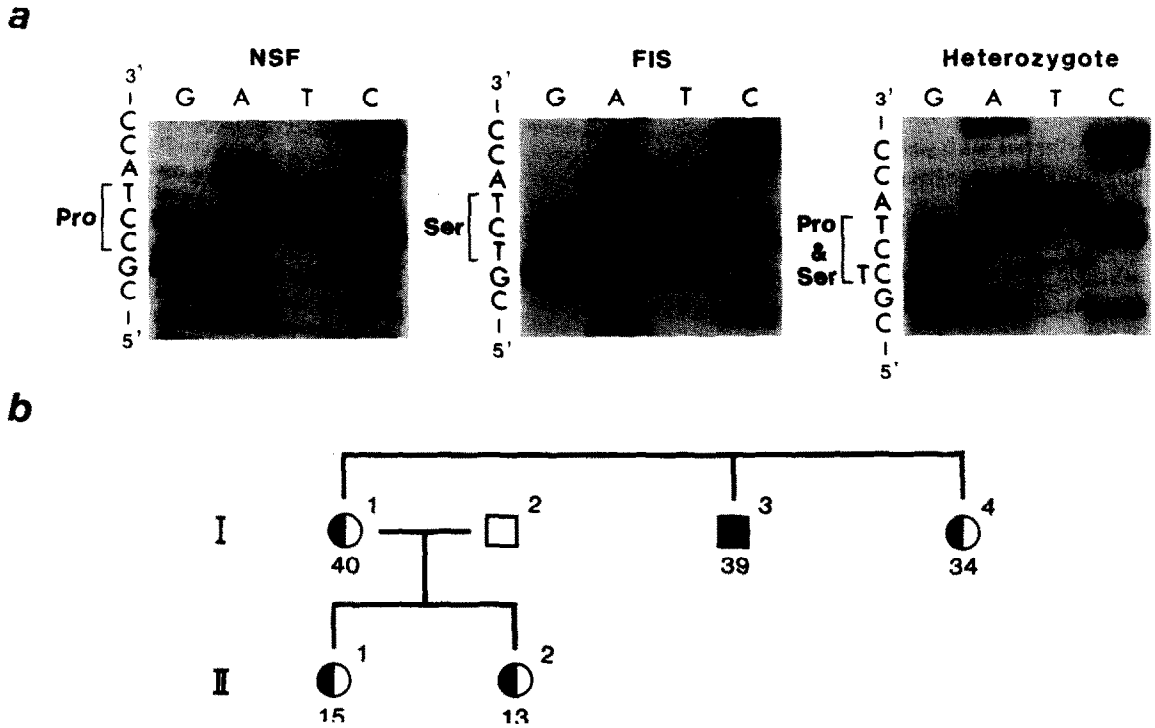

Fig.4. PCR analysis of the α-GalA gene for members of the family of the patient with FD. (a) A 192-bp segment of DNA containing the point mutation found in the α-GalA gene in FIS cells was amplified, using genomic DNAs prepared from NSF cells, FIS cells and from the leukocytes obtained from the members of the patient's family (I-1, I-4, II-1, and II-2), as templates. The reaction mixture, which contained 100 pmol each of PSF1 and PFS2 primers and 1 μg of genomic DNA in a GeneAmp cocktail (Perkin Elmer Cetus) was subjected to 30 temperature cycles which were composed of a 1-min incubation at 93°C, a 2-min incubation at 55°C, and another 2-min incubation at 72°C. The double-stranded reaction products were purified by electrophoresis on acrylamide gels. The single-stranded DNA of the segment was prepared as described previously [6] and then sequenced. Portions of the sequencing ladders for the DNA segments obtained from wild-type NSF cells, mutant FIS cells, and the leukocytes from I-4 (heterozygote) are shown. The arrowheads indicate the bands at nucleotide number 118. (b) Pedigree of the patient's family.

Table 1

Enzymatic activity of α-GalA in transiently expressing cells

Cell	DNA transfected	Type of DNA	Enzymatic activity (nmol/h per mg protein)	
			Mean ± SE*	% of NSF
NSF	None		30.7 ± 0.74	100
FIS	None		0.19 ± 0.02	0.6
	pBa40P	wild-type	3120 ± 712	10200
	pBa40S	FIS mutant	1.14 ± 0.01	3.7
	pBa40T	mutant	1.06 ± 0.14	3.5
	pBa40G	mutant	0.32 ± 0.10	1.0

*Enzymatic activity was determined in triplicate samples. FIS cells were plated 24 h before transfection at 3×10^5 cells per 50 mm plastic dish that contained α-MEM supplemented with 10% FCS. Plasmid DNAs were co-precipitated with calcium phosphate and added to the dishes at a dosage of 20 μg DNA/dish. After incubation at 37°C for 3 h in an atmosphere of 5% CO_2, the precipitates were washed out, and then 0.4 ml of a solution of 15% glycerol in HBS (0.818% NaCl, 0.594% Hepes, 0.02% Na_2HPO_4) were added to the dishes. After a 1-min incubation at room temperature, the glycerol solution was removed and the cells were washed with α-MEM. Then the cells were cultured for 48 h in α-MEM supplemented with 10% FCS. The cells (about 5×10^4 cells) were dissociated by treatment with trypsin and EDTA and collected by low-speed centrifugation. After neutralization of trypsin by washing with α-MEM supplemented with 10% FCS, α-GalA activity was determined.

cells. When FIS cells were transfected with pBa40S, the activity was six times higher than that observed in non-transfected FIS cells, but the value was only 3.7% of the activity observed in NSF cells. In contrast, when FIS cells were transfected with pBa40P, the activity was 102 times higher than that observed in NSF cells. These results indicate that the substitution of Pro-40 by Ser caused the deficiency of α-GalA activity in the patient. In addition, the α-GalA cDNA gene under the control of the β-actin promoter was strongly expressed in human fibroblasts.

Next, we designed two α-GalA cDNA constructs, pBa40T and pBa40G, in which Pro-40 was replaced by Thr and Gly, respectively. According to calculations by the Chou-Fasman program, the protein with Gly-40 (fig.3A,c) should have a gross secondary structure similar to that of the wild-type protein, whereas the protein with Thr-40 (fig.3A,d) should have a gross secondary structure similar to that of the FIS mutant protein. Irrespective of whether FIS cells were transfected with pBa40G or pBa40T, the α-GalA activity observed in the transfectants was very low (only 1.0% and 3.5% of that in NSF cells, respectively). These results suggest that it is not the apparent secondary structure of the α-GalA protein around the 40th amino acid residue, as predicted by the Chou-Fasman program, but it is Pro-40 per se that is critical for the appearance of enzymatic activity.

3.4. *PCR analysis of the α-GalA gene for members of the patient's family*

To trace the transmission of the mutant α-GalA gene among the members of the patient's family, we analyzed genomic DNAs from the female members of the patient's family, two sisters (fig.4b, I-1 and I-4) and two nieces (II-1 and II-2), by PCR and subsequent sequencing. We found that all the females examined had both the wild-type gene and the FIS mutant gene as heterozygotes (fig.4a, heterozygote). These results are consistent with the fact that each of these females, with the exception of II-1 have suffered from some symptoms of FD. The nucleotide sequences determined by control PCR experiments with the genomic DNAs from NSF and FIS cells were consistent with those of the wild-type cDNA [2] and mutant cDNA determined by cloning (fig.4a, NSF and FIS).

Acknowledgements: We are grateful to the patient with FD, to the members of his family, and to his attending doctors of Osaka Prefecture Hospital, M. Fukunami and N. Houki, for their generous cooperation which made this research possible. We thank K. Agata for help in constructing the cDNA library, T. Furuichi and A. Miyawaki for their advice on the transient expression assay, and H. Nakashima for critical comments on the manuscript. This work was supported by a grant provided by the Ichiro Kanehara Foundation and a grant (no. 01570248) from the Japanese Ministry of Education, Science and Culture.

REFERENCES

[1] Desnick, R.J., Klionsky, B. and Sweeley, C.C. (1984) in: The Metabolic Basis of Inherited Disease. Fifth edn. (Stanbury, J.B., Wyngaarden, J.B., Fredrickson, D.S. Goldstein, J.L. and Brown, M.S. eds) pp. 906–944, McGraw-Hill, New York.
[2] Bishop, D.F., Calhoun, D.H., Bernstein, H.S., Hantzopoulos, P., Quinn, M. and Desnick, R.J. (1986) Proc. Natl. Acad. Sci. USA 83, 4859–4863.
[3] Kornreich, R., Desnick, R.J. and Bishop, D.F. (1989) Nucleic Acids Res. 17, 3301–3302.
[4] Bernstein, H.S., Bishop, D.F., Astrin, K.H., Kornreich, R., Eng, C.M., Sakuraba, H. and Desnick, R.J. (1989) J. Clin. Invest. 83, 1390–1399.
[5] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning. A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
[6] Gyllensten, U.B. and Erlich, H.A. (1988) Proc. Natl. Acad. Sci. USA 85, 7652–7656.
[7] Zoller, M.J. and Smith, M. (1987) Methods Enzymol. 154, 329–350.
[8] Kost, T.A., Theodorakis, N. and Hughes, S.H. (1983) Nucleic Acids Res. 11, 8287–8301.
[9] Gorman, C. (1985) in: DNA Cloning, vol. 2 (Glover, D.M. ed.) pp. 143–190, IRL Press, Oxford.
[10] Suzuki, K. (1978) Methods Enzymol. 50, 456–488.
[11] Chou, P.Y. and Fasman, G.D. (1978) Annu. Rev. Biochem. 47, 251–276.