

Protective Protein Gene Mutations in Galactosialidosis

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Abstract

Four different protective protein cDNA mutations, ¹⁴⁶A → G (Q49R), ¹⁹³T → C (W65R), ²⁶⁸⁻²⁶⁹TC → CT (S90L), and ¹¹⁸⁴A → G (Y395C), were identified in six Japanese galactosialidosis patients with various phenotypic manifestations, and another mutation, ⁷⁴⁶T → A (Y249N), in a patient of French-German origin with an atypical clinical course. Y395C was a common mutation in four Japanese patients in infancy and childhood; two juvenile patients were compound heterozygotes of Y395C and another common mutation, SpDEx7, and the other two infants were compound heterozygotes of Y395C and mutant alleles other than SpDEx7. We confirmed these mutations in genomic DNA by direct-sequence analysis or restriction-site analysis. The mutant cDNA clones, transiently expressed in a transformed galactosialidosis cell line, did not restore the secondarily deficient β-galactosidase or α-neuraminidase activity except for the Y249N mutation that expressed some carboxypeptidase activity and restored the two lysosomal enzyme activities. Pulse-chase analysis detected a small amount of the mature form, as well as the precursor, in the cells transfected with the Y249N cDNA. Only precursor proteins were detected, mature proteins not appearing for the other mutant cDNAs. (*J. Clin. Invest.* 1993. 91:2393–2398.) **Key words:** carboxypeptidase • β-galactosidase • neuraminidase • phenotype • genotype

Introduction

Galactosialidosis is an inherited lysosomal storage disease transmitted as an autosomal recessive trait (1). A genetic defect of a multifunctional protein (protective protein) results in marked decreases in two lysosomal enzymes, β-galactosidase (EC 3.2.1.23) and α-neuraminidase (EC 3.2.1.18) (2, 3). It regulates their activities by forming a high molecular weight aggregate (2, 4–6). Furthermore, the cDNA coding for this protein bears homology to that for yeast carboxypeptidase Y (7, 8), and the NH₂-terminal amino acid sequence of its two subunits is identical with that of the enzyme esterase/deamidase purified from human platelets (9). In fact, all these enzyme activities were markedly low or deficient in cultured lymphoblastoid cells and skin fibroblasts from galactosialidosis patients (10–12). We concluded that it is a multifunctional protein with

esterase, deamidase and carboxypeptidase activities, together with activities toward the other two enzyme molecules mentioned above.

The clinical manifestations of this disease are heterogeneous. The age of onset, phenotypic expression, severity, and prognosis are remarkably variable (1, 13, 14). The incidence is high in the Japanese population, and most Japanese patients exhibit almost uniform neurosomatic manifestations in their teens or later. A small number of severe cases in early infancy have also been reported, with edema, ascites, skeletal dysplasia and cherry-red spots (13).

Molecular analysis of Japanese adult patients revealed a common A-to-G substitution at position 3 of the 5' splice donor site of intron 7, causing skipping of exon 7 in protective protein mRNA (SpDEx7) (14, 15). Clinically, homozygotes of this mutation showed less severe manifestations than compound heterozygotes, although individual signs and symptoms were almost indistinguishable from each other (14). Two other different base substitutions have been reported, one in SpDEx7 compound heterozygote siblings of Japanese origin (16) and the other in cases of Italian or Canadian origin reported as "late-infantile type" (17). There has been no report on any other mutations in SpDEx7 compound heterozygotes or in severe infantile-form patients. In this study, four additional base substitutions were identified in Japanese or French-German patients, and furthermore, the Y395C mutation mentioned in our previous report (16) was found to be common in Japanese patients. The expression products of these mutant cDNAs were analyzed in a transformed galactosialidosis cell line.

Methods

Patients. The clinical data for the patients in this study are summarized in Table I. They were unrelated, and described in previous reports (14, 16, 18, 19). Patients 1 and 2 showed severe dysmorphism with hepatosplenomegaly, died of cardiopulmonary failure at 6 and 13 months of age, respectively, and were classified clinically as type I galactosialidosis cases. Patients 4–7 developed audiovisual disturbance or generalized convulsions before 15 years of age, and their subsequent clinical courses were more protracted, although sensorimotor dysfunction and intellectual deterioration were slowly progressive. They had gait disturbance of various degrees at the time of the last examination.

According to a clinical report (19), patient 3 has been neurologically normal, although she has hepatosplenomegaly, stunted growth, and aortic insufficiency at 18 years of age. This clinical course is atypical, in spite of visceromegaly and slight dysmorphism of early onset, compared with the findings for the Japanese cases we analyzed clinically (1, 13, 14).

Cell culture. Fibroblast strains or lymphoblastoid cell lines from normal Japanese subjects and six galactosialidosis fetus/patients were established in our laboratory. Clinical samples for DNA analysis were not available for patient 1, and so fibroblasts from an affected fetus of the next pregnancy were used for study. Lymphoblastoid cell lines from a galactosialidosis family (19) were obtained from the National Institute of General Medical Sciences Human Genetic Mutant Cell Repository (Camden, NJ): GM04305 (patient), GM06092 (father), and GM06091 (mother).

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Table I. Clinical Data for Galactosialidosis Patients

Patient	1	2	3	4	5	6	7
	AN*	TOK	NT	KGo [‡]	TKm	NMi	KTk
Cell No.	(F-429)	L-287	L-195	L-147	L-205	L-211	L-204
Ethnic origin [§]	Japanese	Japanese	F-G	Japanese	Japanese	Japanese	Japanese
Sex	F	M	F	F	M	M	F
Onset							
Age	0 mo	0 mo	3 mo	4 yr	6 yr	7 yr	14 yr
Symptoms	HSM	HF	HSM	VI	HL	VI	GC
Outcome							
Age	6 mo	13 mo	18 yr	17 yr	27 yr	15 yr	20 yr
Symptoms	RF	HF	SG, AI, BD	HSM, AK, CRS	AK, BD, CRS	AK, BD, CRS	AK, BD, CRS
	CNS:SBD (Death)	CNS:SBD (Death)	CNS:normal	AI, AS CNS; MR, Mc, Gp	CNS:MR, Gp	CNS:Mc, Gp	CNS:Mc, Gi
Phenotype [†]	I	I	Var	IIA	IIA	IIA	IIA
Genotype	(Y395C) (S90L)	Y395C ?	Y249N ?	Y395C SpDEx7	Q49R SpDEx7	Y395C SpDEx7	W65R SpDEx7
Ref No (Case No.)	18	14 (1)	19	16 (1)	14 (6)	14 (3)	14 (7)

* The genotype was determined on the basis of the data for an affected fetus of the next pregnancy (F-429). [‡]The genotype was reported previously (16). [§]F-G, French-German ancestry (19). ^{||}AI, aortic insufficiency; AK, angiokeratoma; AS, aortic stenosis; BD, bone dysplasia; CNS, central nervous system; CRS, cherry-red spots; GC, generalized convulsion; Gp, gait possible but unstable; Gi, gait impossible; HF, heart failure; HL, hearing loss; HSM, hepatosplenomegaly; Mc, myoclonus; MR, mental retardation; RF, respiratory failure; SBD, severe brain damage; SG, stunted growth; VI, visual impairment. [†]Var: variant form.

The cells were cultured in Ham's F-10 medium (fibroblasts) or RPMI-1640 (lymphoblastoid cells), supplemented with 10% FCS and antibiotics. Fibroblasts from a galactosialidosis patient (ref 14, case 11) had been transformed with an SV40-adenovirus recombinant according to the method described by van Doren and Gluzman (20), and the transformed cell line, ASVGS-1, was used for expression studies.

Preparation of DNA, RNA and synthetic oligonucleotides. Genomic DNA, total RNA and poly(A)⁺ RNA were prepared as described previously (15). Oligonucleotide primers for polymerase chain reaction (PCR) amplification of cDNA and genomic DNA were synthesized with the DNA Synthesizer (model 381; Applied Biosystems, Foster City, CA). Coordinates of the cDNA and amino acid sequences were numbered for the coding region starting from the initiation codon.

Amplification, sequencing, and restriction site analysis of cDNA and genomic DNA. Details of the procedures were reported in a previous paper (15). The protective protein gene was PCR amplified in two overlapping portions, one directly from genomic DNA covering the 5' end of the open reading frame and the other from cDNA. Each of the products was then subcloned into pUC18 and sequenced. For confirmation of the mutation, the genomic DNA sequence comprising the base substitution was amplified. Restriction site analysis was performed with an appropriate enzyme if the mutation eliminated or created a restriction site. The PCR product without alteration of the restriction site was directly sequenced.

Construction of cDNA clone for expression of protective protein. Protective protein cDNA (PP11) (15) was subcloned into the pUC18 SalI site. This plasmid clone, pUC(PP), was digested with Apal and XbaI to remove the BsmI site in the 3' noncoding region, and then ligated with the SalI linker using a ligation kit (Takara Shuzo, Kyoto, Japan), after the digested fragment had been blunt ended with the Klenow fragment (21). The resulting cDNA clones, pUC(PP) with the complete cDNA sequence and pUC(PPAX) with a partial deletion of the 3' noncoding region (starting from 1594), were digested with SalI, then subcloned into the XhoI site of expression vector pCAGGS (22), and designated as pCAGGS(PP) and pCAGGS(PPAX), respectively. The mutant cDNA fragments in pUC18 were digested with BsmI and MscI, and then subcloned into pUC(PPAX). Finally, the plasmids

containing mutant fragments were digested with SalI and subcloned into the XhoI site of pCAGGS. All mutant sequences were confirmed in the expression vector.

Transfection of cDNA into ASVGS-1 cells. ASVGS-1 cells on 10-cm dishes were transfected with normal or mutant protective protein cDNA in expression vector pCAGGS (30 µg plasmid DNA), by the calcium phosphate-mediated transfection method (21). After 3 h, the transfected cells were treated with 3 ml of 15% glycerol for 60 s, cultured for 60 h, and then harvested by scraping. Carboxypeptidase, β-galactosidase, α-neuraminidase and β-hexosaminidase activities were assayed (12, 23), and the protein concentration was determined by the method of Bradford (24).

Pulse-chase analysis. After transfection with cDNA and glycerol treatment, the cells on 6-cm dishes were cultured for 36 h, and pulse-labeled for 6 h in 5 ml of methionine/cysteine-free Dulbecco's Minimum Essential Medium with Expre³⁵S/³⁵S Protein Labeling Mix ([³⁵S]methionine/[³⁵S]cysteine mixture; 0.1 mCi/dish, 1000 Ci/mmol (Du Pont-NEN Research Products, Boston, MA)). The culture was then continued for various periods (chase). Protein was extracted from the cells that had been harvested by scraping. The culture medium was concentrated and desalted with Centriprep-30 (Amicon Corp., Beverly, MA). These preparations were finally treated with anti-serum against β-galactosidase and protective protein (25), and protein A-Sepharose CL-4B (Pharmacia LKB, Uppsala, Sweden). The proteins were then separated by electrophoresis on a polyacrylamide gradient gel SDS-PAGE Plate 4/20 (Daiichi Pure Chemicals, Tokyo, Japan). Radioactive bands were visualized by fluorography, after the gels had been treated with Amplify (Amersham, Buckinghamshire, UK) and dried.

Results

Mutation analysis of protective protein cDNA. Sequencing of cDNA fragments amplified at two overlapping regions revealed five different base substitutions (Tables I and II). The splice junction mutation SpDEx7 was confirmed by the analytical

Table II. Mutations in Galactosialidosis

Mutation*	Exon [‡]	Base substitution*	Amino acid substitution*	Restriction site [§]	Patient No
Q49R	2	¹⁴⁶ A → G	⁴⁹ Gln → Arg	<i>ScaI</i> (-)	5
W65R	2	¹⁹³ T → C	⁶⁵ Trp → Arg	No change	7
S90L	3	²⁶⁸⁻²⁶⁹ TC → CT	⁹⁰ Ser = Leu	<i>AluI</i> (-)	(1)
SpDEx7	E7/I7	AG/gtatg → AG/gtgtg	Exon 7 skipping	<i>PvuII</i> (+)*	4-7
Y249N	8	⁷⁴⁶ T → A	²⁴⁹ Tyr → Asn	No change	3
Y395C	?	¹¹⁸⁴ A → G	³⁹⁵ Tyr → Cys	<i>SnaBI</i> (+)*	(1), 2, 4, 6

* Amino acid and nucleotide numbers are those starting from the initiation codon. SpDEx7: Splicing junction mutation at the 5' splice donor site (ref. 15). [‡] E7/I7, exon 7/intron 7 junction; ?, exon number not identified. [§](-): restriction site eliminated, (+)*, new restriction site generated by adding a second artificial mutation for SpDEx7 (ref. 15) or for Y395C (ref. 16). ^{||}S90L/Y395C: genotype determined for an affected sibling (fetus) of patient 1.

methods described before (14, 15). All patients were compound heterozygotes of two different mutations. Among them, Q49R, W65R, S90L, and Y249N were the new mutations found in this study. Patients 4-7 had the common mutation, SpDEx7, and patients 2, 4, and 6 had another common mutation, Y395C. The mutation in the second allele was not identified for patient 2 or 3.

The genotype of patient 1 was not directly determined in this study. However, her affected sibling (fetus) was analyzed, and two mutations, Y395C and S90L, were found. The latter (S90L) comprised two base substitutions within the same translation codon, resulting in a single amino acid substitution. Each parent was probably heterozygous for one of these two mutations, but genotype analysis was not performed for them. We concluded that patient 1 also had the same genotype as the sibling fetus.

Sequencing and restriction site analysis of genomic DNA. For confirmation of the mutations described above, genomic DNA prepared from cultured cells was amplified in the region comprising each mutation, and the PCR product was subjected to further analysis. The W65R or Y249N mutation did not create or eliminate any known restriction site of the genomic sequence. The amplified DNA fragments were therefore directly sequenced, and the results for cDNA were confirmed (Fig. 1).

The Y249N mutation was confirmed in one of the alleles for the cells from patient 3. Further analysis of the parents in this region revealed the same mutation in one of the father's alleles and a normal sequence in the mother. In this case, the second mutation was not identified.

The S90L mutation eliminated an *AluI* restriction site. The 175-bp PCR product of the normal gene sequence was digested into a 144-bp fragment by the enzyme. The fetal sibling of patient 1 showed two bands, digested (144 bp) and undigested (175 bp) (Fig. 2 A). The Q49R mutation in patient 5 eliminated a *ScaI* restriction site, giving both digested (233 bp) and undigested (295 bp) bands, which were consistent with the diagnosis of a heterozygote for this mutation (Fig. 2 B). *SnaBI* site analysis was positive for the Y395C mutation as reported previously (16).

Enzyme activities in cells transfected with mutant cDNA. To assess the effect of an amino acid substitution on the protective protein function, normal and mutant cDNAs were transiently expressed in galactosialidosis-derived ASVGS-1 cells

with markedly low endogenous carboxypeptidase, β -galactosidase and neuraminidase activities (Table III). The cells transfected with normal full-length cDNA, pCAGGS(PP), or normal cDNA with a partial deletion, pCAGGS(PPAX), showed high carboxypeptidase activity, and restored β -galactosidase and α -neuraminidase activities. The mutant cDNAs did not affect the enzyme activities except for the Y249N mutation, which expressed significant amounts of carboxypeptidase, β -galactosidase and α -neuraminidase activities.

Pulse-chase analysis. After transfection of normal cDNA and a 6-h pulse, the 54-kD precursor was synthesized and converted rapidly to mature proteins (32 and 20 kD) (Fig. 3). The mutated cDNAs expressed the precursor that was detected as a band of apparently higher intensity than that for normal cDNA. The mature proteins did not appear in the cells transfected with mutant cDNAs except for those with Y249N. The Y249N expression of the mature protein was much lower than that of normal cDNA.

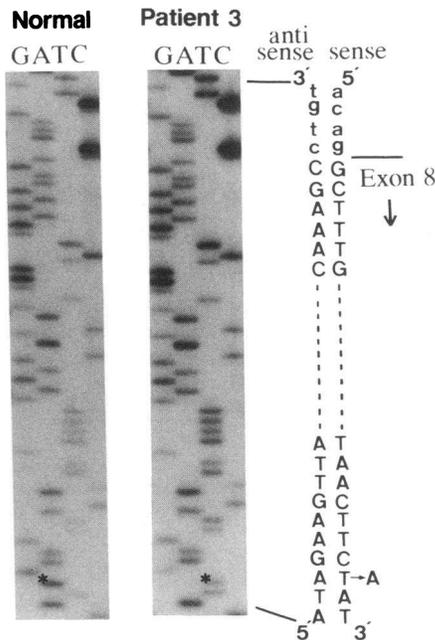
The precursor was detected in the culture medium only for the normal and W65R cDNAs. The cells transfected with other cDNAs did not secrete an immunoprecipitable precursor into the medium (data not shown).

Discussion

Galactosialidosis is a disease with variable phenotypic expression that has been classified into a few clinical types. In a previous report, we divided Japanese galactosialidosis patients into two major groups (14), type I with generalized and severe manifestations of neonatal or early infantile onset, and type II mainly with neurological manifestations of later onset. The latter was further divided into subtypes IIA (relatively severe) and IIB (relatively mild). The type II Japanese patients had a common gene mutation, SpDEx7. Subtype IIB corresponded roughly to homozygotes for this mutation and subtype IIA to compound heterozygotes (14). The clinical course of patient 3 in this study did not conform to any clinical types for Japanese cases, and thus was tentatively designated as a variant type.

In this study, we identified five different base substitutions. One of them, ¹¹⁸⁴A → G (Y395C), was found in four patients with various clinical manifestations that had been classified as either type I or type IIA. Probably the second mutations are responsible for this clinical heterogeneity. Another common mutation, SpDEx7, induces alternative splicing of the protec-

A



B

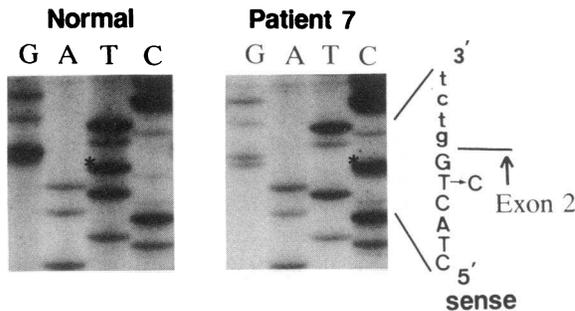
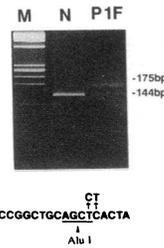


Figure 1. Sequence analysis of the PCR-amplified protective protein genomic DNA. Exon sequences are indicated in capitals and intron sequences in small letters. Asterisk indicates the point of mutation. (A) Y249N mutation in patient 3. PCR amplification (30 cycles) of genomic DNA (1 μ g): denaturation at 94°C for 1 min, annealing at 54°C for 2 min, and extension at 72°C for 3 min. Primers: 5'-AAT-AAGCTTGAATTCGTCATGCAGGATCCCAGCAT-3' (sense) and 5'-TGAGAATTCACGCATTCCAGGTCTTT-3' (antisense). (B) W65R mutation in patient 7. PCR amplification: denaturation at 94°C for 1 min, annealing at 58°C for 2 min, and extension at 72°C for 2 min. Primers: 5'-CCGGTCGACGTCCCCGAGGCGAGGCAG-3' (sense) and 5'-TGAGAATTCGTGAGCTGCAGCCGGGACCCC-CATTGAG-3' (antisense). Each primer has SalI or EcoRI site and a few additional nucleotides at its 5' end for subsequent subcloning.

tive protein gene, and the presence of a small amount of normal mRNA probably reflects mild phenotypic expression (Shimmoto, M., et al., unpublished data). The Y395C/SpDEx7 compound heterozygotes had more severe manifestations than the SpDEx7 homozygotes (type IIB patients), and presented with the type IIA phenotype. The compound heterozygote Y395C/S90L (patient 1) showed the most severe (type I) clinical manifestations among those with the Y395C mutation in one of the alleles.

A



B

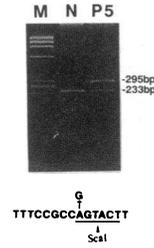


Figure 2. Restriction site analysis of PCR-amplified protective protein genomic DNA. Electrophoresis was performed on 3% agarose gel. (A) S90L mutation in the sibling (fetus) of patient 1. PCR amplification (30 cycles) of genomic DNA (1 μ g): denaturation at 94°C for 1 min, annealing at 54°C for 3 min, and extension at 72°C for 1 min. Primers: 5'-ATTGTCGACGGAGCCGGGAGGGCTGGAAA-3' (sense) and 5'-ACTGAATTCTGTGAGGAGCCCATCTA-3' (anti-sense). The products were digested with AluI. (B) Q49R mutation in patient 5. PCR amplification: denaturation at 94°C for 1 min, annealing at 60°C for 2 min, and extension at 72°C for 1 min. Primers: 5'-ATTGAATTCGAGGCGCCGAGCAACT-3' (sense) and 5'-ATTGTCGACGAATTCACCGATGAGGAGGTGCTTG-3' (anti-sense). The amplification product was digested with ScaI. M, molecular size markers (ϕ X 174HaeIII digest); N, normal control; P1F, sibling (fetus) of patient 1; P5, patient 5.

Neither mutation Y249N in a patient of French-German ancestry (patient 3), nor mutation ⁴¹²Phe \rightarrow Val (F440V, our nomenclature system) in Italian or Canadian patients (17) has been detected in Japanese patients. Conversely, no report of the SpDEx7 mutation common to Japanese patients has appeared for other ethnic populations. Different gene mutations seem to be present in different ethnic groups in this disease.

In spite of early onset with dysmorphia and visceromegaly, patient 3, at 18 years of age has not yet developed any recognizable central nervous system involvement. However, aortic insufficiency necessitated valvular surgery (19). This French-German case may represent a special variant ("late-infantile" form) with a protracted course, which is different from the

Table III. Expression of Enzyme Activities in Galactosialidosis Fibroblasts Transfected with Mutant cDNA

Plasmid	Mutation	Carb-pept	β -Gal	Neur	β -Hex
None	—	<300	21.5	5.0	506
pCAGGS	Mock	<300	23.8	4.9	816
pCAGGS(PP)	Normal	3,636	74.9	26.4	658
pCAGGS(PPAX)	3'-Del*	2,146	61.1	18.0	536
pCAGGS(PPAX49)	Q49R	<300	19.4	4.2	646
pCAGGS(PPAX65)	W65R	<300	18.0	4.9	680
pCAGGS(PPAX90)	S90L	<300	18.1	2.2	610
pCAGGS(PPAX249)	Y249N	800	46.6	12.5	697
pCAGGS(PPAX395)	Y395C	<300	17.5	2.8	747
Normal fibroblasts		1,732	415.5	59.5	887
GS fibroblasts [‡]		<300	29.2	4.0	664

* Normal cDNA with partial deletion of the 3' noncoding region. [‡] GS: galactosialidosis. Carb-pept, carboxypeptidase; β -Gal, β -galactosidase; Neur, α -neuraminidase; β -Hex, β -hexosaminidase. Enzyme activities are expressed as nmol/mg protein/h.

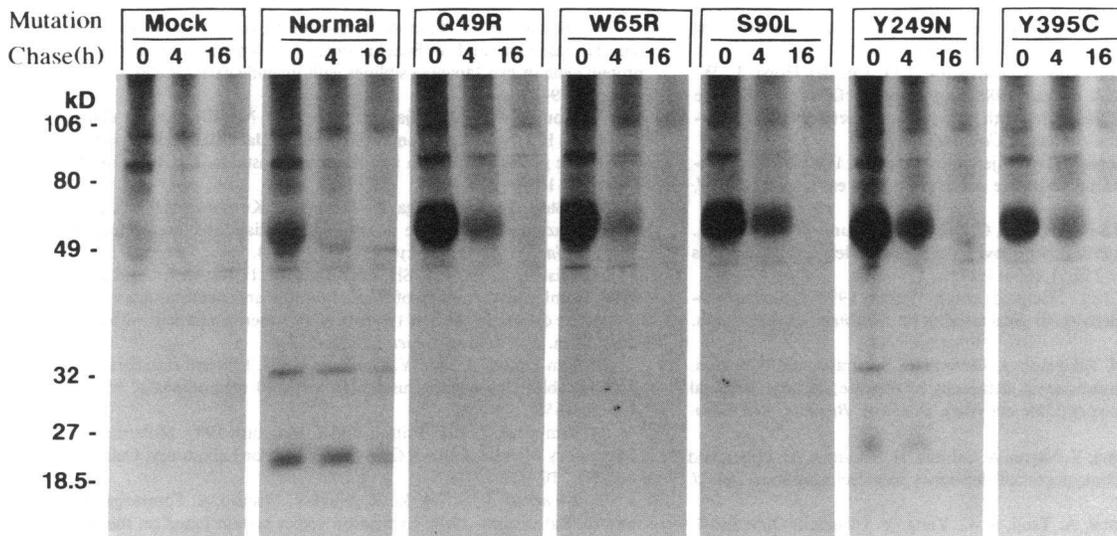


Figure 3. Pulse-chase analysis of the expressed protective protein. ASVGS-1 cells were transfected with each mutant cDNA in expression vector pCAGGS. Newly synthesized proteins were radiolabeled for 6 h with [³⁵S]methionine and [³⁵S]cysteine, and then chased for the time indicated above each lane. The labeled protein was immunoprecipitated from the cell extract or culture medium with the antibody against the β -galactosidase-protective protein complex. Electrophoresis was performed on a sodium dodecyl sulfate-polyacrylamide gradient gel. In this experiment, normal cDNA with partial deletion of the 3' noncoding region was used as a reference control, which expressed a normal amount of enzyme activity (see Table III).

phenotypes of the Japanese patients. She was heterozygous for the Y249N mutation; her father was a healthy carrier, but Y249N was not detected in the mother. The Y249N mutation transiently expressed a small amount of carboxypeptidase catalytic activity, and slightly restored β -galactosidase and α -neuraminidase activities. The second unknown mutation probably resulted in a marked decrease in mRNA, as we could not detect mRNA without the Y249N mutation in this patient. Taken together, we concluded that the protein produced by Y249N retained its function to some extent, protected against more destructive pathological lesions, and contributed to the mild phenotypic expression.

There was no difference in enzyme activities, including that of carboxypeptidase, between the fibroblasts from patient 3 and those from other patients in this study (Shimmoto, M., et al., unpublished data). However, expression of the mutated genes distinguished clearly Y249N and the others. The former showed relatively high intracellular catalytic activity, as compared with the latter, and produced a small amount of the mature protein. It has been reported that the formation of a precursor homodimer is a prerequisite for the proper targeting and a stable conformation of protective protein (17). A mutated gene, F440V, failed to form this precursor homodimer, and thus the mature two-chain form protein was not detected (17). The mutant gene Y249N may have expressed a limited amount of the mature enzyme in some tissues and resulted in a unique phenotypic manifestation in the patient.

At this stage, we conclude that the mutation SpDEx7, producing a small amount of normally spliced mRNA, is closely related to the mild expression of galactosialidosis, and Y395C without expression of any enzyme activity is related to relatively severe expression of this disease. A combination of these two mutations in an individual seems to express clinical manifestations of intermediate degree. The mutation Y249N that expressed a small amount of carboxypeptidase activity may be

responsible for the intermediate phenotype. Further structural analysis of mutated proteins is necessary for elucidation of the molecular pathogenesis in galactosialidosis.

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