ORIGINAL

Elastogenesis in cultured dermal fibroblasts from patients with lysosomal β-galactosidase, protective protein/cathepsin A and neuraminidase-1 deficiencies

Yutaka Tatano[a,d], Naohiro Takeuchi[a], Jun Kuwahara[a], Hitoshi Sakuraba[b,d], Tsutomu Takahashi[c], Goro Takada[c], Kohji Itoh[a,d]

[a]*Department of Medicinal Biotechnology, Institute for Medicinal Resources, Graduate School of Pharmaceutical Sciences, The University of Tokushima, Tokushima , Japan;* [b]*Department of Clinical Genetics, The Tokyo Metropolitan Institute of Medical Science, Tokyo Metropolitan Organization for Medical Research, Tokyo, Japan; and* [c]*Department of Pediatrics, Akita University of Medical School, Akita, Japan; and* [d]*CREST, JST, Saitama, Japan*

Abstract : The human *GLB1* gene encodes a lysosomal β-galactosidase (β-Gal) and an elastin-binding protein (EBP). Defect of the EBP as a chaperon for tropoelastin and a component of receptor complex among neuraminidase-1 (NEU1) and protective protein/cathepsin A (PPCA) is suggested responsible for impaired elastogenesis in autosomal recessive β-Gal, PPCA and NEU1 deficiencies. The purpose of this study is to determine effects of *GLB1, PPCA* and *NEU1* gene mutations on elastogenesis in skin fibroblasts. Elastic fiber formation and the EBP mRNA expression were examined by immunofluorescence with an anti-tropoelastin antibody and RT-PCR selective for EBP in skin fibroblasts with these lysosomal enzyme deficiencies. Apparently normal elastogenesis and EBP mRNA expression were observed for fibroblasts from Morquio B disease cases with the *GLB1* gene alleles (W273L/W273L, W273L/R482H and W273L/W509C substitutions, respectively), a galactosialidosis case with the *PPCA* allele (IVS7+3A/IVS7+3A) and a sialidosis case with the *NEU1* allele (V217M/G243R) as well as normal subject. In this study, the W273L substitution in the EBP could impossibly cause the proposed defect of elastogenesis, and the typical *PPCA* splicing mutation and the V217M/G243R substitutions in the NEU1 might hardly have effects on elastic fiber formation in the dermal fibroblasts. J. Med. Invest. 53 : 103-112, February, 2006

Keywords **:** *elastin-binding protein, lysosomal β-galactosidase gene, lysosomal enzyme deficiencies, morquio B disease, costello syndrome*

INTRODUCTION

GM1-gangliosidosis and Morquio B disease are autosomal recessive lysosomal storage diseases caused by primary defects of the *GLB1* gene, which is located on chromosome 3 p 21.33 (1,2). GM1-gangliosidosis comprises neurosomatic clinical phenotypes accompanied by the accumulation of GM1-ganglioside in the central nervous system (CNS) and β-galactosyl glycoconjugates in visceral organs. Morquio B disease

ABBREVIATIONS

GLB 1 , lysosomal β-galactosidase gene ; β-Gal, lysosomal β-galactosidase protein ; EBP, elastin-binding protein ; NEU 1, lysosomal neuraminidase-1; PPCA, lysosomal protective protein/cathepsin A ; RT-PCR, reverse transcription-polymerase chain reaction ; CNS, central nervous system ; FBS, fetal bovine ser um ; PBS, phosphate-buffered saline; DEPC, diethylpyridylchloride.

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Address correspondence and reprint requests to Kohji Itoh, Ph. D, Department of Medicinal Biotechnology, Institute for Medicinal Resources, Graduate School of Pharmaceutical Sciences, The University of Tokushima, Sho-machi Tokushima 770-8505, Japan and Fax : +81-88-633-7290.

is characterized by generalized skeletal dysplasia without CNS involvement. The patients exhibit reduced catalytic activity toward keratan sulfate and oligosaccharides but normal activity toward GM1-ganglioside. These patients may also exhibit cardiac-valve malformations, aortic stenosis, and intimal thickening in the coronary arteries and pulmonary artery (3, 4).

Lysosomal protective protein/cathepsin A (PPCA; EC 3.2.1.14) is a serine carboxypeptidase that forms a high molecular weight complex with β-galactosidase (β-Gal; EC 3.2.1.23) and neuraminidase-1 (NEU1; EC 3.2.1.18), and it stabilizes the former enzyme and activates the latter one in lysosomes (protective function). A genetic defect of *PPCA* causes an autosomal recessive inherited metabolic disorder, galactosialidosis, associated with a combined deficiency of cathepsin A, β-Gal, and NEU1 and neurosomatic clinical manifestations (5).

Sialidosis is an autosomal recessive metabolic inborn error caused by a genetic defect of *NEU1*, which is accompanied by accumulation in tissues and urinary excretion of sialyloligosaccharides and neurosomatic phenotypes (6).

Elastic fibers in connective tissues and blood vessel walls are made of polymeric tropoelastin and are placed on a scaffold of microfibrils consisting of glycoproteins (e.g. fibrillins and microfibril-associated glycoproteins)(7, 8). Several functional proteins, including elastin-binding protein (EBP) as a tropoelastin chaperone and lysyl oxidase involved in cross-linking, have also been demonstrated to contribute to elastic fiber formation (9-12). Impaired elastogenesis has been reported in various human diseases, including Marfan syndrome (13), William syndrome (14), and Costello syndrome (15, 16), accompanied by connective tissue abnormalities and cardiovascular manifestations. Hinek *et al*. have demonstrated that an enzymatically inactive, alternatively spliced variant of the *GLB1* gene product functions as an EBP to facilitate intracellular transport and extracellular assembly of tropoelastin into elastic fibers (17,18). The 67-kDa EBP with a different stretch of 32 amino acids from β-Gal has also been reported to be a major component of the non-integrin cell surface receptor complex composed of PPCA and NEU1 for elastic peptides involved in the signal transduction for proliferation and migration of fibroblasts, smooth muscle cells, chondroblasts, leukocytes, and certain cancer cell types (12, 19, 20). In recent years, Mochizuki *et al*. have also hypothesized that the 67-kDa EBP might associate with PPCA and NEU1 to form a secondary complex different from the lysosomal multienzymic complex consisting of β- Gal, PPCA and NEU1, and the secondary complex might interact with an intracellular signaling system including G-protein coupled receptor and effectors (21). Additionally, Malvagia *et al*. reported that new *PPCA* gene mutations (c60delG and IVS 2+1 G>T) that lead to a frameshift and a premature stop codon, identified in a galactosialidosis patient, reduce the 67-kDa EBP protein expressed in the patient's fibroblasts accompanied by an absence of elastic fibers deposition (22, 23), suggesting the PPCA might be essential for the integrity of the secondary complex.

The *GLB1*, *PPCA* and *NEU1* gene mutations have been identified in patients with these lysosomal enzyme deficiencies, and the genotype-phenotype relationship has been analyzed (5, 24-27). On the other hand, dysfunction of EBP as an alternative pathogenic mechanism has been proposed to contribute to the connective tissue and cardiovascular malformations in β- Gal deficiencies as well as Costello syndrome (19, 28-30), although the relation between the defect in EBP and the abnormalities has not been established yet.

In this study, we analyzed elastic fiber formation in cultures of skin fibroblasts from patients with β-Gal, PPCA and NEU 1 deficiencies as well as Costello syndrome, and discuss the relation between the effects of each gene mutation on the expression of EBP and elastogenesis.

METHODS

Human skin fibroblasts derived from patients and control subjects ; F430, a juvenile GM1-gangliosidosis with missense mutations R201C/R201C in the *GLB1* gene (31) ; F435, a juvenile GM1-gangliosidosis with R201H/N318H (32) ; F350, a Morquio B disease with W273L/R482H in the *GLB1* gene (32) ; F 351, a Morquio B disease with W273L/W509C (33) ; F 451, a Morquio B disease with W273L/W273L (32) ; F622, a galactosialidosis with a splicing mutation in the *PPCA* gene (IVS7+3aA/IVS7+3a)(unpublished case); F319, type1 sialidosis with missense mutations in the *NEU1* gene (V217M/G243R) (27); F642, Costello syndrome(unpublished case) ; and F592, a normal subject, were cultured in Ham's F-10 medium supplemented with 10% fetal bovine serum (FBS) and antibiotics at 37°C under 5% CO_2. The research was carried out in accordance with the Declaration of Helsinki of the World Medical Association, and was approved by the ethical committee of the institution in which

the work was performed.

Elastic fiber formation on the surface of skin fibroblasts was assayed as immunofluorescence with the anti-fetal bovine tropoelastin serum (Elastin Products Company, Inc., Owensville, MO). The fibroblasts were plated on 8-well chamber slides (Nunc., Pittsburgh, PA), and then cultured for 10 days to confluency. The cells were fixed with cold 2% paraformaldehyde/phosphate buffered-saline (PBS) overnight at 4°C, and then immunostained by a two-step incubation method; first with a 1 : 500 dilution of the anti-fetal bovine tropoelastin serum, and then with a 1 : 1000 dilution of goat anti-rabbit IgG-F(ab')$_2$ fragment conjugated with rhodamine (Biosource International, Camarillo, CA). After inclusion, elastic fiber formation was observed under a confocal laser scanning microscope (LSM5Pascal-V2.8 ; Zeiss, Oberkochen, Germany). Morphometric analysis of the immunostained fibroblasts was also performed by standard digital imaging software attached to the confocal microscopic system. The relative amount of the deposited elastic fiber over the fibroblast was expressed as the immunofluorescence intensity index in each image. Relative fluorescence intensity was graded, ranging from 0 to 255 in this system. The fluorescence intensity linearly correlated with the number of pixels showing a positive signal. In each optic field, the immunofluorescent area with more than 100 intensity was estimated as elastic fiber-positive area, and the relative index (mean±SD) was expressed as a percentage of the total optical field area. The number (n) of optical fields ranged from 2 to 7.

For RT-PCR analysis, fibroblasts (approx. 5×10^5 on 60-mm plastic dishes) were rinsed with cold PBS, and then homogenized manually in 1 ml of TRIZOL reagent (Invitrogen, Carlsbad, CA) on ice. RNA was isolated according to the manufacturer's protocol. Briefly, 0.2 ml of $CHCl_3$ was added to the homogenate, and followed by centrifugation at 20,600× g for 15 min. An equal volume of 2-propanol was added to the resultant supernatant to precipitate RNA. After centrifugation, the pellet was rinsed with 75% ethanol/diethylpyridylchloride (DEPC)-treated water, and then dried. The pellet was dissolved in an appropriate volume of DEPC-treated water as the total RNA fraction. For reverse transcription (RT), 2 μg RNA from each sample was transcribed at 37°C for 1 h in the presence of 200U of Molony leukemia virus reverse transcriptase (Promega, Madison, WI), Oligo(dT)$_{12\text{-}18}$ primer (Invitrogen), 0.5 mM dNTPs and 50U of RNaseOUT (Invitrogen). The PCRs for β-Gal, EBP and β- actin were performed within the linear range of amplification using the following selective primer sets: β-Gal (Accession No. M27507), forward 5'-GGACCGGCTGCTGAAGATGA-3', reverse 5'-TTGATGGGCCCAGAGGGACA-3'; EBP (Accession No. M27508), forward 5'-CCATCCAGACATTACCTGGC-3', reverse 5'-TTGATGGGCCCAGAGGGACA-3'; β-actin (Accession No. NM 001101), forward 5'-GACAACGGCTCCGGCATGTG-3', reverse 5'-CCT TCTGCATCCTGTCGGCA-3'.

β- actin was amplified for 30 cycles, and β- Gal and EBP for 30～35 cycles. The PCR products were analyzed by 1.5% agarose gel electrophoresis and stained with ethidium bromide (Sigma). As a control, RT was also performed in the absence of Molony leukemia virus reverse transcriptase. In another experiment, plasmid DNAs including human β- Gal and EBP cDNAs, respectively, were used as templates as positive controls.

Real-time PCR analysis of human β- Gal and EBP mRNA relative to β-actin mRNA was performed by means of the SYBR Green I (Roche, Mannheim, Germany) assay for comparison. The β-Gal, EBP and β-actin mRNAs were amplified by using a GeneAmp 5700 Sequence Detection System (PE Applied Biosystem, Foster City, CA, USA) in the presence of cDNAs, as templates, prepared from the fibroblasts, as described above, 0.25 mM each primer, a 1/10 vol. of 10×FastStart DNA master SYBR Green I, and 1.5 mM $MgCl_2$ using the following protocol : 95°C for 10 min, then 45 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 60 s. For standardization of quantification, β-actin was amplified simultaneously. The change of reporter fluorescence in each reaction tube was monitored by a GeneAmp 5700 Sequence Detection System. The threshold cycle (Ct) for each mRNA was determined as PCR cycle number at which the reporter fluorescence reached above 10-fold of the standard deviation of the baseline signal. The fold change of the EBP or β- Gal mRNA relative to that of β-actin was determined as follows :

Fold chang = 2^{-ddCt}, where $dCt=Ct_{EBP\ or\ \beta\text{-}Gal} - Ct_{\beta\text{-}actin}$, and $ddCt=dCt_{target} - dCt_{F592}$

β-Gal activity was measured in cell extracts of cultured skin fibroblasts using 4-methylumbelliferyl β-D-galactoside (34). Protein determination was performed with the DC protein assay kit (Bio-Rad Laboratories, Richmond, CA) with bovine serum albumin (BSA) as the standard.

RESULTS

We have studied five patients from unrelatede the juvenile form of GM1-gangliosidoisis, and three patients had Morquio B disease. We also examined one patient affected by galactosialidosis (a PPCA deficiency), and one patient with sialidosis (a NEU1 deficiency). Table 1 summarizes the clinical phenotypes, intracellular β-Gal activities and genotypes. A marked decrease in β-Gal activity was confirmed in the cases of GM1-gangliosidosis and Morquio B disease, and a partial β-Gal deficiency was observed in the galactosialidosis with a splicing mutation in the *PPCA* gene allele (IVS7+3a/IVS7+3a), based on the enhanced proteolytic degradation. In contrast, the β-Gal activities in fibroblasts from the patients with Costello syndrome and sialidosis with missense mutations in the *NEU1* gene allele (V217 M/G243R) were normal.

Table 1. Phenotypes and genotypes of Lysosomal enzyme dificiencies and Costello syndorome

case	Phenotype	Genotype	% of normal activity[a)]
F 592	Normal		100
F 350	Morquio B	W 273 L/R 482 H	2.1
F 351	Morquio B	W 273 L/W 509 C	3.6
F 451	Morquio B	W 273 L/W 273 L	3.1
F 430	Juvenile GM 1-gangliosidosis	R 201 C[b)]/R 201 C	2.8
F 435	Juvenile GM 1-gangliosidosis	R 201 H[b)]/N 318 H	3.1
F 319	Sialidosis(Type 1)	V 217 M[c)]/G 243 R[c)]	100
F 622	Galactosialidosis	IVS 7+3 a[d)]/IVS 7+3 a[d]	19.6
F 642	Costello syndrome		86.6

a) Relative β-Gal activity to that of nomal subject was indicated.
b) The amino acid position is spliced out in EBP amino acid sequence.
c) The genotype for *NEU1* gene is indicated.
d) The genotype for *PPCA* gene is indicated.

Figure 1 shows the location of point mutations in *GLB* gene identified in the present GM1-gangliosidosis and Morquio B disease cases. The *GLB1* gene mutations causing W273L, N318H, R482H and W509C amino acid substitutions located in the exon 8, 14 and 15, respectively, possibly have effects on expression and functions of the mutant EBP while those causing the R201C and R201H substitutions located in the exon 6 impossibly have effects on the EBP expression and functions because the mutated portion should be spliced out in the resultant EBP mRNA. Therefore, normal EBP expression in the juvenile GM1-gangliosidosis cases (F430 and F435) with the *GLB1* genotypes (R201C/R201C and R201H/N318H, respectively) is expected, although the difference in gene dosage effect on normal EBP expression might be exhibited between the two cases.

As shown in Fig. 2a, immunofluorescence analysis with anti-bovine tropoelastin antibodies that cross-react with the human counterpart revealed that extracellular elastic fiber formation occurred in the fibroblasts from all the patients affected with β-Gal deficiencies, including the juvenile forms of GM1-gangliosidosis (F 430 and F 435), and the Morquio B syndrome (F350, F351 and F451), as well as in the normal subject (F592), although there might be a slight shape change of elastic fiber in the F451 case. The fibroblasts (F430) from the GM1-gangliosidosis patient with a homozygous mutation causing amino acid substitution R201C showed reasonable elastic fiber formation, this substitution being absent in the amino acid sequence of EBP and not having an effect on the normal EBP function. The fibroblasts (F435) from the GM1-gangliosidosis patient with a compound heterozygous mutated allele causing amino acid substitutions R201C and N318H also exhibited the elastic fiber formation. A significant

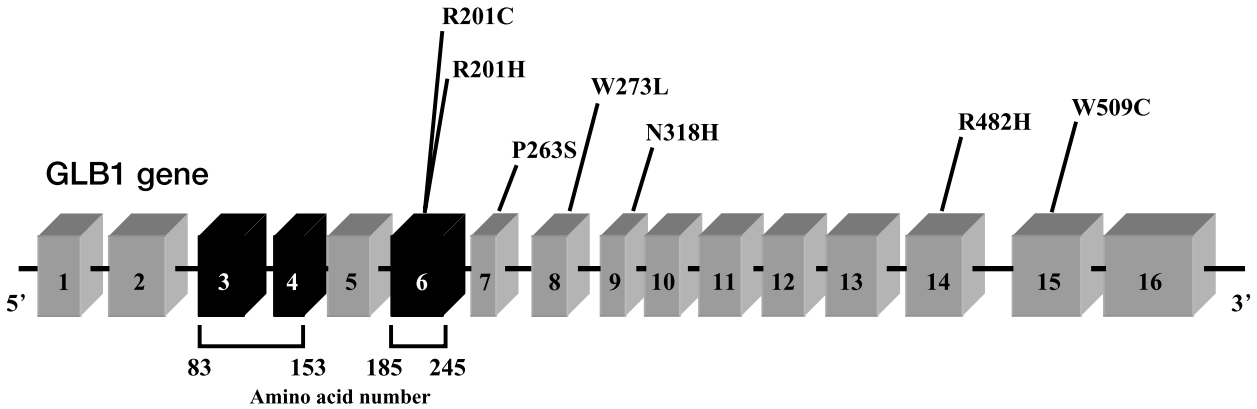
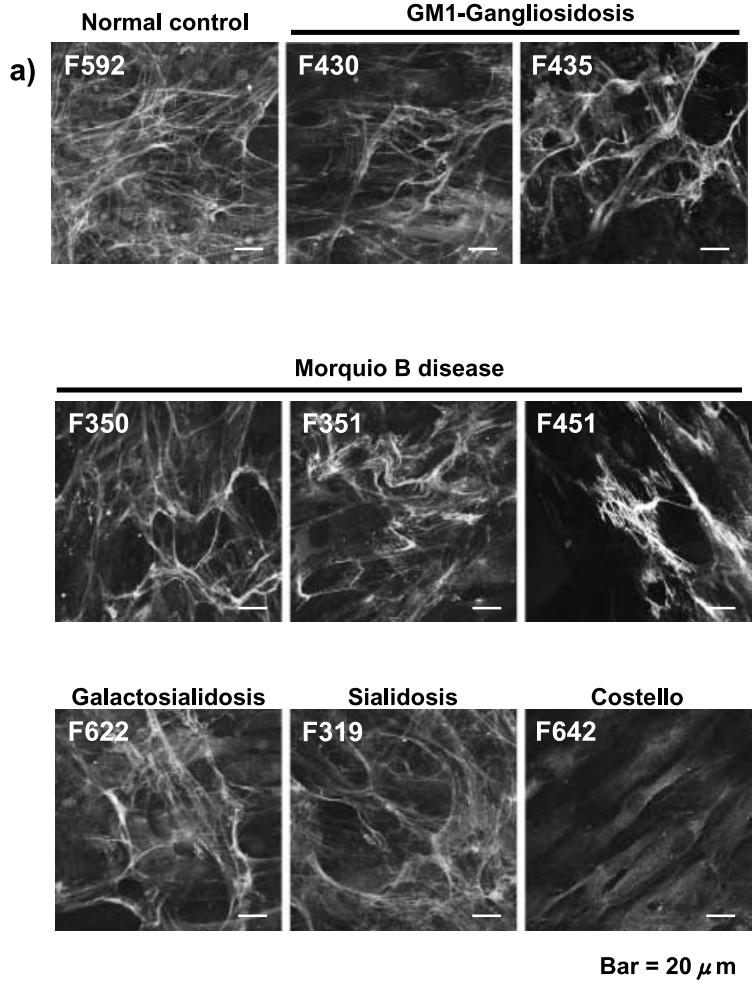


Fig. 1. Schematic representation of the human β-galactosidase (*GLB1*) gene containing the relative position of the sixteen exons and listing the identified mutations in the present clinical cases. Amino acid numbers spliced out from the amino acid sequence of the human β- galactosidase precursor protein are also indicated

gene dosage effect on fiber formation was not also observed (F430 and F435). We also examined the elastic fiber formation in the fibroblasts derived from an infantile (24, 25) and a juvenile (24, 25) GM1-gangliosidosis case, in which the *GLB1* gene mutations and genotypes were not determined, and also demonstrated the normal elastogenesis in these cases (data not shown). The patients affected with Morquio B disease comprised a homozygote and compound heterozygotes with mutated alleles causing W273L/W273L, W273L/R482H and W273L/W509C amino acid substitutions, respectively. In particular, the fibroblasts (F451) from the Morquio B patient with the W273L/W273L allele exhibited elastic fiber formation. Moreover, fibroblasts from the patients with galactosialidosis and sialidosis also showed extracellular elastic fibers (F622 and F319) as well as a normal subject (F592). In contrast, elastic fiber formation was hardly observed in fibroblasts from the patient with Costello syndrome (F642).

We also performed semi-quantitative evaluation for elastogenesis by means of the immunofluorescent imaging. As shown in Fig.2b, the degree of elastic fiber formation expressed as the relative immunofluorescence index was not significantly changed among normal subject and the patients with GM1-gangliosidosis, Morquio B disease, galactosialidosis and sialidosis, while the index value of Costello syndrome was very low.

We next examined the differential expression of mRNAs encoding β-Gal precursor protein and EBP, respectively, by RT-PCR using selective primers that discriminate each alternative splicing product. As shown in Fig. 3, significant transcriptional expression of β-Gal precursor mRNA was confirmed in fibroblasts from patients with GM1-gangliosidosis and Morquio B disease as well as in controls, including ones with galactosialidosis and sialidosis (panel a), as previously reported (24, 25). On the other hand, a significant level of EBP mRNA was observed in all of the fibroblasts from patients affected with GM1-gangliosidosis and Morquio B disease (panel a).



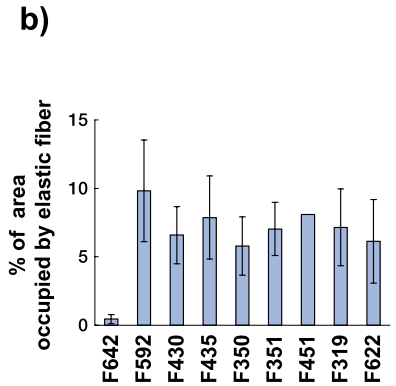


Fig. 2. Immunofluorescence analysis for elastic fibers in fibroblasts. (a) Elastic fiber formation was observed under a fluorescence microscope. Normal control, F 592 ; juvenile GM1-gangliosidosis, F430 and F435 ; Morquio B disease, F350, F351 and F451; juvenile/adult galactosialidosis, F 622 ; type I sialidosis, F319;Costello syndrome, F642. Bar, 20 mm. (b) Morphometric analysis of elastic fiber formation by means of anti-tropoelastin antibody. In each optic field, the immunofluorescent area with more than 100 intensity was estimated as elastic fiber-positive area, and the relative index (mean±SD) was expressed as a percentage of the total optical field area. n=2～7.

Expression of EBP was also observed in the cells from the patient with Costello syndrome (panel b).

To evaluate the semi-quantitative expression of β-Gal and EBP mRNAs in the fibroblasts with lysosomal enzyme deficiencies and Costello syndrome, the gene expression ratios of β-Gal and EBP mRNAs relative to β-actin, respectively, were also measured by real-time PCR analysis. As shown in Figure 4a, the relative fold change in expression level of EBP mRNA in the fibroblasts with lysosomal enzyme deficiencies and Costello syndrome ranged from 0.4 to 1.3, compared to that of normal subject (F592). Figure 4b shows the results for β-Gal mRNA. The relative fold change ranged from 0.7 to 2.2 among the fibroblasts.

The expression levels of EBP and β-Gal mRNAs did not correlate with the elastic fiber formation in the fibroblasts, although those of EBP mRNA in the GM1-gangliosidosis cases (F430 and F435) and a sialidosis case (F319) were about half of the normal control. These results suggest that there might not be a remarkable defect in the expression of EBP mRNA in the present β-Gal deficiencies as well as Costello syndrome.

DISCUSSION

The human *GLB1* gene encodes two gene products (1, 2). In human fibroblasts, two kinds are synthesized; one is a larger (2.4 kb) and more abundant mRNA encoding the full length lysosomal β-Gal precursor protein of 677 amino acids, and the other is a minor form (2.0 kb) produced through alternative splicing (whereby exons 3, 4, and 6 are deleted) which encodes an enzymatically inactive variant of 546 amino acids (35). The former *GLB1* product is co-translationally modified into a glycosylated 88-kDa precursor and then processed into a 64-kDa mature enzyme that associates with PPCA ; and NEU1 to form a stable multienzymic complex in lysosomes (5, 36), which

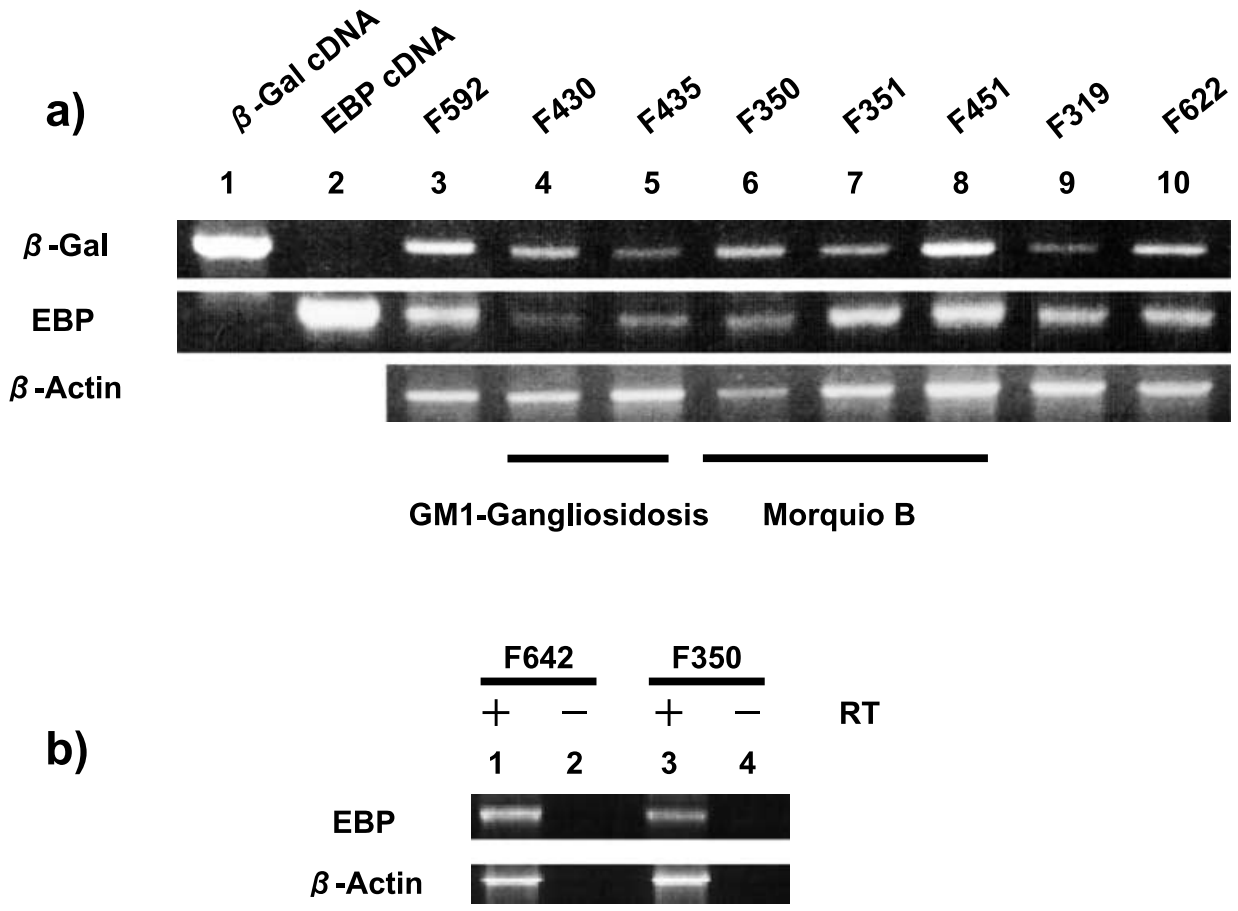


Fig. 3. Expression analysis for EBP mRNA in fibroblasts by RT-PCR. (a) RT-PCR for EBP, β-Gal and β-actin mRNAs was performed as described under "Methods". Lane 1, plasmid DNA carrying the β-Gal cDNA as a positive control ; lane 2, plasmid DNA carrying the EBP cDNA as a positive control ; lane 3, F 592 ; lane 4, F 430 ; lane 5, F 435 ; lane 6, F 350 ; lane 7, F 351 ; lane 8, F 451 ; lane 9, F 319 ; lane 10, F 622. (b) RT-PCR analysis of fibroblasts (F 642) derived from the Costello syndrome patient. Lanes 1 and 2, F 642 in the presence and absence of reverse transcriptase, respectively ; lanes 3 and 4, F 350 in the presence and absence of reverse transcriptase, respectively.

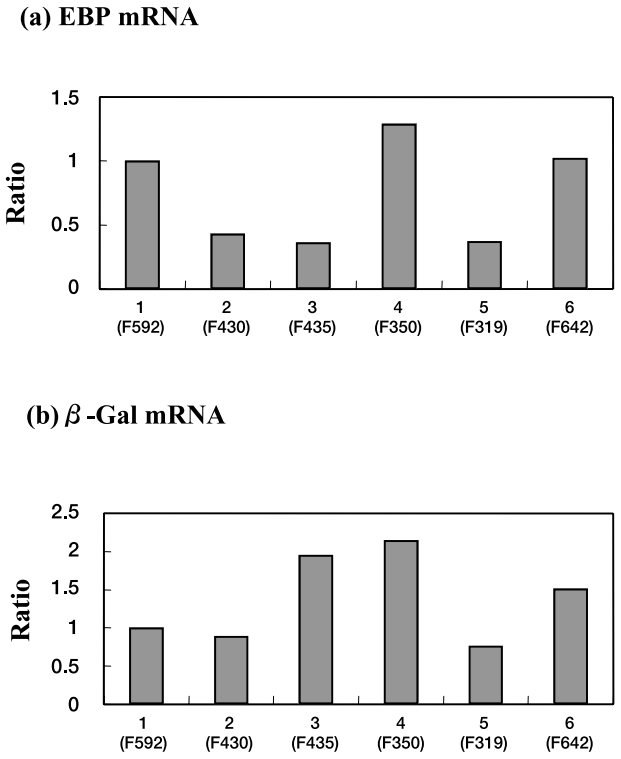


Fig. 4. Semi-quantitative evaluation of EBP and β- Gal mRNAs expressed in fibroblasts. Relative fold changes for expression of (a) EBP and (b) β- Gal mRNAs against β- actin mRNA were measured by quantitative RT-PCR as described under" Methods ". Column 1, F 592 ; column 2, F 430 ; column 3, F 435 ; column 4, F 350, column 5, F 319 ; column 6, F 622.

degrades glycoconjugates carrying β-galactose residues at their non-reducing termini, including GM1-ganglioside, keratan sulfate, and glycoproteins(1). The latter product is the 67-kDa EBP that functions as a molecular chaperon for tropoelastin in elastogenesis (17, 18). In recent years the EBP has also been suggested to form a cell surface receptor complex for elastic peptides with NEU1 and PPCA (20) that might be involved in cell proliferation and migration via signal transduction coupled with G-proteins (21).

GM1-gangliosidosis caused by the *GLB1* gene mutation is classified into three clinical forms : infantile (type 1), late infantile/juvenile (type 2), and adult/chronic (type 3). The most severe infantile patients develop rapidly progressive CNS involvement, skeletal abnormalities, and visceromegaly. A few patients have also been reported to show cardiomyopathy (3, 4, 37, 38). The late infantile/juvenile form is characterized by slowly progressive neurological symptoms. The rare adult/chronic form shows mild extrapyramidal manifestations, such as dystonia (1). On the other hand, Morquio B disease patients develop generalized skeletal dysplasia without CNS involvement. These phenotypes are believed to be governed by the kind of *GLB1* mutation and the residual β-Gal activity toward natural substrates. It has been reported that impaired elastogenesis might be caused by a genetic defect of 67-kDa EBP in the β- Gal deficiencies, including GM1-gangliosidosis and Morquio B disease, which should be responsible for some specific clinical manifestations in these disorders, including cardiac involvement and dysfunctions in connective tissues (19, 29).

However, we demonstrated in this study that significantly impaired elastogenesis was hardly observed in cultured skin fibroblasts derived from the six examined patients affected by β- Gal deficiencies, including infantile and juvenile GM1-gangliosidosis as well as Morquio B disease. Among the GM1-gangliosidosis patients, it may be reasonable that the two cases with the R201C/R201C and R201H/N482H mutations exhibited normal elastic fiber formation because the codon encoding the R 201 residue present in the exon 6 should have been spliced out from the wild-type EBP mRNA, which does not prevent the expression of normal EBP derived from at least a *GLB1* gene allele. On the other hand, the three of the examined Morquio B disease cases had the genotypes W273L/W273L, W273L/R482H and W273L/W509C that involve in the coding region of EBP, respectively. These fibroblasts with Morquio B disease also showed elastic fiber formation, although difference in the shape of elastic fiber was observed in the F451 fibroblasts with the W273L/W273L genotype. However, the difference was thought due to the cellular state of the F451 cells rather than genetic defect. Mecham R.P. suggested that elastin production should be extremely unstable *in vitro* culture system and care must be taken to adjust culture conditions to optimize for elastin production, including cell density, passage number and concentration of FBS, although the elastin phenotype shows greater stability in dermal fibroblasts in culture than most other cell types including vascular smooth muscle cells and ligament fibroblasts (39). We also revealed that there was a significant amount of EBP-specific mRNA in skin fibroblasts from the patients with β- Gal deficiencies. These results suggested that the *GLB1* gene mutations identified in the present cases might neither cause the defect in EBP mRNA expression nor the dysfunction of EBP leading to impaired elastogenesis in cultured skin fibroblasts.

Costello syndrome is a connective tissue disorder associated with sparse, thin, and fragmented elastic fibers in tissues, and characterized by polyhydramnios, a severely short stature, and a distinctive appearance

with craniofacial and dermatological manifestations, including soft skin, curly hair, hyperextensibility of the digits, pigmented nevi, vascular birthmarks and papillomata. (15, 16). About 60% of the patients also develop various cardiac abnormalities. We observed remarkably impaired elastic fiber formation in fibroblasts derived from the present Costello syndrome case.

Recently, Hinek *et al*. has reported that some proteoglycans regulate elastic fiber formation in fibroblasts via EBP shedding on the cell surface (28, 30). The" lectin-like "domain of EBP has been suggested to contribute to the regulation of assembly-disassembly between EBP and tropoelastin through binding the β-galactosugar-bearing moieties on the extracellular glycoconjugates, such as microfibrillar glycoproteins, and glycosaminoglycans including chondroitin sulfate and dermatan sulfate (28, 30, 40). The W273 residue of the human EBP is one of the conserved amino acids in the" lectin-like "domain putatively involving in binding to β-galactosugar-bearing glycoconjugates. However, we demonstrated the elastic fiber formation in fibroblasts derived from the Morqiuo B disease patient with the W273L/W 273L genotype, while the elastic fiber formation in fibroblasts derived from the present Costello syndrome patient was impaired. These results suggest the W273L substitution in the EBP might have little influence on interaction with an extracellular glycosaminoglycan carrying β-galactose residues and EBP shedding.

We showed in this study that elastogenesis in skin fibroblasts derived from patients with galactosialidosis and sialidosis was also not significantly impaired. Galactosialidosis, a PPCA deficiency, is classified into three clinical groups, that is, a severe early infantile form, a late infantile form and a juvenile/adult form (5, 26). The present galactosialidosis patient with the homozygous PPCA gene allele (IVS7+3A/IVS 7+3A) causing splicing mutation is the most typical for the juvenile/adult Japanese cases, in which the expression level of normal PPCA mRNA was reported to be about 10% of that expressed in normal fibroblasts (26). Therefore, a small amount of PPCA protein also exists in the galactosialidosis case, that is considered enough for normal elastogenesis in the skin fibroblasts.

The clinical phenotypes of sialidosis are classified in two major groups according to the age of onset and severity: late-onset type 1 sialidosis is a relatively mild form characterized by macular cherry-red spots and myoclonus, and early infantile-onset type 2 sialidosis is a severe condition distinguished by the appearance of dysmorphic manifestations in addition to neurological symptoms and cherry-red spots. Apparently normal elastogenesis was also observed in the fibroblast derived from a mild type1 sialidosis case with the compound heterozygous *NEU1* gene allele (V217M/G243R substitutions). Our previous study showed a small amount of the *NEU1* product with V217M substitution and a slight residual enzyme activity were expressed from the mutated *NEU1* allele, that might determine the mild phenotype of the type 1 sialidosis patient, while the G243R product hardly exhibited the normal *NEU1* functions (27). These results also suggested that at least these amino acid substitutions in *NEU1* product might not significantly affect the elastic fiber formation in the sialidosis fibroblast.

Thus, the biological and clinical effects of functional expression of the 67-kDa non-integrin EBP was demonstrated to vary on β-Gal deficiencies, the related genetic diseases and Costello syndrome. These findings also suggest that putative dysfunction of EBP and secondary complex with PPCA and NEU1 might not be a dominant cause of impaired elastogenesis at least in skin fibroblasts derived from the patients with these deficiencies. Further detail and careful study is necessary to elucidate the difference in elastogenesis among these diseases as well as the interaction among the components (EBP, PPCA and NEU1) of the putative secondary complex involving in the elastic fiber formation.

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