amount of mutant enzyme activity and protein was detectable in the endoplasmic reticulum (ER)/endosomal fractions, although the protein remained unprocessed. By contrast, the kinetically impaired mutation E59K was found to be normally processed to the lysosomes in transfected COS-7 cells. These results suggested that excessive degradation of these mutant proteins occurred within the ER.

Because mutant proteins with a misfolded conformation would be subject to rapid degradation in the ERAD [33], purified mutant proteins are expected to be fully folded and have a conformation similar to that of the residual enzyme under physiological conditions. A protein with a stable conformation typically resists denaturation, whereas those proteins with a fragile conformational structure are often intolerant to thermo- or pH-denaturation. To assess conformational stability of purified mutant enzymes, we performed thermo- and pH-denaturations with these enzymes [32]. Compared to the wild-type enzyme, most mutant proteins were found to be stable only over a narrow pH range. Noticeably, the mutant proteins maintained stability similar to that of the wild-type enzyme at a pH environment similar to that in lysosomes, suggesting that the folded conformation of mutant proteins is stable in lysosomes. All mutant proteins were less stable compared to the wild-type enzyme at neutral pH. These results suggest that the substitution of an amino acid residue in missense mutant α-Gal A enzymes could alter conformational stability, creating a more fragile molecular structure under neutral pH conditions.

The folding process of temporarily misfolded glycoproteins in the ER is subject to two dynamic competitive events, in which the calnexin/calreticulin system and glucosidases I and II promote refolding, whereas ER α-mannosidases and the ER degradation enhancing α-mannosidase I-like protein are involved in retrotranslocation and degradation of misfolded proteins in the process of ERAD [34]. Removal of a mannose residue from Man9 N-linked oligosaccharides by ER α-mannosidase I is a critical luminal event for preventing proteins from reentering the refolding process, and serves as a signal for targeted ERAD. Inhibition of ER α-mannosidase I often delays the degradation of glycoproteins in the ERAD in favor of protein refolding. When kifunensine, a selective inhibitor of the ER α-mannosidase I, was added to the culture medium of transfected cells, the amount of all mutant proteins (except E59K) appeared to increase (Fig. 2), suggesting that the degradation of mutant enzymes was partially inhibited. This result provided clear evidence that degradation of misfolded mutant α-Gal A enzymes occurred by ERAD as the result of misfolding of mutant proteins.

Protein misfolding is recognized as an important cause of protein deficiency in various inherited disorders [35]. Despite the widespread occurrence of protein misfolding, supported by the fact that individual cases of misfolding exist in a variety of diseases, the significance of protein misfolding in each genetic disorder has not been well addressed except in a few examples, such as the ΔF508 mutation that causes misfolding of cystic fibrosis transmembrane regulator and is responsible for the majority of cystic fibrosis patients [36]. The results obtained from a large set of Fabry missense mutant proteins also provide evidence that protein misfolding is a primary cause of protein deficiency not limited to a few mutations, but rather is a generalized pathophysiological phenomenon that occurs as the result of many missense mutations in a single

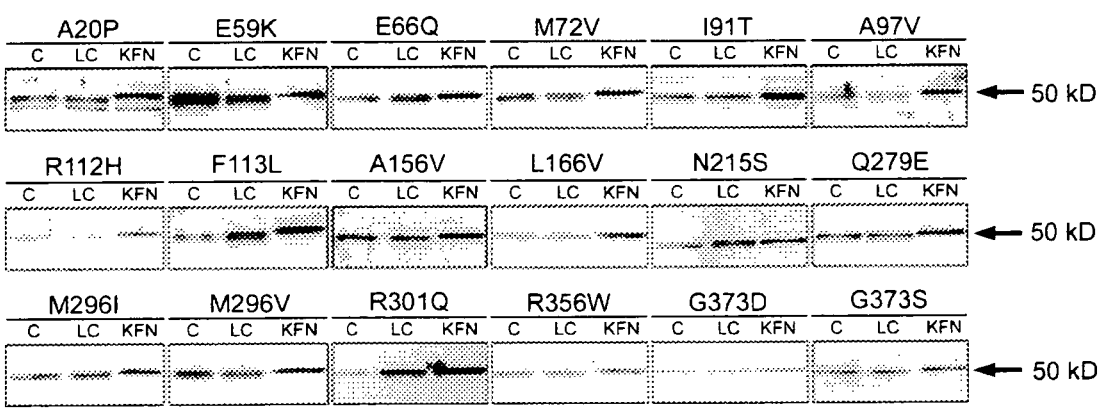

Fig. 2. Effects of ERAD inhibitors on the amount of mutant α-Gal A expressed in COS-7 cells. Wild-type, or mutant α-Gal A enzymes were transiently expressed in COS-7 cells. Cells were treated with 2 μM lactacystin (LC), or 0.2 mM kifunensine (KFN) 5 h after transfection. Upon harvest, western blot analyses of cell lysates were performed. (C) Control. Reproduced permission from Ishii *et al.* [32].

genetic disorder. The development of strategies that specifically rescue such misfolded mutant proteins from the ERAD could be significant in battling various inherited protein deficiencies.

Development of ASSC therapy for Fabry disease

The strategy of using competitive inhibitors as ASSCs began with DGJ for increasing residual α-Gal A activity in the lymphoblasts established from Fabry patients [18,19]. Prior to this, studies of the residual activities of mutant enzymes in many Fabry patients showed that some of them had kinetic properties similar to those for wild-type α-Gal A [3,30,37]. The biosynthetic processing was delayed in the cultured fibroblasts of a Fabry patient [38], and over-expressed mutant protein formed aggregates in the ER of transfected COS-1 cells [39], suggesting that enzyme deficiency in some mutants may primarily be caused by an aborted exit from the ER. Upon the realization that the deficiency of α-Gal A activity could be the direct consequence of mutant protein misfolding within the ER, we purposely took a chemical biology approach to seek active-site directed competitive inhibitors for the enhancement of residual enzyme activity. Enzyme substrates and substrate analogues have been historically used as enzyme stabilizers *in vitro*. If the hypothesis were true, potent enzyme inhibitors could serve as a folding template in the ER to modify the dynamics of protein folding in favor of proper folding, thereby increasing intracellular enzyme activity (Fig. 3). Retrospectively, these enzyme inhibitors could be useful tools for probing and assessing the folding status of a mutant protein. To gain therapeutic benefits, the rescued mutant enzyme needs to be active and free of inhibitors in the lysosomes. Competitive inhibitors have, contradictorily, potential to fulfill such requirements *in vivo*. Massive storage of glycolipid substrates would replace chaperone inhibitors in lysosomes to permit the catalytic function of enzymes. In addition, dynamic exclusion of small molecules *in vivo* could be an additional advantage in stripping off the inhibitors from the mutant enzymes. If necessary, this could be accomplished by an alternate scheduled dose in patients (e.g. a 1-week dose of the chaperone drugs to permit the accumulation of mutant enzymes in lysosomes, followed by a halt in drug administration the

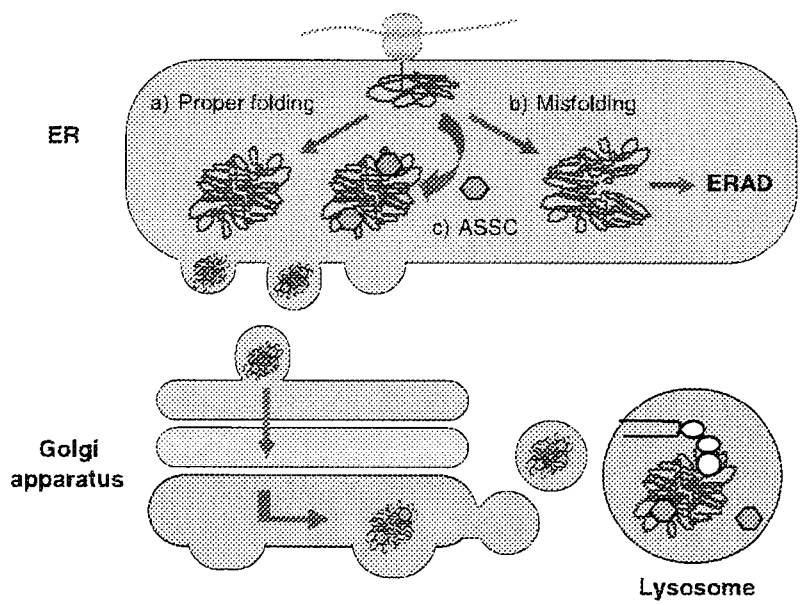

Fig. 3. Consequence of misfolded α-Gal A in the ER and active-site-specific chaperone therapy. Synthesis of proteins takes place at ribosomes, and newly synthesized proteins are secreted to the lumen of the ER. The ER has developed a 'quality-control system' to ensure the full integrity of each protein. This system is enforced by several molecular chaperones and folding-assistant enzymes. (a) Appropriately folded proteins are transported out of the ER, whereas (b) misfolded and unfolded mutant proteins are retained in the ER and are eventually degraded by ERAD. (c) ASSCs (red hexagons) bind to the active-sites of mutant enzymes and induce their properly folded conformation. As a result, this prevents excessive degradation of the mutant proteins within ERAD and promotes their smooth transport to the Golgi apparatus. Once the mutant protein/ASSC complex reaches lysosomes, ASSCs are replaced by massive storage of substrates to allow the catalytic function of the mutant enzymes.

following week to accelerate dissociation of inhibitors from the enzymes), permitting reduction of substrate storage by the mutant enzymes. As a result, DGJ was discovered as an ASSC specifically effective for Fabry disease [18].

DGJ is a small molecular iminosugar that resembles an α-galactose residue when bound to the active-site of α-Gal A. DGJ is one of the most potent competitive inhibitors for α-Gal A [40]. Based upon active-site interactions observed in the crystal structure of α-galactose bound to α-Gal A, a model of DGJ binding to α-Gal A shows many favorable interactions: the imino group on DGJ is expected to interact with D170; the hydroxyl groups of DGJ form hydrogen bounds with D92, D93, K168, E203, R227, and E231; and a hydrophobic surface on DGJ makes van der Waals interactions with W47 (Fig. 4). The binding between DGJ and the protein would fix the active-site involving the five loops β1-α1, β2-α2, β4-α4, β5-α5, and β6-α6. The initial folding process in the ER is a thermodynamic equilibrium based upon the amino acid sequence of the peptide. A firm binding between DGJ and the fragile enzyme could dramatically shift the folding process toward normal folding, conferring the correct conformation on mutant enzymes that would otherwise be largely misfolded.

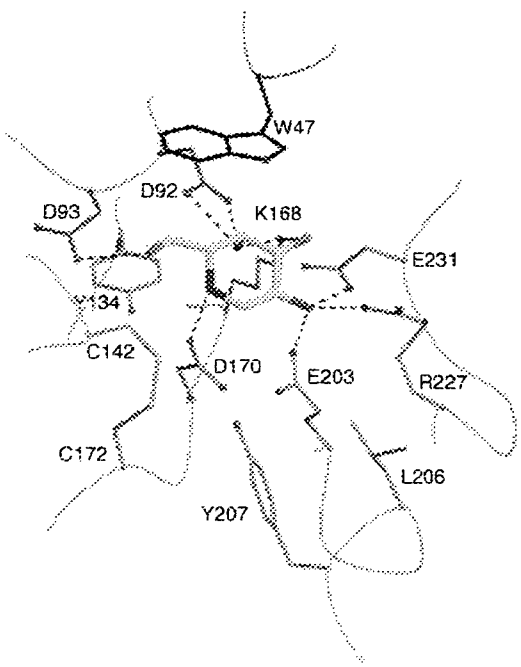

Fig. 4. Predicted interactions between DGJ and the active-site of α-Gal A. DGJ is a known active-site directed competitive inhibitor of α-Gal A. Interactions of α-Gal A with DGJ were modeled based upon the crystal structure of α-Gal A with bound α-galactose. The key interactions with the 2-, 3-, 4-, and 6-hydroxyls on the ligand are maintained when either α-galactose or DGJ bind to the active site. One key interaction between E231 on the enzyme and the anomeric hydroxyl of α-galactose is lost when DGJ binds. Modified from Ishii *et al.* [32].

Cellular enhancement of mutant α-Gal A activity with DGJ

ASSC activity is derived from a combination of affinity to the targeted protein, cellular permeability, and ER accessibility. An ASSC is required to cross both the plasma and ER membranes, and be deliverable to the ER where it binds to and rescues its counterpart. Although an *in vitro* enzyme inhibitory assay could be an efficient initial screening of ASSCs, a cell-based enhancement assay was performed to evaluate the ASSC activity of DGJ [41]. In an attempt to rescue misfolded mutant enzyme from excessive degradation, we demonstrated that DGJ effectively increased residual α-Gal A activity in Fabry lymphoblasts derived from hemizygous Fabry patients with the R301Q or Q279E mutations. These cells were treated with concentrations lower than that usually required for intracellular inhibition of the enzyme [18,40]. The enzyme activity in R301Q or Q279E lymphoblasts increased by eight- or seven-fold, respectively, after cultivation with DGJ at 20 μM for 4 days, and the increase was dose-dependent at concentrations that were not intracellularly inhibitory. DGJ was α-Gal A specific, and did not affect misfolded mutant proteins in fibroblasts from other lysosomal storage disease patients at the concentrations effective for α-Gal A [40]. Upon treatment with DGJ of transfected COS-7 cells, R301Q and L166V mutant enzymes were apparently trafficked into lysosomes in a processed mature form [32]. Independent studies by Yam *et al.* [42] in transgenic mouse fibroblasts that overexpress human R301Q α-Gal A confirmed that the mutant enzyme was retained in the ER and not correctly folded, as demonstrated by the formation of complexes with BiP. Cultivation of the cells with DGJ significantly reduced these complexes, indicating that DGJ exerts a chaperone-like effect on enzyme conformation. In human Fabry R301Q and Q357X fibroblasts, DGJ treatment resulted in clearance of lysosomal storage, accompanied by the disappearance of multilamellar lysosomal inclusions. Genes involved in cell stress signaling, heat shock response, unfolded protein response, and ERAD show no apparent difference in expression between untreated and DGJ-treated fibroblasts [43], indicating that DGJ does not directly affect the ERAD system.

Schiffmann and colleagues have used a T-cell based system to determine whether the activity of 11 Fabry disease enzyme mutants can be enhanced using DGJ. When patient-derived T cells were grown in the presence of DGJ, α-Gal A activity increased to more than 50% of normal for several mutations, including A97V, R112H, R112C, A143T, and L300P [44]. We recently tested DGJ enhancement in patient fibroblasts and lymphoblasts expressing a variety of disease-causing α-Gal A missense mutations. The results showed that residual enzyme activity could be specifically increased 20% above normal after incubating the cultured cells with DGJ at 20 μM for 5 days [32].

Interestingly, the effect of DGJ does not appear to be limited to mutations that primarily cause protein misfolding. After treatment with DGJ, residual enzyme activity increased by eight-fold in the cultured fibroblasts of a Fabry patient with the E59K mutation. This mutation has been shown to confer compromised kinetic properties, and protein misfolding is not a major obstacle to enzyme activity [32]. It has been proposed that retention and degradation of misfolded proteins entering the secretory pathway may not be restricted to mutant proteins [45]. Protein folding is not a perfect process even with wild-type proteins. A large fraction of newly synthesized proteins never attain their native structure, and are ubiquitinylated before being degraded by cytosolic proteasomes. Small molecular ligands have also been shown to be effective at increasing maturation of the wild-type δ-opiod receptor [46]. Evidence obtained from our study indicates that DGJ enhancement could be clinically beneficial for a broad range of missense mutations that not only cause protein misfolding, but also other types of protein defects.

Enhancement of mutant α-Gal A activity with DGJ in transgenic mice

To examine the effect of DGJ enhancement *in vivo*, we generated transgenic mice expressing human mutant α-Gal A (R301Q) in an endogenous null background [47]. Because the expression level of the transgene is substantially higher than that of the endogenous gene, these mice are clinically healthy, and do not present a clinical phenotype. Because the mice exclusively express human mutant enzyme in all major tissues including the heart, kidneys, and brain (the main organs affected by Fabry disease in man), they are an excellent biochemical animal model for *in vivo* proof-of-concept, and allow the pharmacokinetics of DGJ to be studied. Oral administration of DGJ to transgenic mice led a dose-dependent increase in α-Gal A activity in the major tissues of the mice. Enzyme activities increased by 13-, 3.3-, 3.9-, 2.6-, and 2.3-fold in heart, kidneys, spleen, liver, and brain, respectively, in mice that were fed with DGJ at approximately 3 $mg \cdot g^{-1}$ body $weight \cdot day^{-1}$ for 2 weeks [47]. No apparent toxic effects were observed in transgenic mice treated with DGJ for 140 days, indicating that DGJ is well tolerated in mice.

ASSC therapy for Fabry disease in humans

The clinical proof-of-concept for ASSC therapy has been investigated in cardiac Fabry disease by Frustaci and colleagues [48]. Galactose, a less effective inhibitor of α-Gal A compared to DGJ, was administered to a cardiac Fabry patient by intravenous infusion at 1 $g \cdot kg^{-1}$ three times weekly. After a 3-month treatment period, remarkable improvements in the increase in the left ventricular ejection fraction (from 32% to 51%), and reduction in ventricular wall thickness (from 18 mm to 15 mm) were observed. The patient who had severe myocardial disease no longer required a cardiac transplant, and returned to full-time work after 2 years of treatment. Although galactose is not considered to be a viable therapeutic agent for Fabry disease because it requires an excessive amount of intravenous infusions every other day to sustain its therapeutic effect, the concept of ASSC was confirmed as an effective therapeutic approach in humans.

DGJ is approximately 120 000-fold more potent than galactose. Upon completion of preclinical safety tests in rats and monkeys, clinical phase I trails for DGJ (Amigal™) were conducted in healthy volunteers for safety and pharmacokinetics (http://www.amicustherapeutics.com). Currently, several phase II clinical trials for Amigal are being conducted with male and female Fabry patients who harbor a variety of missense mutations.

How much residual enzyme activity is enough?

A full level of lysosomal enzyme activity is not required to prevent the storage of substrate. Many lysosomal storage disease patients with a significant level of residual enzyme activity are asymptomatic, indicating that clinical symptoms develop in patients only when the level of residual enzyme activity falls to a critical threshold [49]. In Fabry disease, the critical threshold for residual enzyme activity could vary between individuals. However, based on the fact that the majority of diagnosed variant patients retain residual enzyme activity at

5–10% the level of normal, and that a hemizygote patient with less than 3% of the normal level is likely to present classic symptoms, one would assume that residual enzyme activity greater than 10% of normal in hemizygote patients might be sufficient at reducing the majority of clinical symptoms. Even for patients whose residual enzyme activity cannot be increased over approximately 10% of normal, any increase in activity is still considered to be clinically beneficial because it may dramatically modify the clinical phenotype and reduce clinical manifestations that affect quality of life.

Perspective of DGJ treatment for Fabry disease

To date, ERT is the only available Food and Drug Administration approved therapy for Fabry disease. ERT has clear advantages in that it can be administered to a full clinical spectrum of patients, including those with nonsense mutations and missense mutations that result in total disruption of the catalytic domain. For them, DGJ would not be effective. On the other hand, DGJ is expected to be highly effective for patients who have missense mutations that primarily lead to misfolding of the mutant protein. DGJ could also be useful as an adjunct therapy with ERT for patients whose residual enzyme activity cannot be increased by DGJ alone to a level that reverses disease development. This could potentially reduce the overall therapeutic cost and add convenience for patients. Compared to the protein macromolecule that is administered through intravenous infusion every other week, DGJ is an orally active small molecule drug. This would provide undeniable advantages of convenience, cost savings, and ease of accessibility by the drug to tissues, including the central nervous system. Because a large proportion of mutant enzymes in Fabry patients with missense mutations are kinetically active, ASSC therapy using DGJ may be broadly applicable to Fabry patients with various missense mutations.

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Mutant α-galactosidase A enzymes identified in Fabry disease patients with residual enzyme activity: biochemical characterization and restoration of normal intracellular processing by 1-deoxygalactonojirimycin

Satoshi ISHII*†, Hui-Hwa CHANG*, Kunito KAWASAKI†, Kayo YASUDA*, Hui-Li WU*, Scott C. GARMAN‡ and Jian-Qiang FAN*[1]

*Department of Human Genetics, Mount Sinai School of Medicine, Fifth Avenue at 100th Street, New York, NY 10029, U.S.A., †Department of Agricultural and Life Sciences, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, 080-8555, Japan, and ‡Department of Biochemistry and Molecular Biology, University of Massachusetts, 710 North Pleasant Street, Amherst, MA 01003, U.S.A.

Fabry disease is a lysosomal storage disorder caused by the deficiency of α-Gal A (α-galactosidase A) activity. In order to understand the molecular mechanism underlying α-Gal A deficiency in Fabry disease patients with residual enzyme activity, enzymes with different missense mutations were purified from transfected COS-7 cells and the biochemical properties were characterized. The mutant enzymes detected in variant patients (A20P, E66Q, M72V, I91T, R112H, F113L, N215S, Q279E, M296I, M296V and R301Q), and those found mostly in mild classic patients (A97V, A156V, L166V and R356W) appeared to have normal K_m and V_{max} values. The degradation of all mutants (except E59K) was partially inhibited by treatment with kifunensine, a selective inhibitor of ER (endoplasmic reticulum) α-mannosidase I. Metabolic labelling and subcellular fractionation studies in COS-7 cells expressing the L166V and R301Q α-Gal A mutants indicated that the mutant protein was retained in the ER and degraded without processing. Addition of DGJ (1-deoxygalactonojirimycin) to the culture medium of COS-7 cells transfected with a large set of missense mutant α-Gal A cDNAs effectively increased both enzyme activity and protein yield. DGJ was capable of normalizing intracellular processing of mutant α-Gal A found in both classic (L166V) and variant (R301Q) Fabry disease patients. In addition, the residual enzyme activity in fibroblasts or lymphoblasts from both classic and variant hemizygous Fabry disease patients carrying a variety of missense mutations could be substantially increased by cultivation of the cells with DGJ. These results indicate that a large proportion of mutant enzymes in patients with residual enzyme activity are kinetically active. Excessive degradation in the ER could be responsible for the deficiency of enzyme activity *in vivo*, and the DGJ approach may be broadly applicable to Fabry disease patients with missense mutations.

Key words: active-site-specific chaperone (ASSC), 1-deoxygalactonojirimycin (DGJ), endoplasmic reticulum-associated degradation (ERAD), Fabry disease, α-galactosidase A, protein misfolding.

INTRODUCTION

Fabry disease is an X-linked inherited lysosomal storage disorder resulting from the deficient activity of α-Gal A (α-galactosidase A), an enzyme responsible for the catabolism of neutral glycosphingolipids that have an α-galactose residue at their non-reducing terminus [1]. Deficient enzyme activity results in the progressive deposition of glycosphingolipids, predominantly globotriaosylceramide, in the lysosomes of vascular endothelial cells. The disease is classified into two major subtypes based upon clinical manifestations. Patients with little or no detectable enzyme activity have early onset, or classic, Fabry disease. Clinical symptoms are severe and range from angiokeratomas, acroparesthesia, hypohidrosis, corneal opacity in the early teens, and progressive vascular disease of the heart, kidneys and central nervous system [2]. Without medical intervention, death typically occurs in the fourth or fifth decade of life, as a result of renal failure, cardiac disease or cerebrovascular disease [3,4]. In contrast, patients with residual enzyme activity have a late-onset phenotype, and include atypical cardiac or renal variants. These patients are usually asymptomatic until their late thirties, and their clinical manifestations are often limited to the heart [5,6] or kidneys [7]. Without treatment, patients eventually suffer from heart failure or end-stage renal failure in the fifth or sixth decade of life [8].

To date, more than 400 mutations have been identified in the α-Gal A gene (termed GLA; Human Gene Mutation Database), and more than 57 % of mutations are missense. The majority of mutations are private, occurring in only one or a few families. The correlation between genotype and residual enzyme activity (measured primarily in leucocytes) is not strong, and presumably depends upon the nature of the mutation and additional genetic or non-genetic factors. However, it has been clearly demonstrated that a higher level of residual enzyme activity results in a milder disease phenotype, typically involving a few or monosymptomatic clinical manifestations, and are less likely to cause the classic form of the disease [9].

The mature α-Gal A enzyme contains 398 amino acid residues once the signal peptide at the 31st amino acid residue (an alanine residue) has been cleaved [10]. From the X-ray crystal structural information [11], 13 amino acid residues were predicated to be directly involved in the interaction with α-galactose; mutations occurring at any of these amino acids result in the severe classic phenotype of Fabry disease [11,12]. Structural studies also revealed that the majority of amino acids within the mutant proteins do not directly contribute to the catalytic function of the

Abbreviations used: ASSC, active-site-specific chaperone; DGJ, 1-deoxygalactonojirimycin; DMEM, Dulbecco's modified Eagle's medium; ER, endoplasmic reticulum; ERAD, endoplasmic reticulum-associated degradation; FCS, fetal calf serum; α-Gal A, α-galactosidase A; GLA, α-Gal A gene; 4MU, 4-methylumbelliferyl; 4MU-α-Gal, 4MU α-D-galactopyranoside.

[1] To whom correspondence should be addressed (email jian-qiang.fan@mssm.edu).

enzyme, but rather to the maintenance of the tertiary structure. Despite the fact that many mutations have been identified in Fabry disease patients, with a few exceptions [13–15], the biochemical and molecular defects of mutant enzymes in patients who retain residual enzyme activity are not well understood.

Protein misfolding has been recognized as an important pathophysiological cause in many inherited disorders, including cystic fibrosis, α1-antitrypsin deficiency, familial hypercholesterolaemia and Alzheimer's disease, in which mutant proteins are not properly folded [16–18]. Improperly folded proteins are retained in the ER (endoplasmic reticulum) and subject to ERAD (ER-associated degradation), resulting in a protein deficiency. Despite the broadness of protein misfolding, which has been supported by the fact that individual cases of misfolding exist in a variety of diseases, the significant impact of protein misfolding in each genetic disorder has not yet been well-addressed except in a few examples, such as cystic fibrosis, in which one mutation (ΔF508) causing misfolding of CFTR (cystic fibrosis transmembrane conductance regulator), is responsible for the majority of patients [19]. Although powerful computer modelling techniques can predict the folding status of many mutations once the crystal structure is available, direct experimental evidence would still be important. Therefore it is important to examine protein misfolding in a variety of affected mutations within clearly defined subsets of patients; such a study can provide evidence that protein misfolding can be pathogenetically significant, not only in individual mutations, but also in clinical subsets of diseases.

Previously we have shown that addition of DGJ (1-deoxygalactonojirimycin), a competitive inhibitor of α-Gal A, at subinhibitory concentrations, to cultured lymphoblasts established from Fabry disease patients with the R301Q or Q279E mutation, substantially increases residual enzyme activity [20]. A reduction of large-size lysosomes and loss of characteristic multilamellar lysosomal inclusions were demonstrated upon DGJ treatment [21]. The proposed mechanism for this observation is that DGJ is an ASSC (active-site-specific chaperone) and serves as a folding template by which the mutant protein is induced to attain the proper conformation, thus preventing excessive degradation by ERAD [22]. Administration of DGJ to transgenic mice that express human R301Q α-Gal A in a null background results in a substantial increase in enzyme activity in various tissues of the heart, kidneys, liver and spleen, suggesting that DGJ could have a therapeutic effect in treating certain Fabry disease patients, particularly those with a cardiac phenotype [23]. Currently, DGJ is under phase II clinical evaluation for Fabry disease.

In the present study, we generated 19 mutant α-Gal A cDNA constructs with various missense mutations found in Fabry disease patients with residual enzyme activity, including nine variant mutations, five classic mutations, one presymptomatic mutation and four mutations causing both variant and classic phenotypes. Sixteen mutant enzymes were efficiently purified from transfected COS-7 cells, and their enzymatic and biochemical properties were characterized. These present studies demonstrated that a significant number of missense mutations give rise to enzymes which are defective with respect to conformational stability, but which retain full or partial catalytic activity. These findings apply to a relatively large set of missense mutations identified in both classic and variant Fabry disease patients, and suggest that protein misfolding may be more widespread than previously thought. The results also provide a molecular understanding of the therapeutic effect of ASSCs in Fabry disease patients and indicate that DGJ treatment may be effective for a large number of Fabry disease patients with missense mutations regardless of their clinical phenotypes.

MATERIALS AND METHODS

Cells from patients

Fabry disease lymphoblasts and fibroblasts were supplied by Dr S. Nakao (Kagoshima Prefectural Kanoya Hospital, Japan), Dr R.O. Brady of the NIH (National Institutes of Health, Bethesda, MD, U.S.A.) and Dr R.J. Desnick (Mount Sinai School of Medicine, New York, NY, U.S.A.). G_{M1}-gangliosidosis fibroblasts were established from an adult patient with the homozygous I51T mutation, and Gaucher fibroblasts were obtained from a type I patient with the homozygous N370S mutation (purchased from the Coriell Institute, Camden, NJ, U.S.A.).

Cell culture

Human lymphoblasts were maintained in RPMI-1640 (Mediatech) supplemented with 10% FCS (fetal calf serum; Mediatech) and 1% penicillin/streptomycin (Invitrogen). Human fibroblasts and COS-7 cells were cultured in DMEM (Dulbecco's modified Eagle's medium; Mediatech) supplemented with 10% FCS and 1% penicillin/streptomycin. All cells were incubated in a water-jacket incubator at 37 °C under 5% CO_2. DGJ (Toronto Research Chemicals) was added to the culture medium when needed to increase residual enzyme activity.

Site-directed mutagenesis of α-Gal A

Expression vectors containing α-Gal A mutations were generated by site-directed PCR mutagenesis [24] using the QuikChange® Site-Directed Mutagenesis Kit (Stratagene). Nineteen amino acid substitutions were individually introduced by PCR amplification using PfuTurbo DNA polymerase, the wild-type α-Gal A cDNA sequence as a template and a 35-mer primer set, with sense and antisense primers harbouring one of the nucleotide substitutions in the middle of their sequence. Each mutant cDNA was verified by DNA sequencing before being subcloned into the expression vector pCXN2 [25].

Transient expression of mutant enzymes in COS-7 cells

Transient expression of mutant enzymes in COS-7 cells was carried out using FuGENE™ 6 transfection reagent (Roche Molecular Biochemicals) or Lipofectamine™ 2000 reagent (Invitrogen), according to the manufacturer's protocol. Typically, prior to transfection, a mixture of plasmid DNA (0.5 μg) and FuGENE™ 6 transfection reagent (1.5 μl) in 100 μl of serum-free DMEM medium was incubated at room temperature (25 °C) for 20 min, followed by mixing with 400 μl of the complete medium. The transfection mixture was added to COS-7 cells grown in six-well culture plates, and the cells were then incubated at 37 °C. Additional complete medium (1 ml) was added to each well the following day, and cells were further incubated until harvest.

Purification of mutant α-Gal A enzymes expressed in COS-7 cells

Mutant α-Gal A enzymes were typically harvested from homogenates of transfected COS-7 cells grown on 10-cm diameter culture dishes, and purified as described previously [26]. The purified protein appeared as a single band after SDS/PAGE and visualization using a silver stain kit (Bio-Rad Laboratories).

Enzyme assay and protein determination

Cell pellets obtained from cell cultures were homogenized in water using a micro homogenizer. The supernatant collected after centrifugation of the homogenate at 10000 *g* for 5 min was used in enzyme assays.

All 4MU (4-methylumbelliferyl) substrates were obtained from Sigma. The α-Gal A activity was assayed with a mixture (60 μl) of 4MU-α-D-galactopyranoside (4MU-α-Gal, 5 mM) and *N*-acetyl-D-galactosamine (75 mM) in 0.1 M sodium citrate buffer (pH 4.6) as described previously [20]. β-Hexosaminidase activity was determined with 5 mM 4MU-*N*-acetyl-β-D-glucosaminide as substrate in the same buffer. β-Galactosidase activity was assayed with 1 mM 4MU-β-D-galactopyranoside in 0.1 M sodium citrate and 0.2 M disodium hydrogen phosphate buffer (pH 4.6). The activity of glucocerebrosidase was determined with 3 mM 4MU-β-D-glucopyranoside in the presence of 0.25 % (w/v) sodium taurocholate and 0.1 % (v/v) Triton X-100 in McIlvaine buffer (0.1 M sodium citrate and 0.2 M disodium hydrogen phosphate buffer, pH 5.2). All enzyme reactions were terminated by the addition of 1.2 ml of 0.1 M glycine buffer (pH 10.7), prepared by adjusting the pH using 0.1 M NaOH. The released 4MU was determined by fluorescence measurement at 360 and 450 nm as the excitation and emission wavelengths respectively. One unit of enzyme activity is defined as the amount of enzyme that releases 1 nmol of 4MU per h. The protein concentration was determined by using a DC Protein Assay kit (Bio-Rad Laboratories), using BSA as a standard.

Kinetic properties of mutant enzymes

The kinetic properties were determined with 4MU-α-Gal at various concentrations as substrate in 0.1 M sodium citrate buffer (pH 4.6) at 37 °C for 10 min. The K_m and V_{max} values of each mutant enzyme were obtained from Lineweaver–Burk plots.

Measurement of optimal pH and pH stability of purified mutant α-Gal A enzymes

The optimal pH of mutant enzymes was determined by assaying the enzyme activity in 0.1 M McIlvaine buffer at various pHs (pH 3.5–5.5). The pH stability of purified enzymes was assayed as described previously [13]. In order to determine the relative stability of the mutant enzymes at neutral pH, the enzyme was pre-incubated in 2.5 mM phosphate buffer (pH 7.5; prepared by mixing 2.5 mM sodium dihydrogen phosphate and 2.5 mM disodium hydrogen phosphate) at 37 °C for various times. After incubation, an aliquot was diluted with a 4-fold volume of 0.2 M sodium citrate buffer (pH 4.6), and α-Gal A activity was assayed immediately.

Western blot analysis

Western blot analysis for the detection of α-Gal A protein was performed using an anti-α-Gal A polyclonal antibody produced in rabbit and a HRP (horseradish peroxidase)-conjugated anti-rabbit IgG antibody produced in goat (Pierce Biothechnology). An anti-Bip monoclonal antibody purchased from StressGen Biotechnologies and a HRP-conjugated anti-mouse IgG antibody (Pierce Biotechnology) were used for the detection of Bip. After SDS/PAGE, proteins were transferred electorophoretically to a PVDF (Immobilon P) membrane (Millipore). The membrane was blocked with 5 % (w/v) non-fat dried skimmed milk in blot solution [10 mM Tris/HCl (pH 7.5) with 0.25 M NaCl and 0.05 % Tween 20] at 4 °C overnight, and then treated with a primary antibody diluted in a milk/blot solution [1 % (w/v) non-fat dried skimmed milk in blot solution] for 1 h at room temperature with mild shaking. After washing with an excess volume of the milk/blot solution, the membrane was treated for 1 h at room temperature with a secondary antibody diluted in the milk/blot solution. Following extensive washing with the milk/blot solution, protein bands were visualized with SuperSignal® Chemiluminescent Substrate (Pierce Biotechnology).

Subcellular fractionation of expressed mutant α-Gal A enzymes in COS-7 cells

COS-7 cells plated on to four 10-cm diameter dishes were transfected with plasmid DNA containing mutant α-Gal A cDNA. Cells were harvested in PBS on the third day after transfection, and cell pellets were resuspended in Suc Buffer (0.25 M sucrose, 10 mM Hepes and 1 mM EDTA, pH 7.4) on ice. Cells were homogenized in the same buffer using a Potter–Elvehjem Teflon-glass homogenizer. Following centrifugation at 1000 *g* for 5 min at 4 °C, the supernatant (1 ml) was pooled as a postnuclear fraction, and layered on top of a 9 ml 40 % Percoll solution in Suc Buffer. Subcellular fractionation was performed by centrifugation at 25 000 *g* for 1 h. Each fraction containing approx. 0.5 ml was collected from the bottom of the centrifugation tube and used for the enzyme assay and Western blot analysis.

Metabolic labelling of mutant α-Gal A enzymes expressed in COS-7 cells

COS-7 cells transfected with the wild-type or mutant α-Gal A cDNAs were washed with PBS and incubated in 0.4 ml of methionine- and cystine-free DMEM containing 10 % dialysed FCS for 30 min. A 4 μl portion of EXPRE^{35}S^{35}S[^{35}S]Protein labelling mix (293 MBq/ml; DuPont) was added to each well, and the cells further incubated for 2 h. After washing the cells with PBS, complete culture medium (2 ml DMEM with 10 % FCS) was added, and the cells further incubated at 37 °C, 5 % CO_2 for various times as the chasing period. After washing with PBS, the cell pellets were resuspended in 10 mM Tris/HCl buffer (pH 7.5) containing 40 mM KCl, 0.1 % Triton X-100, 62.5 μM EDTA, and 2.5 μg/ml leupeptin, and incubated at 4 °C for 30 min. Final cellular samples were prepared by centrifugation at 10 000 *g* for 5 min.

Immunoprecipitation of α-Gal A enzymes

A polyclonal anti-α-Gal A antibody was added to the sample and incubated at 4 °C overnight. Protein A Sepharose CL-4B (Amersham Biosciences) suspended in 10 mM Tris/HCl buffer (pH 7.5) containing 40 mM KCl, 0.1 % Triton X-100, 62.5 μM EDTA and 2.5 μg/ml leupeptin was added to each sample which were then incubated at 4 °C for 1 h. The precipitate was collected by centrifugation at 10 000 *g* for 5 min and washed three times with 25 mM Tris/HCl buffer (pH 7.5) containing 0.5 M NaCl, 0.5 % Triton X-100, 0.1 % SDS and 1 mM EDTA. After resuspending the sample in 50 mM Tris/HCl buffer (pH 6.8) containing 2 % SDS, 10 % glycerol, 0.01 % Bromophenol Blue and 5 % 2-mercaptoethanol, the protein was recovered by boiling the sample for 5 min, followed by SDS/PAGE and visualization by autoradiography.

RESULTS

Expression and purification of mutant α-Gal A enzymes in COS-7 cells

Nineteen α-Gal A cDNAs that encode various missense mutations found in Fabry disease patients with residual enzyme activity were generated to study the biochemical and molecular properties of the mutant proteins. Among them, nine mutations (A20P, E66Q, M72V, I91T, F113L, N215S, Q279E, M296I and M296V) were found in variant Fabry disease patients [6,7,27–31] and five mutations (E59K, A156V, L166V, R356W and G373S) [32–34]

Table 1 Kinetic properties of human mutant α-Gal A enzymes expressed in COS-7 cells.

Human α-Gal A enzymes with various missense mutations were purified by affinity column chromatography of the homogenates of COS-7 cells. The kinetic properties of mutant enzymes were determined using 4MU-α-Gal at variable concentrations. The K_m and V_{max} values of each mutant enzyme were obtained from Lineweaver–Burk plots.

Mutation	K_m (mM)	V_{max} (mmol/h per mg of protein)
Wild-type	2.8	2.56
A20P	2.8	2.67
E59K	16.3	6.13
E66Q	3.1	4.10
M72V	3.2	4.56
I91T	3.4	0.99
A97V	3.7	4.80
R112H	5.8	1.52
F113L	3.6	2.57
A156V	3.4	3.93
L166V	2.6	6.80
N215S	2.8	5.29
Q279E	1.8	2.84
M296I	2.7	3.14
M296V	2.4	4.06
R301Q	1.9	2.56
R356W	2.0	2.46

were identified in classic Fabry disease patients. Four mutations (A97V, R112H, R301Q and G373D) [7,28,32,35–37] were found in both mild classic and variant patients, and P146S was found in a presymptomatic patients [38].

COS-7 cells were transfected with expression vectors encoding various mutant α-Gal A cDNAs and cultivated in the presence of DGJ (to ensure maximum production of the mutant enzymes). The expression of variant mutant enzymes was generally higher (median value of 3870 units/mg) than that of classic mutant enzymes (median value of 2090 units/mg), in line with observations that variant patients have higher residual enzyme activity. Since the purification strategy for these enzymes may not permit total separation of expressed exogenous human mutant enzyme from the endogenous enzyme in COS-7 cells, only those mutant enzymes with activities at least 10-fold higher than that of endogenous enzyme activity were used for further characterization. The expression of the P146S, G373D and G373S enzyme mutants was relatively low (2- to 5-fold above the endogenous level), therefore these mutant enzymes were excluded from further studies. This precaution ensured that the kinetic and biochemical results generated from COS-7 cell-derived enzymes are comparable with those of the human mutant enzyme, and contamination by endogenous enzymes from COS-7 cells was kept to a minimum.

Kinetic properties of human mutant α-Gal A enzymes purified from transfected COS-7 cells

K_m and V_{max} values of purified mutant α-Gal A enzymes were determined (Table 1). The K_m and V_{max} values of the wild-type enzyme were 2.8 mM and 2.56 mmol/h per mg of protein respectively, in agreement with earlier findings [39]. Except for the E59K mutant, most mutant enzymes had K_m and V_{max} values similar to those of the wild-type enzyme (K_m of 1.8–5.8 mM, and V_{max} of 0.99–6.80 mmol/h/mg of protein). The K_m and V_{max} values for the E59K mutant were 16.3 mM and 6.13 mmol/h/mg of protein respectively, indicating that this mutation may cause impaired kinetic ability. These results suggest that the majority of missense mutant enzymes studied here are catalytically active, regardless of the clinical phenotype with which they are associated.

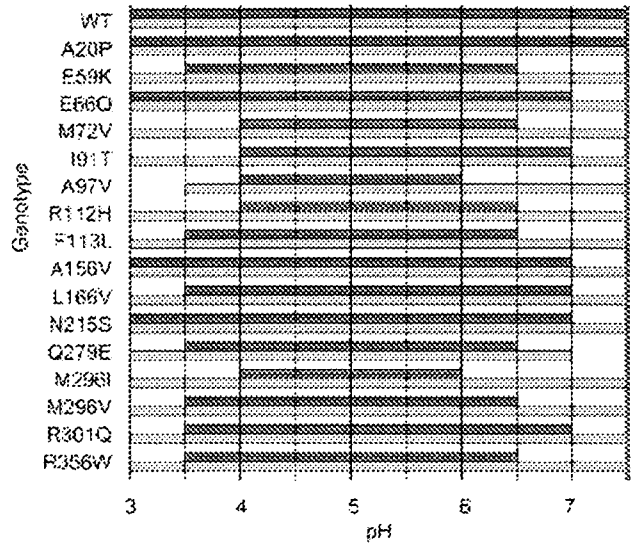

Figure 1 pH Stability of wild-type and mutant α-Gal A enzymes

The enzymes were purified from cell lysates of COS-7 cells transfected with expression plasmids encoding various mutant α-Gal A enzymes. The stable pH range is defined as the pH in which more than 50% enzyme activity remained after incubation at 37 °C for 1 h in the absence (dark grey bar) or presence (light grey bar) of 1 μM DGJ.

Optimal pH for activity and pH stability of human missense mutant α-Gal A enzymes

The optimal pH for enzyme activity was found to be the same (pH 4.0–4.5) for both the wild-type enzyme and all purified mutant enzymes. This indicated that these mutant enzymes were likely to be enzymatically and physically active within the lysosomes. Compared with the wild-type enzyme, which is relatively stable between pH 3.0 and 7.5, most mutant enzymes were only stable over a narrow pH range, typically pH 3.5–6.5 (Figure 1). Since the A20P mutation occurs within the signal peptide, the purified A20P enzyme was expected to be identical to the wild-type enzyme. Therefore it was not surprising that its stability profile was the same as that of the wild-type protein. The pH stability of almost all of the mutant enzymes was restored to normal by the addition of 1 μM DGJ to the pre-incubation buffer. The exception was with the Q279E mutant, the stability of which nevertheless improved.

Because the environment of the ER has a neutral pH, the stability of the mutant enzymes at pH 7.5 was monitored in a time-dependent fashion. The wild-type enzyme retained more than half of its activity after pre-incubation at pH 7.5 and 37 °C for 90 min (Table 2), indicating that the protein is relatively stable within the ER. However, all mutants lost more than 50% of their enzyme activity within a 45-min pre-incubation period. More strikingly, five variant mutants (M72V, A97V, R112H, F113L and Q279E) completely lost their enzyme activity within 15 min under the same conditions, clearly indicating that these mutants are not physically stable at neutral pH, which resembles the condition within the ER. The stability of the mutant enzymes could be partially or completely restored with the addition of 1 μM DGJ to the pre-incubation buffer, indicating that DGJ is effective at stabilizing the conformation of these missense mutant enzymes at neutral pH.

Table 2 Stability of mutant α-Gal A at pH 7.5

The enzyme was pre-incubated in 2.5 mM phosphate buffer (pH 7.5) in the presence or absence of DGJ (1 μM) for various times, and the remaining enzyme activity determined in 0.2 M citrate buffer (pH 4.5) using 4MU-α-Gal as a substrate. Enzyme stability was measured in terms of the length of incubation time that results in more than 50% remaining enzyme activity.

Mutation	$Time_{50\%,\ pH\ 7.5}$ (min)	
	−DGJ	+DGJ
Wild-type	90	90
A20P	90	90
E59K	15	90
E66Q	30	90
M72V	< 15	90
I91T	30	60
A97V	< 15	90
R112H	< 15	90
F113L	< 15	90
A156V	30	90
L166V	45	90
N215S	45	90
Q279E	< 15	45
M296I	30	90
M296V	15	90
R301Q	30	90
R356W	30	90

Effects of ERAD inhibitors on mutant α-Gal A

To investigate the intracellular degradation of mutant α-Gal A, the effects of lactacystin (a proteasome inhibitor) and kifunensine (an inhibitor of ER α-mannosidase I) on the processing of mutant α-Gal A were determined (Figure 2). After treatment with kifunensine, a substantial increase in the enzyme protein was observed in M72V, I91T, A97V, R112H, F113L, L166V, N215S, Q279E and R301Q mutations, and a modest increase was also found in A20P, E66Q, A156V, M296I, M296V, R356W, G373D and G373S mutations. The amount of E59K protein was not affected by this treatment. The protein amount of P146S was too low to be detectable by Western blot analysis, even in the presence of kifunensine (results not shown). In contrast with the kifunensine-effect, the enzyme amount was increased by treatment with lactacystin in E66Q, F113L, N215S, M296I and R301Q mutations, but not in other mutations. This result suggested that the final degradation of mutant α-Gal A protein may involve different proteases.

Effect of DGJ on mutant α-Gal A enzymes

In order to assess whether the enzyme activity of mutant enzymes could effectively be increased by DGJ, DGJ was added to the culture medium of transfected COS-7 cells. The intracellular enzyme activities of all mutant enzymes were substantially higher (1.7- to 10-fold) in cells incubated with DGJ, as compared with those of transfected cells not treated with DGJ (Figure 3). Western blotting determined that the amount of mature α-Gal A increased accordingly in cells treated with DGJ. These results indicate that the mutant enzymes studied here are conformational amendable, and that DGJ is an effective agent for the rescue of these mutations.

Intracellular processing and trafficking of mutant α-Gal A enzymes found in classic Fabry disease patients

It has been demonstrated that abnormal processing and maturation of α-Gal A occurred in mutations causing the Fabry disease cardiac phenotype [20]. To confirm that abnormal protein folding that affects trafficking and processing can occur in mutant enzymes in classic Fabry disease patients, the E59K and L166V mutants were subjected to further cellular processing studies.

In order to assess the intracellular processing of mutant proteins, metabolic labelling of proteins was performed in COS-7 cells transfected with vectors expressing mutant α-Gal A cDNA (E59K, L166V or R301Q), followed by immunoprecipitation with an anti-α-Gal A antibody. The wild-type enzyme showed partial maturation after 6 h, and complete maturation after 12 h (Figure 4), evidenced by the transition of the band from 50 kDa (ER and Golgi forms) to 46 kDa (mature lysosomal form) [40]. Maturation of the E59K mutant was also complete after 12 h, suggesting that this mutation does not perturb processing and maturation of the protein. On the other hand, both the L166V and R301Q mutant α-Gal A enzymes remained in the non-processed 50 kDa form, even after a 24 h chase, and showed rapid decay after 6 h, indicating that enzyme processing was defective. Upon incubation of the cells with DGJ, the mutant R301Q α-Gal A enzyme was processed after 12 h, and the processing was complete after 24 h. Processing of the L166V mutant α-Gal A progressed slowly over a 24 h period following incubation of cells with DGJ, suggesting that DGJ treatment also partially corrects this mutation, and improves processing during the maturation of mutant proteins.

To examine the intracellular trafficking of the E59K and L166V mutant α-Gal A enzymes, subcellular fractions of both mutant enzymes cultured in the absence or presence of DGJ were

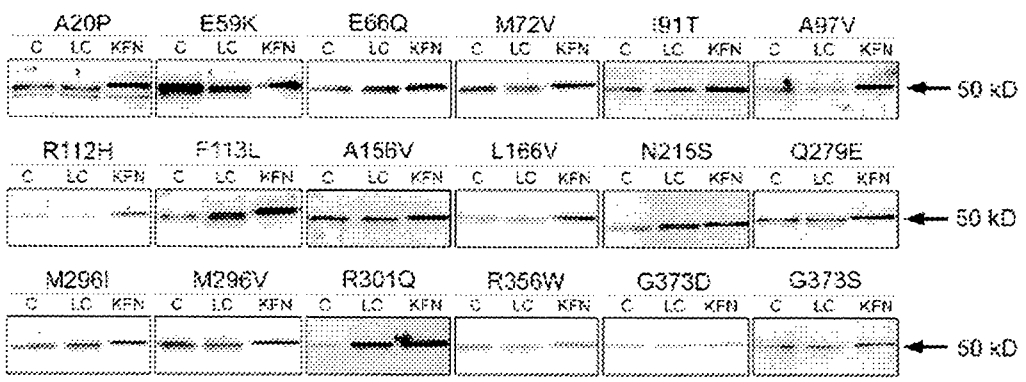

Figure 2 Effects of ERAD inhibitors on the amount of mutant α-Gal A expressed in COS-7 cells

Wild-type or mutant α-Gal A enzymes were transiently expressed with FuGENE™ 6 transfection reagent in COS-7 cells. At 5 h after transfection, cells were treated with 2 μM lactacystin (LC), 0.2 mM kifunensine (KFN) or nothing as a control (C), and harvested at 48 h. Western blot analyses of cell lysates from transfected COS-7 cells were performed.

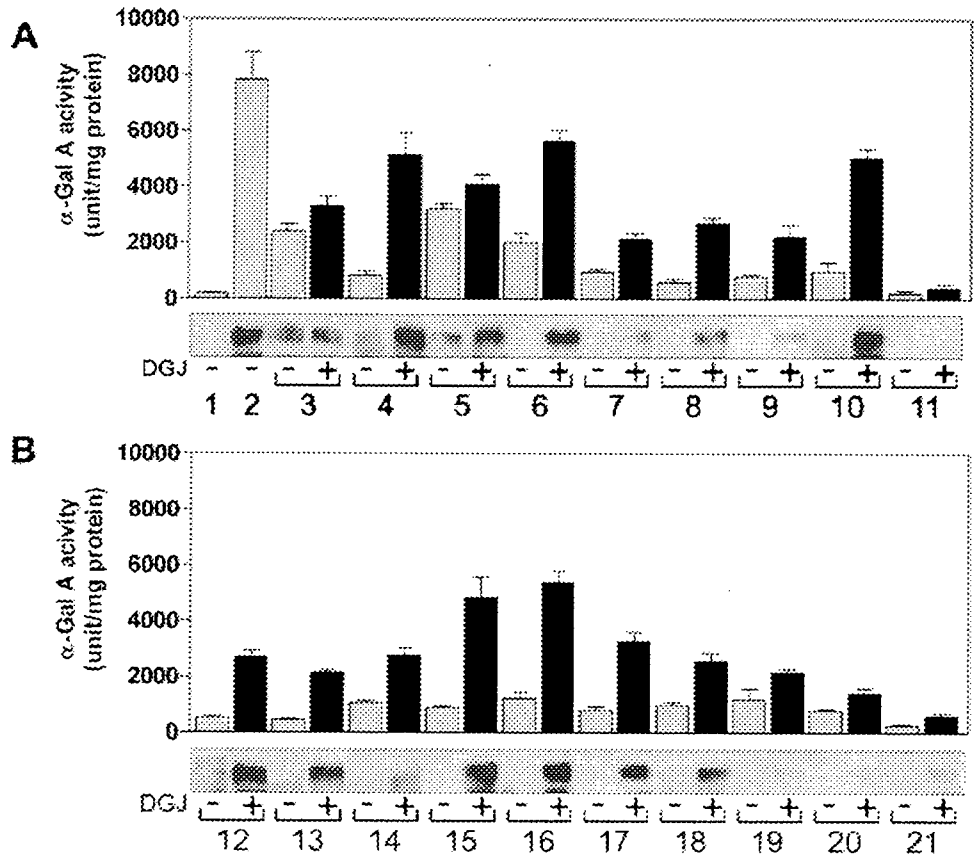

Figure 3 Effect of DGJ treatment on various mutant α-Gal A enzymes expressed in COS-7 cells

COS-7 cells were re-plated on six-well plates and transfected with expression constructs containing various mutant α-Gal A cDNAs using Lipofectamine™ 2000 reagent. Cells were cultured in complete medium for 3 days with or without 20 μM DGJ. α-Gal A activity and Western blot analyses of cell lysates from transfected COS-7 cells were performed as described in the Materials and methods section. Lane 1, Mock transfection; lane 2, wild-type α-Gal A; lane 3, A20P; lane 4, E59K; lane 5, E66Q; lane 6, M72V; lane 7, I91T; lane 8, A97V; lane 9, R112H; lane 10, F113L; lane 11, P146S; lane 12, A156V; lane 13, L166V; lane 14, N215S; lane 15, Q279E; lane 16, M296I; lane 17, M296V; lane 18, R301Q; lane 19, R356W; lane 20, G373D; and lane 21, G373S.

examined by Percoll density gradient centrifugation of COS-7 cells transfected with the mutant enzyme expression plasmids. The mature form of the wild-type α-Gal A was recovered in both high-density (#1–5) and low-density (#14–18) fractions (Figure 5A) that comprised the lysosomal and ER/endosomal fractions respectively [41], in line with the pattern of β-hexosaminidase as a lysosomal marker enzyme. Mutant E59K enzyme activity was also recovered in both high- and low-density fractions, although at a reduced level, indicating that the mutation does not compromise normal trafficking of the mutant enzyme. The mutant protein recovered from the lysosomal fractions showed a reduced size (Figure 5B), indicating that it is processed normally to the mature form. In contrast, neither enzyme activity nor protein could be detected in the lysosomal fractions of COS-7 cells transfected with the L166V or R301Q expression plasmid. Only trace amounts of L166V or R301Q enzyme activity and protein were detected in the ER/endosomal fractions, although the protein remained unprocessed. The results clearly indicate that the E59K mutation alters the catalytic capability of the mutant enzyme, and has little effect on processing, maturation and trafficking, whereas disruption of maturation and trafficking occurred in the L166V and R301Q mutations. The results also indicate that the abnormal processing and trafficking can be a primary cause for the protein deficiency in mutations associated with classic Fabry disease. After incubation of the cells with DGJ for 3 days, the fully mature lysosomal form of both mutant enzymes (46 kDa) could be recovered in the lysosomal fractions (Figures 5A and 5B). These results indicate that DGJ treatment can at least partially correct for the trafficking defect caused by both mutations.

Lysosomal enzyme activities in the cells of patients with lysosomal storage diseases cultured with DGJ

To rule out the possibility that DGJ may effect ERAD by some unknown mechanism, we investigated the effect of DGJ on human fibroblasts established from patients with G_{M1}-gangliosidosis or Gaucher disease, in which there is a deficiency of lysosomal β-galactosidase or glucocerebrosidase (acid β-glucosidase) respectively. The mutations identified in the G_{M1}-gangliosidosis and Gaucher fibroblasts were homozygous I51T in the β-galactosidase gene (GLB1) and N370S in the glucocerebrosidase gene (GBA) respectively. Both mutations are known to result in protein trafficking defects [42,43], and residual enzyme activities of these mutant enzymes can be partially restored by other small molecules [44].

After cultivation of Fabry disease lymphoblasts with DGJ at 20 μM for 5 days, a 3.8-fold increase in α-Gal A activity was observed, whereas the activities of β-galactosidase and glucocerebrosidase, which were normal in the cultured lymphoblasts,

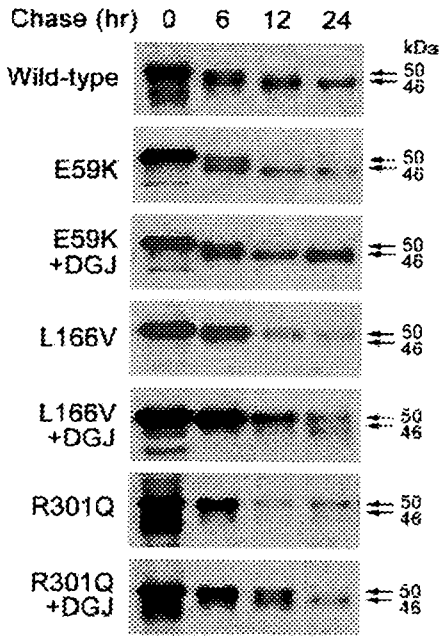

Figure 4 Metabolic labelling of mutant α-Gal A expressed in COS-7 cells

COS-7 cells were transfected in six-well plates with expression plasmids coding for the wild-type, E59K, L166V or R301Q α-Gal A enzymes respectively, and cultured in the absence or presence of 20 μM DGJ. The cells were exposed to 4 μl of [^{35}S]Protein labelling mix for 2 h. After washing the cells with PBS, the labelled proteins were chased by replacement of medium for the indicated period. Following immunoprecipitation with a polyclonal anti-α-Gal A antibody, an aliquot (one-fifth of the sample) was analysed by SDS/PAGE (10 % gels) and visualized by fluorography.

were not significantly affected (Table 3). The activities of both mutant β-galactosidase in G_{M1}-gangliosidosis fibroblasts and mutant glucocerebrosidase in Gaucher fibroblasts did not increase after DGJ-treatment, indicating that DGJ at such concentrations had little or no effect on these mutant proteins. Our result indicates that DGJ does not directly affect the ERAD machinery, and at 20 μM concentrations, its effect is limited specifically to mutant α-Gal A.

Increase in residual enzyme activity in the cells of Fabry disease patients by DGJ

Human lymphoblasts and fibroblasts established from hemizygous Fabry disease patients with a variety of disease-causing GLA mutations were incubated with DGJ. Residual enzyme activity increased substantially in cells incubated for 5 days with DGJ at 20 μM, regardless of the clinical phenotype that the mutation conferred (Table 4). Both lymphoblasts and fibroblasts that harboured the same mutation (e.g. A97V and R301Q) responded to DGJ treatment, though the response in lymphoblasts was higher than those in fibroblasts, which could be attributed to differences in the permeability of DGJ. Noticeably, residual enzyme activity in all cells following DGJ treatment was increased by over 20 % of those in normal subjects, a level of enzyme activity that would be expected to have a significant impact upon disease progression [45], suggesting that the DGJ treatment would be therapeutically beneficial for patients with these genotypes.

DISCUSSION

The consequence of genetic errors that lead to the dysfunction of coding proteins involved in genetic disorders can be various. Nonsense, frame-shift mutations, splicing mutations and missense mutations involving the substitution of critical amino acids often result in the biosynthesis of mutant proteins that are non-functional. In other cases, missense mutations or small in-frame deletions/insertions could have little or no impact on the biological activity of the mutant protein, but may cause misfolding and an altered tertiary structure of the protein. The ER lumen is a cellular compartment where newly synthesized proteins fold into their tertiary structure to gain biological functionality. To maintain the integrity of each synthesized protein, cells have evolved an efficient 'quality control' system, termed ERAD, in which only properly folded and assembled proteins are transported to the Golgi complex for further maturation, and those improperly folded proteins are retained by molecular chaperones in the ER for subsequent degradation [46]. Although this process is essential for normal cellular function, the process may also contribute significantly to protein deficiency in many inherited disorders. It is clear that the correction of the conformation of a mutant protein could be a therapeutic option for protein deficiencies. Since protein folding is a thermodynamic process, even a slight shift in favour of proper folding could have a significant impact on the increase of enzyme activity in treating the disease.

A significant number of patients with missense mutations have residual α-Gal A activity ranging from 1 to 10 % of normal individuals, and they are usually associated with less severe phenotypes, either a milder classic phenotype or an atypical cardiac or renal variant. The low level of residual enzyme activity could be the result of either normal processing of mutant enzyme with an altered catalytic site, producing an enzyme with reduced catalytic activity, or a small amount of correctly folded mutant enzyme that escapes ERAD. To assess the direct cause responsible for deficient enzyme activity in Fabry disease, we studied mutations that have been identified in patients who present a significant level of residual enzyme activity, regardless of clinical phenotypes. In most cases, patient's fibroblasts or lymphoblasts harbouring the same mutations were available to us for studies at an endogenous level. Based on the X-ray crystallography structure of α-Gal A [11], twelve mutated amino acids were used in the present study (Glu^{66}, Met^{72}, Ile^{91}, Ala^{97}, Arg^{112}, Phe^{113}, Pro^{146}, Ala^{156}, Leu^{166}, Gln^{279}, Met^{296} and Gly^{373}) which are normally buried inside the tertiary structure and maintain the gross folding state of the molecule. Three amino acids (Glu^{59}, Arg^{301} and Arg^{356}) are involved in the formation of ion pairs with other amino acids that could contribute to protein folding and/or catalytic function. The Arg^{20} residue is located in the signal peptide, and the Asn^{215} residue is one of the N-linked carbohydrate-attachment sites.

Because mutant proteins with a misfolded conformation would be subject to rapid degradation by ERAD [47], the conformation of residual enzyme in lysosomes is considered to be fully folded. The conformation of the mutant proteins purified in the present study are expected to be fully folded and have a conformation similar to that of the residual enzyme in the physiological condition. In order to assess the conformational stability of these purified mutant enzymes, they were subjected to thermo- and pH-denaturation. A protein with a stable conformation typically resists denaturation, whereas those proteins with a fragile conformational structure are often intolerant to thermo- or pH-denaturation. The wild-type enzyme is relatively stable over a wider range of pH and particularly at neutral pH (Figure 1 and Table 2), indicating that its structure is well-folded and maintained. Most mutant proteins were found to be stable only over a narrower pH range (typically pH 4–6.5). Noticeably, these mutant proteins were stable at pH 4.5–5.0, the pH similar to that of the environment in the lysosomes, suggesting that the folded conformation of mutant proteins is stable in lysosomes.

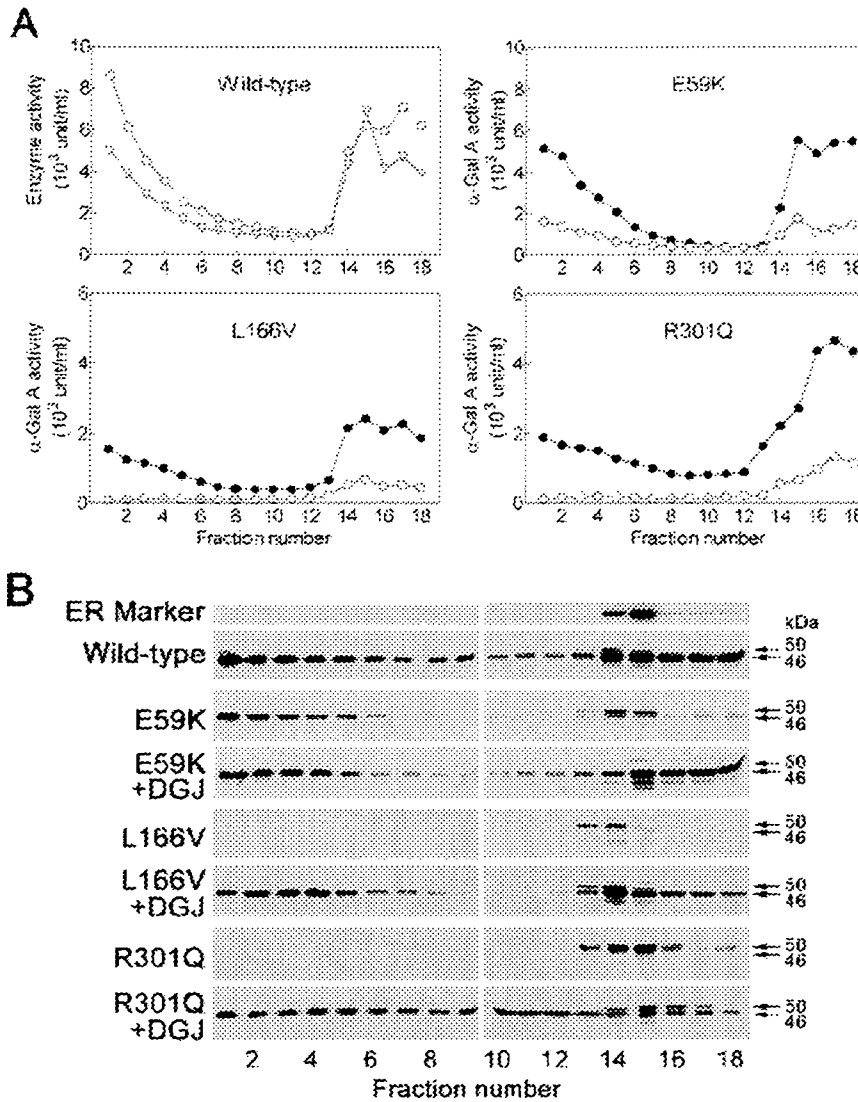

Figure 5 Subcellular fractionation of the wild-type or mutant α-Gal A expressed in COS-7 cells

COS-7 cells were transfected with expression plasmids pCXN2-GLA, pCXN2-GLA-E59K, pCXN2-GLA-L166V or pCNX2-GLA-R301Q respectively. Subcellular fractionation was performed by Percoll gradient centrifugation at 25000 ***g*** for 1 h. Each fraction was collected, and the density determined using density markers (Fraction 2, 1.11 g/ml; fraction 4, 1.07 g/ml; fraction 7, 1.056 g/ml; fraction 12, 1.053 g/ml; and fraction 16, 1.041 g/ml). (**A**) α-Gal A (○) and β-hexosaminidase (▽) activities in each fraction of wild type α-Gal A. α-Gal A activities of E59K, L166V and R301Q in cells treated with 20 μM DGJ (●) or without (○) in each fraction. (**B**) Western blot analyses with an antibody against human α-Gal A. Bip was used as an ER marker protein.

Table 3 Residual enzyme activity in the cells of patients with various lysosomal storage diseases after DGJ treatment

Residual enzyme activities in respective cells of patients were in the range of 3–8 % of normal. All other enzyme activities in the cells of patients were within a normal range. All cells were cultivated with DGJ at 20 μM for 5 days prior to the enzyme assay. Enzyme activities in cells treated with DGJ were compared with those without DGJ treatment. All values are the average of three parallel assays and have a S.D. of less than 15 %.

	Fold increase in enzyme activity		
Patient cells	α-Gal A	β-Galactosidase	Glucocerebrosidase
Fabry lymphoblasts (R301Q)	3.8	1.1	1.3
G_{M1}-gangliosidosis fibroblasts (I51T)	1.3	1.2	1.1
Gaucher fibroblasts (N370S)	1.1	1.3	1.0

All mutant proteins showed less stability compared with the wild-type enzyme at pH 7.0–7.5, a value similar to that in the ER, indicating that they do not conform well at neutral pH. In the presence of DGJ at pH 7.5, the instability of the mutant proteins was alleviated to a certain degree, suggesting that DGJ helps to stabilize the conformation of the protein molecule. These results indicate that the substitution of an amino acid residue in missense mutant α-Gal A enzymes alters conformational stability, creating a more fragile molecular structure under extreme pH conditions.

Folding and refolding of glycoproteins in the ER involves the calnexin/calreticulin system and glucosidases I and II, whereas ER α-mannosidases and EDEM (ER degradation enhancing α-mannosidase I-like protein) are involved in retrotranslocation and degradation of misfolded proteins in the process of ERAD [48]. Removal of a mannose residue from Man9 N-linked

Table 4 Increase in residual enzyme activity in cells from Fabry disease patients cultivated with DGJ

Fibroblasts and lymphoblasts were obtained from hemizygous Fabry disease patients with one of the mutations listed. All cells were cultivated in the presence or absence of DGJ at 20 μM for 5 days before their residual enzyme activity was determined. The values are the average of three independent assays and have a S.D. of less than 10%.

Mutation	Cell type	Residual enzyme activity (% of normal)	
		−DGJ	+DGJ
E59K	Fibroblasts	6.0	47.6
E66Q	Lymphoblasts	29.9	43.2
I91T	Fibroblasts	6.9	22.6
A97V	Lymphoblasts	7.5	61.5
	Fibroblasts	11.1	32.2
R112H	Fibroblasts	5.2	31.9
F113L	Fibroblasts	1.8	35.0
N215S	Fibroblasts	3.7	21.2
Q279E	Lymphoblasts	3.6	45.0
M296I	Lymphoblasts	11.9	62.6
M296V	Fibroblasts	11.5	71.9
R301Q	Lymphoblasts	4.0	48.0
	Fibroblasts	6.3	26.3

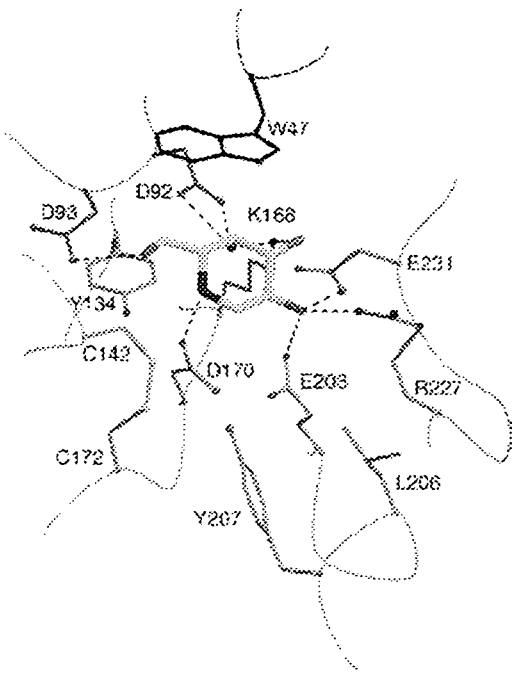

Figure 6 Predicted interactions between DGJ and the active site of α-Gal A

Based upon the crystal structure of α-Gal A with bound α-galactose, we modelled the interactions of α-Gal A with DGJ. The DGJ is shown bound to the active-site of the enzyme, in a manner very similar to α-galactose binding. The key interactions to the 2, 3, 4, and 6 hydroxy groups on the ligand are maintained when either α-galactose or DGJ binds to the active-site. One key interaction between Glu^{231} on the enzyme and the 1 hydroxy group of α-galactose is lost when DGJ binds, because DGJ lacks a functional group at the 1 position. At acidic pH, DGJ becomes protonated and positively charged, which may increase its affinity for the highly negatively charged enzyme.

oligosaccharides by the ER α-mannosidase I is a critical luminal event for preventing proteins re-entering the refolding process and serves as a signal for targeted ERAD. Therefore inhibition of the ER α-mannosidase I often delays the degradation of glycoproteins in ERAD in favour of protein refolding. When kifunensin, a selective inhibitor of the ER α-mannosidase I, was added to the culture medium, the amount of all mutant proteins (except E59K) appeared to be increased (Figure 2), suggesting the degradation of mutants was partially inhibited. This result indicates that degradation of mutant α-Gal A enzymes occurred within the ERAD as the result of misfolding of the mutant proteins. Protein misfolding has been recognized as having an important role in the protein deficiency in various inherited disorders [16]. The results obtained from a large set of missense mutant proteins further provide evidence that protein misfolding as a primary cause of protein deficiency is not only limited in a few mutations, but is rather a generalized cause that exists in many missense mutations in a single genetic disorder.

Fabry disease patients with an atypical variant cardiac phenotype often retain a measurable level of residual enzyme activity, and the main cause of the depletion of enzyme activity has been demonstrated to be caused by impaired processing and transport from the ER, as exemplified by the R301Q mutation [20]. Certain milder classic patients also present residual enzyme activity, often at a lower level. In the present study, five mutations (E59K, A97V, A156V, L166V and R356W) causing classic Fabry disease were examined for their effect on the kinetic properties of α-Gal A. All of the mutant enzymes, except for the E59K mutant, retained kinetic properties similar to those of the wild-type enzyme, suggesting that the primary reason for the disruption in enzyme activity does not involve reduced catalytic ability. To further characterize the direct cause for the deficiency in enzyme activity in classic Fabry disease patients, the E59K and L166V mutant enzymes were chosen as examples to study their processing and trafficking. The E59K mutant protein demonstrated normal processing and trafficking, suggesting that the primary cause for the deficiency in enzyme activity is the alteration in kinetic activity. On the other hand, subcellular fractionation (Figure 5) and metabolic labelling (Figure 4) studies showed that the L166V mutant has perturbed processing and stalled trafficking profiles, comparable with those of the R301Q mutant enzyme, indicating that the primary biochemical defect responsible for diminished enzyme activity is abnormal processing and trafficking. Since four of the five classic mutations retained the normal catalytic capability similar to the L166V mutation, deficiencies in enzyme activity caused by abnormal processing and trafficking may be more widespread in Fabry disease patients with residual enzyme activity than those previously thought to be limited to the variant phenotype.

Human α-Gal A is a homodimeric glycoprotein with each monomer composed of two domains, a $(\beta/\alpha)_8$ domain formed with amino acid residues 32–330 and a C-terminal domain (amino acid residues 331–429) containing eight antiparallel β-strands on two sheets in a β-sandwich [11]. The first domain contains the active site formed by the C-terminal ends of the β-strands at the centre of a barrel. Thirty residues from loops β1–α1, β6–α6, β7–α7, β8–α8, β11–β12 and β15–β16 of each monomer contribute to the dimer interface. DGJ is an ASSC specifically serving as a folding template for mutant enzymes that have a fragile conformational structure. Based upon active-site interactions seen in the crystal structure of α-galactose bound to α-Gal A, a model of DGJ binding to α-Gal A shows many favourable interactions. The imino group on DGJ is expected to interact with Asp^{170}, the hydroxy groups of DGJ form hydrogen bonds with Asp^{92}, Asp^{93}, Lys^{168}, Glu^{203}, Arg^{227} and Glu^{231}, and a hydrophobic surface on DGJ makes van der Waals interactions with Trp^{47} (Figure 6). A binding between DGJ and the protein would fix the active site involving five loops β1–α1, β2–α2, β4–α4, β5–α5 and β6–α6.

Mutations at the amino acid residues involving the interaction resulted in severe classic phenotypes [11] and are predicted to be reluctant to the DGJ rescue. On the other hand, all of the mutations investigated in the present study are not involved with the proposed interactions between the protein and DGJ, thus permitting the rescue effect.

The E59K mutant enzyme was found to have compromised kinetic properties, and abnormal trafficking was not a major obstacle for expression of this mutation. Nevertheless, residual enzyme activity in cultured fibroblasts expressing the E59K mutant α-Gal A enzyme increased 8-fold in the presence of DGJ, and reached a level approx. 48% of normal enzyme activity (Table 4). It has been proposed that retention and degradation of misfolded proteins entering the secretory pathway may not be restricted to mutant proteins [49]. Protein folding is not a perfect process even with wild-type proteins. A large fraction of newly synthesized proteins never attain their native structure, and are ubiquitinylated before being degraded by cytosolic proteasomes. In the present study we have demonstrated that α-Gal A activity in both normal human lymphoblasts and COS-7 cells transfected with wild-type GLA can be raised (approx. 10–20%) by DGJ treatment [20]. Small molecular ligands termed pharmacological chaperones have also been shown to be effective at increasing maturation of the wild-type δ-opioid receptor [50]. Evidence obtained from the E59K mutant enzyme indicate that chaperone-like enhancement may be effective at correcting a broad range of mutations beyond those mutations that mainly cause protein misfolding.

Currently, enzyme replacment therapy is the only FDA (Food and Drug Administration) approved therapy for Fabry disease. The effect of infusion with exogenous wild-type α-Gal A on patients has been well-documented. Compared with protein macromolecules that could be difficult to deliver to tissues, DGJ is a small molecule and therefore has the advantage of being able to be delivered to various tissues. In the present study we also demonstrated that residual enzyme activity could be increased by DGJ treatment in a variety of fibroblast and lymphoblast cell lines established from Fabry disease patients with missense mutations (Table 4). Since a large proportion of mutant forms of α-Gal A that are found in patients with missense mutations may have folding defects, DGJ could conceivably be effective at treating a broad number of Fabry disease patients with missense mutations regardless of the clinical phenotype of the patients.

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Endosomal Accumulation of Toll-Like Receptor 4 Causes Constitutive Secretion of Cytokines and Activation of Signal Transducers and Activators of Transcription in Niemann–Pick Disease Type C (NPC) Fibroblasts: A Potential Basis for Glial Cell Activation in the NPC Brain

Michitaka Suzuki,[1] Yuko Sugimoto,[1] Yuki Ohsaki,[1] Makoto Ueno,[2] Shinsuke Kato,[3] Yukisato Kitamura,[4] Hiroshi Hosokawa,[5] Joanna P. Davies,[6] Yiannis A. Ioannou,[6] Marie T. Vanier,[7] Kousaku Ohno,[2] and Haruaki Ninomiya[1]

Departments of [1]Neurobiology, [2]Child Neurology, [3]Neuropathology, and [4]Pathology, Tottori University Faculty of Medicine, Yonago 683-8503, Japan, [5]Department of Intelligence Science and Technology, Graduate School of Informatics, Kyoto University, Kyoto 606-8501, Japan, [6]Department of Human Genetics, Mount Sinai School of Medicine, New York, New York 10029, and [7]Institut National de la Santé et de la Recherche Médicale, Unité 499, Université Lyon 1, Faculté de Médecine R. T. H. Laënnec, F-69372 Lyon, France

Niemann–Pick disease type C (NPC) is an inherited lipid storage disorder caused by mutations in *NPC1* or *NPC2* genes. Loss of function of either protein results in the endosomal accumulation of cholesterol and other lipids, progressive neurodegeneration, and robust glial cell activation. Here, we report that cultured human NPC fibroblasts secrete interferon-β, interleukin-6 (IL-6), and IL-8, and contain increased levels of signal transducers and activators of transcription (STATs). These cells also contained increased levels of Toll-like receptor 4 (TLR4) that accumulated in cholesterol-enriched endosomes/lysosomes, and small interfering RNA knockdown of this receptor reduced cytokine secretion. In the $NPC1^{-/-}$ mouse brain, glial cells expressed TLR4 and IL-6, whereas both glial and neuronal cells expressed STATs. Genetic deletion of TLR4 in $NPC1^{-/-}$ mice reduced IL-6 secretion by cultured fibroblasts but failed to alter STAT levels or glial cell activation in the brain. In contrast, genetic deletion of IL-6 normalized STAT levels and suppressed glial cell activation. These findings indicate that constitutive cytokine secretion leads to activation of STATs in NPC fibroblasts and that this secretion is partly caused by an endosomal accumulation of TLR4. These results also suggest that similar signaling events may underlie glial cell activation in the $NPC1^{-/-}$ mouse brain.

Key words: Niemann–Pick; cholesterol; Toll-like receptor; IL-6; STAT; glia

Introduction

Niemann–Pick disease type C (NPC) is an inherited lipid storage disorder caused by mutations in *NPC1* or *NPC2* genes (Carstea et al., 1997; Naureckiene et al., 2000). NPC1 is a membrane protein that contains a sterol-sensing domain and resides primarily in late endosomes, whereas NPC2 is a soluble protein that contains an MD-2-related lipid recognition domain and resides primarily in lysosomes. The loss of function of either protein results in aberrant endosomal membrane flow that leads to an accumulation of cholesterol and other lipids in the endosomal/lysosomal compartment. The main clinical feature of NPC is progressive neurological deterioration that is eventually fatal. NPC is typically a childhood disease, but its manifestations can be variable, and depending on the onset, it is classified as infantile, juvenile, and adult forms (Patterson et al., 2001).

BALB/c NPC mice contain a retroposon insertion in *NPC1* (Loftus et al., 1997). $NPC1^{-/-}$ mice show symptoms similar to those of human patients and survive for <3 months. Brain tissues from these mice show lipid accumulation, progressive neuronal cell loss, predominantly affecting Purkinje cells, and robust activation of astroglial and microglial cells (Higashi et al., 1993; German et al., 2002; Walkley and Suzuki, 2004). Recently, administration of the neurosteroid allopregnanolone has been shown to suppress Purkinje cell loss and glial cell activation, and increase the lifespan of these mice (Griffin et al., 2004; Ahmad et al., 2005).

The prevailing hypothesis for glial cell activation in the $NPC1^{-/-}$ brain is that its occurrence is secondary to neuronal damage, resulting from activation of their phagocytic function to eliminate degenerating neurons. However, histochemical studies have shown that, in the $NPC1^{-/-}$ mouse brain, glial cell activa-

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Correspondence should be addressed to Haruaki Ninomiya, Department of Neurobiology, Tottori University Faculty of Medicine, Yonago 683-8503, Japan. E-mail: ninomiya@grape.med.tottori-u.ac.jp.

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tion preceded neuronal cell loss (Baudry et al., 2003) and that, in the monkey brain, NPC1 was predominantly localized in perisynaptic astrocytes (Patel et al., 1999). These findings raise the possibility that glial cell activation is a cell-autonomous event and is causally related to neurodegeneration, although the biochemical basis for the activation remains unknown. A recent study using chimeric mice that have both *NPC1*$^{-/-}$ and *NPC1*$^{+/+}$ Purkinje cells revealed survival of *NPC1*$^{+/+}$ Purkinje cells surrounded by infiltrating glial cells (Ko et al., 2005). These findings exclude the possibility that any cytokines or toxic substances secreted by glial cells are the primary cause of neuronal death, but do not exclude the possibility that such factors may trigger and/or accelerate degeneration of *NPC1*$^{-/-}$ cells.

The involvement of cytokines in the brain pathology of *NPC1*$^{-/-}$ mice has been suggested by several studies showing expression of tumor necrosis factor α (TNFα) mRNA by neurons and astrocytes (Wu et al., 2005), expression of interleukin-1β (IL-1β) by astrocytes (Baudry et al., 2003), and deregulation of nuclear factor-κB (NFκB) activity in neurons and astrocytes (Bi et al., 2005).

To gain additional insight into NPC pathogenesis, we screened for cytokines that were secreted by cultured human NPC fibroblasts and found increased secretion of interferon-β (IFN-β), IL-6, and IL-8. The purpose of the current study was to reveal the biochemical basis for the secretion and their role in NPC pathogenesis.

Materials and Methods

Cell culture. Human skin fibroblasts were from control subjects (H1, H2, H5, H11, H17, H34), NPC1 patients (UCH, YON, KUR, MUR, OHS, SAS, END, KAI) (Yamamoto et al., 2000), an NPC2 patient (81027) (Millat et al., 2001), and patients with Niemann–Pick disease type A (NPA) (GM0112) or B (NPB) (GM0252). All cells were passaged 5–15 times. Human fibroblasts, HepG2 and HEK293 cells were maintained in DMEM/10% bovine calf serum (BCS) at 37°C in a humidified atmosphere with 5% CO_2. HEK293 cells that stably expressed human Toll-like receptor 4 (TLR4) were obtained from InvivoGen (San Diego, CA). CHO cells were maintained in the same manner except for the use of Ham's F12 instead of DMEM. CHO/NPC1(−) cells that do not express any NPC1 protein and "knock-in" cells that express human NPC1 have been described previously (Sugimoto et al., 2001). For preparation of conditioned medium, cells were seeded on six-well dishes (10^4/well), and 24 h later, the medium was changed to serum-free DMEM. After a 24 h incubation, the medium was collected and filtered through 0.22 μm pore filters. To stimulate H11 cells or HepG2 cells, conditioned medium was concentrated 100× (v/v) by using Ultrafree-15 Biomax-30 (Millipore, Bedford, MA), and applied at 1/10 (v/v). For the assessment of IL-6 secretion by H34 cells, cells were cultured for 24 h, and then incubated for 12 h in serum-free DMEM containing lipopolysaccharide (LPS) (from *Escherichia coli*; Sigma, St. Louis, MO), polyinosinic:polycytidylic acid [poly(I:C)] (Sigma), macrophage-activating lipopeptide-2 (MALP-2) (from *Mycoplasma fermentans*; Alexis Biochemicals, San Diego, CA), or recombinant TNFα (Peprotech, Rocky Hill, NJ).

Peritoneal exudates and skin fibroblasts were isolated from 6-week-old mice. Briefly, animals were killed with diethyl ether, and 5 ml of ice-cold PBS was injected to the peritoneal cavity. Cells were recovered by gentle massage of the peritoneum and suspended in DMEM/10% BCS. To enrich for macrophages, cells were plated on serum-coated dishes for 15 min, and nonadherent cells were removed by washing with PBS. Cells that adhered to the dishes were maintained in DMEM/10% BCS. Fibroblasts that migrated out of a piece of skin in DMEM/10% BCS were maintained in the same medium.

Expression plasmids. pEFBOS expression plasmids of human TLR4 and MD-2 were a kind gift from Dr. M. Kimoto (Saga Medical University, Saga, Japan). The entire coding sequence of TLR4 was subcloned into pEGFP-N1 in-frame with green fluorescent protein (GFP) to generate a TLR4-GFP fusion protein. Cells were transfected by using FuGENE Transfection Reagent (Roche, Indianapolis, IN).

Small interfering RNA. Predesigned, double-stranded small interfering RNA (siRNA) against human TLR4 (sense sequence, 5′-GGUCUGGCUGGUUUAGAAG-3′) and control siRNA were purchased from Ambion (Austin, TX). They were introduced to human fibroblasts by electroporation using a Human Dermal Fibroblast Nucleofector kit (Amaxa, Gaithersburg, MD), according to the manufacturer's recommendations. At 48 h after electroporation, cells were lysed for Western blotting, or further incubated for 24 h in serum-free DMEM for preparation of conditioned medium.

Western blotting. Triton X-100 (1%) extracts from cells and tissues were used unless otherwise indicated. Cells were cultured for 48 h. Cell pellets were resuspended in buffer (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 30 mM $Na_4P_2O_7$, 50 mM NaF, 2 mM Na orthovanadate) supplemented with a protease inhibitor mixture (Boehringer, Bagnolet, France) and lysed by sonication. The lysates were mixed with Triton X-100 (1% v/v) and incubated on ice for 30 min, and insoluble materials were removed by brief centrifugation. To prepare 1% Triton X-100 insoluble fractions, the lysates were centrifuged at 100,000 × *g* for 30 min, and the pellet was suspended in the same buffer containing 1% Triton X-100 and 0.1% SDS. Protein concentrations were measured using the BCA protein assay kit (Bio-Rad, Hercules, CA). Brains were removed from 6-week-old mice that were killed with diethyl ether. Tissues were homogenized with Polytron in 10× (w/v) of the same buffer, and homogenates were processed as described above. SDS-PAGE, Western transfer, and immunoblotting were performed as described previously (Sugimoto et al., 2001), and bound antibodies were detected using an ECL detection kit (Amersham Biosciences, Piscataway, NJ). The following antibodies were used: myxovirus resistance A (MxA) (mouse monoclonal; kindly provided by Dr. K. Nagata, Tsukuba University, Ibaraki, Japan), signal transducers and activators of transcription 1~6 (STAT-1~STAT-6), tyrosine-701-phosphorylated STAT-1, IFN-stimulated gene factor 3γ (ISGF3γ) (mouse monoclonal; Transduction Laboratories, Lexington, KY), GFAP (rabbit polyclonal; Santa Cruz Biotechnology, Santa Cruz, CA), TLR4 (goat polyclonal; Santa Cruz Biotechnology) and β-actin (mouse monoclonal; Calbiochem, La Jolla, CA). Blot densitometric analysis was performed by using the NIH Image J software.

Cytochemistry. Cells cultured on coverslips for 24 h were fixed with 3.7% paraformaldehyde/PBS and permeabilized with 0.5% Triton X-100. After blocking in BlockAce (Dainippon, Osaka, Japan), they were incubated with a primary antibody (STAT-1, 1:100; MxA, 1:200) overnight at 4°C. Bound antibodies were visualized with an Alexa 488-conjugated secondary antibody, and images were obtained using a Bio-Rad MRC 1024 confocal microscope. Procedures for cellular uptake of Alexa 546-conjugated cholera toxin B subunit and the filipin staining of fixed cells have been described previously (Sugimoto et al., 2001).

Histochemistry. Mice were anesthetized with sodium pentobarbital (0.1 ml/100 g body weight) and perfused with 0.01% heparin/saline followed by 4% paraformaldehyde/0.1 M phosphate buffer, pH 7.4. Brains were embedded in OCT compound, and 10 μm sections were cut using a cryostat. Sections were incubated in 0.3% H_2O_2, blocked with BlockeAce, and incubated with primary antibodies at 4°C overnight. We used rabbit polyclonal antibodies against TLR4, GFAP, STAT-1, -3, and -6, proliferating cell nuclear antigen (PCNA), goat polyclonal antibodies against IL-6, GFAP (all at 1:100; Santa Cruz Biotechnology), and rat antibody against MAC1 (1:200; Chemicon, Temecula, CA). Bound antibodies were detected by HRP-conjugated secondary antibodies and visualized with DAB, and the sections were counterstained with hematoxylin. For immunofluorescence costaining, sections were incubated with antibodies against TLR4, IL-6, STATs together with antibodies against GFAP or MAC1. Bound antibodies were visualized with Alexa 488- or 568-conjugated secondary antibodies and imaged using a confocal microscope.

Luciferase reporter gene assay. HepG2 cells were cultured for 24 h and transfected with a luciferase reporter gene construct (pISRE-Luc, pSTAT3-Luc, or pGAS-Luc; Clontech, Cambridge, UK) together with pEGFP (Clontech) for normalization of transfection efficiency. After incubation in DMEM/0.5% BCS for 24 h, cells were further incubated for