Biochimica et Biophysica Acta 1782 (2008) 408–413

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbadis

Rescue of mutant α-galactosidase A in the endoplasmic reticulum by 1-deoxygalactonojirimycin leads to trafficking to lysosomes

Ryoji Hamanaka [a], Tetsuji Shinohara [a], Shinji Yano [b], Miki Nakamura [a], Aiko Yasuda [c], Shigeo Yokoyama [b], Jian-Qiang Fan [d], Kunito Kawasaki [e], Makoto Watanabe [a], Satoshi Ishii [e,*]

[a] Department of Anatomy, Biology and Medicine, Faculty of Medicine, Oita University, Oita, Japan
[b] Department of Oncological Science, Faculty of Medicine, Oita University, Oita, Japan
[c] Institute of Scientific Research, Faculty of Medicine, Oita University, Oita, Japan
[d] Department of Human Genetics, Mount Sinai School of Medicine, New York, USA
[e] Department of Agricultural and Life Science, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido, 080-8555, Japan

ARTICLE INFO

Article history:
Received 24 December 2007
Received in revised form 5 March 2008
Accepted 5 March 2008
Available online 12 March 2008

Keywords:
α-galactosidase A
Active-site-specific chaperone
Fabry disease
In vitro translation
Immunoelectron microscopy

ABSTRACT

Active-site-specific chaperone therapy for Fabry disease is a genotype-specific therapy using a competitive inhibitor, 1-deoxygalactonojirimycin (DGJ). To elucidate the mechanism of enhancing α-galactosidase A (α-Gal A) activity by DGJ-treatment, we studied the degradation of a mutant protein and the effect of DGJ in the endoplasmic reticulum (ER). We first established an *in vitro* translation and translocation system using rabbit reticulocyte lysates and canine pancreas microsomal vesicles for a study on the stability of mutant α-Gal A with an amino acid substitution (R301Q) in the ER. R301Q was rapidly degraded, but no degradation of wild-type α-Gal A was observed when microsomal vesicles containing wild-type or R301Q α-Gal A were isolated and incubated. A pulse-chase experiment on R301Q-expressing TgM/KO mouse fibroblasts showed rapid degradation of R301Q, and its degradation was blocked by the addition of lactacystin, indicating that R301Q was degraded by ER-associated degradation (ERAD). Rapid degradation of R301Q was also observed in TgM/KO mouse fibroblasts treated with brefeldin A, and the amount of R301Q enzyme markedly increased by pretreatment with DGJ starting 12 h prior to addition of brefeldin A. The enhancement of α-Gal A activity and its protein level by DGJ-treatment was selectively observed in brefeldin A-treated COS-7 cells expressing R301Q but not in cells expressing the wild-type α-Gal A. Observation by immunoelectron microscopy showed that the localization of R301Q in COS-7 cells was in the lysosomes, not the ER. These data suggest that the rescue of R301Q from ERAD is a key step for normalization of intracellular trafficking of R301Q.

1. Introduction

Fabry disease is a lysosomal storage disorder caused by deficient lysosomal α-galactosidase A (α-Gal A, EC 3.2.1.22) activity, resulting in progressive accumulation of neutral glycosphingolipids in vascular endothelial cells [1]. This disorder is classified into two major subtypes based on clinical manifestations. Patients with classic early onset Fabry disease show various clinical symptoms, such as angiokeratoma, acroparathesis, hypohidrosis, corneal and lenticular opacities, and progressive vasculopathy of the kidney, heart, and central nervous system. In contrast, atypical variants with residual α-Gal A activity show late onset cardiomyopathy with left ventricular hypertrophy but not the classic symptoms [2].

Currently, enzyme replacement therapy (ERT) is the only approved therapy for Fabry disease [3,4]. ERT has been well-tolerated by patients, as characterized by improvement in gastrointestinal and neurological manifestations (acroparaesthesia, hypohidrosis, vasomotion) and in quality of life [5,6]. However, results from treating variant Fabry disease patients have been mixed, suggesting that ERT may be inefficient for treating severe late-stage patients, presumably as a result of insufficient delivery of the enzyme to particular tissue [7,8]. ERT is expensive, and it could therefore be an economic burden for patients, so it is necessary to develop alternative affordable therapies for treating patients with Fabry disease.

We have shown that residual enzyme activity in lymphoblasts established from atypical Fabry patients with R301Q or Q279E mutations can be increased by cultivating cells with 1-deoxygalactonojirimycin (DGJ), a potent competitive inhibitor of α-Gal A [9]. We designated our therapeutic strategy as active-site-specific chaperone

* Corresponding author. Inada-cho, Obihiro, Hokkaido 080-8555, Japan. Fax: +81 155 49 5577.
E-mail address: ishiis@obihiro.ac.jp (S. Ishii).

Abbreviations: α-Gal A, α-galactosidase A; DGJ, 1-deoxygalactonojirimycin; ASSC, active-site-specific chaperone; ER, endoplasmic reticulum; DTT, dithiothreitol; Endo H, endoglycosidase H; ERAD, ER-associated degradation; ERT, enzyme replacement therapy; TEA, triethanolamine; RM, rough microsomes; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; BSA, bovine serum albumin; HRP, horseradish peroxidase; EDEM, ER degradation enhancing α-mannosidase-like protein

0925-4439/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbadis.2008.03.001

(ASSC) therapy because we hypothesized that DGJ binds to the active site of the enzyme, works as a folding template of misfold proteins, and enhances the enzyme activity [10,11]. Our recent study showed that the majority of missense mutant enzymes, identified in patients with residual enzyme activity, retain the catalytic activity and fragile conformation resulting in excessive degradation of affected mutant protein in endoplasmic reticulum-associated degradation (ERAD). This is the main molecular cause for the deficiency of the protein and broadness of the DGJ effect in the treatment of both classic and variant Fabry patients with residual enzyme activity [12]. We proposed that DGJ acts for those mutant proteins with a fragile conformation to facilitate proper folding, thus avoiding retardation and excessive degradation of these mutant enzymes in the endoplasmic reticulum (ER) [13].

To prove our hypothesis, in this study we focused on the behavior of mutant α-Gal A in the ER of mammalian cells and provided the evidence that DGJ acts in the ER. To determine the manner of degradation of mutant α-Gal A in the ER and the enhancement of enzyme activity of mutant α-Gal A by the treatment of DGJ in the ER, we used *in vitro* translation, translocation, and cells cultured in the presence of brefeldin A, which is an inhibitor of transport from the ER to the *cis*-Golgi [14,15]. We also provided the evidence that the majority of mutant α-Gal A was retained in the ER when it was expressed in COS-7 cells, whereas, in contrast, the rescued mutant α-Gal A by the treatment of DGJ was mainly located in lysosomes.

2. Materials and methods

2.1. Cell culture and transient expression

Murine fibroblasts (TNK and TMK2 cells) had been established from heterozygous transgenic mice expressing human wild-type and mutant α-Gal A (R301Q), respectively, in an endogenous murine α-Gal A knock-out background [16]. TNK, TMK2, and COS-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM, Sigma Chemicals, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS, Invitrogen Corp., Carlsbad, CA) and antibiotics (penicillin and streptomycin) at 37 °C under 5% CO_2. For the preparation of COS-7 cells expressing wild-type or mutant α-Gal A, 2 μg of plasmid DNA (pCXN2-GLA or pCXN2-GLA-R301Q, respectively) [17] were transfected into the COS-7 cells with Lipofectamine 2000 (Invitrogen Corp.) according to the manufacturer's instructions. DGJ (Toronto Research Chemicals, North York, ON, Canada) was added to the culture medium.

2.2. Antibodies and inhibitors

Polyclonal antibody against human α-Gal A [18] was purified by ImmunoPure Immobilized Protein A Gel (Pierce Biotechnology, Rockford, IL) for immunoprecipitation and immunoelectron microscopic study. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody (Pierce Biotechnology), gold-conjugated goat anti-rabbit IgG (GE Healthcare Bio-Sciences Corp., Piscataway, NJ), lactacystin (kindly provided by Dr. S. Omura, The Kitasato Institute, Tokyo, Japan) and brefeldin A (Sigma Chemicals) were obtained from indicated sources.

2.3. In vitro translation and translocation, and degradation assay

The pGEM-9Zf(−)9 vector containing either human wild-type or R301Q α-Gal A cDNA was linearized beyond the 3' end of cDNA using Xba I (New England Biolabs, Beverly, MA). Capped mRNAs were prepared using mMESSAGE mMACHINE kit (Ambion, Austin, TX). Wild-type and R301Q α-Gal A polypeptides were synthesized from 0.2 μg mRNA and labeled with [^{35}S]-methionine in the rabbit reticulocyte lysate cell-free system (Promega, Madison, WI) with canine pancreas microsomal vesicles prepared as previously described by Walter and Blobel [19], according to the manufacturer's protocol. Rough microsomes (RM) containing synthesized proteins were sedimented by centrifugation for 5 min at 100,000 ×g through a cushion of 50 mM triethanolamine (TEA) (pH 7.4) buffer containing 0.5 M sucrose and 1 mM dithiothreitol (DTT). Resulting vesicles were subjected to trypsin-digestion or endoglycosidase H (Endo H, New England Biolabs) digestion as follows. Trypsin (25 μg/ml) was added to RM in the presence of 1% Triton X-100. Following incubation for 10 min on ice, the reaction was terminated by the addition of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (50 mM Tris–HCl buffer, pH 6.8, containing 2% SDS, 10% glycerol, 0.01% bromophenol blue, and 3% 2-mercaptoethanol). Endo H digestion was carried out according to manufacturer's protocol (CALBIOCHEM, San Diego, CA). For the degradation assay, the vesicles were washed with a high-salt buffer (0.5 M potassium acetate, 5 mM magnesium acetate) and subjected to ultracentrifugation through a 0.5 M sucrose cushion to remove the reticulocyte lysates. The isolated vesicles were resuspended in a chase buffer (20 mM HEPES, pH 7.5, 50 mM KCl, 1 mM $CaCl_2$) and incubated with or without 20 μM DGJ at 37 °C for 0.5, 1, 2, and 4 h. Each vesicle was subjected to SDS-PAGE and quantified by a BAS 2000 phosphoimage analyzer (Fuji Film, Tokyo, Japan).

2.4. Pulse-chase experiments and immunoprecipitation

TNK and TMK2 cells were preincubated in serum/Met/Cys-free DMEM (Sigma Chemicals) for 30 min and then radiolabeled for 30 min by the addition of [^{35}S]-Met/Cys (ICN Biomedicals Inc., Costa Mesa, CA). Following the labeling period, the cells were cultured in complete DMEM containing excessive amounts of cold methionine/cysteine, 10% FBS, and 2 μM lactacystin or 5 μg/ml brefeldin A with or without 20 μM DGJ. At 1 and 2 h, the cells were washed with phosphate buffered saline (PBS) and lysed in cold buffer (100 mM Tris–HCl, pH 7.5, 0.4 M KCl, 1% Triton X-100, 1 mM EDTA, 0.1 mg/ml leupeptin). Cell debris was removed by sedimentation at top speed in a microfuge for 15 min. The indicated antibodies absorbed by protein A-sepharose CL-4B (GE Healthcare Bio-Sciences) were then added to the supernatants from medium and cell lysates for immunoprecipitation, which was carried out with a slight modification as previously reported [20]. The immunoprecipitates were briefly washed five times in a washing buffer (25 mM Tris–HCl, pH 7.5, 0.5 M NaCl, 0.1% Triton X-100 and 0.1% SDS) to remove nonspecifically absorbed protein. Bound antigen was eluted from the beads by boiling in SDS-PAGE sample buffer and then analyzed by SDS-PAGE. The gels were quantified in the BAS 2000 phosphoimage analyzer.

2.5. Enzyme assay and protein determination

α-Gal A activity was assayed with 4-methylumbelliferyl α-D-galactopyranoside (4MU-α-Gal, Sigma Chemicals) as a substrate, as described previously [18]. One unit of enzyme activity was defined as the amount of enzyme that releases 1 nmol of 4-methylumbelliferone per hour. Protein concentration was determined using a DC Protein Assay kit (Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin (BSA, Sigma Chemicals) as a standard.

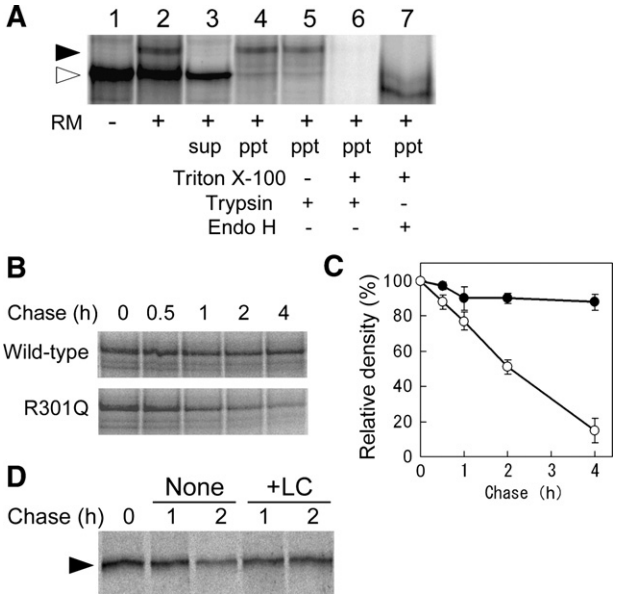

Fig. 1. *In vitro* translation, translocation, and degradation of R301Q by ERAD. (A) R301Q mRNA was translated in rabbit reticulocyte lysates with RM (lanes 2–7) or without RM (lane 1) as described in Materials and methods. After the reaction, the mixture was separated into supernatant (lane 3) and pellet (lane 4) by centrifugation, and aliquots of the mixtures were treated with trypsin in the absence (lane 5) or presence (lane 6) of 1% Triton X-100, and treated with Endo H in the presence of 1% Triton X-100 (lane 7). All the products were subjected to SDS-PAGE. Closed and open arrowheads indicate the glycosylated precursor form (50 kDa) and non-glycosylated precursor form (46 kDa), respectively. (B) Stability of *in vitro* translated and translocated wild-type or R301Q α-Gal A. The vesicles were washed with a high-salt buffer and subjected to sedimentation, then resuspended in a chase buffer and incubated at 37 °C for indicated times. Same amount of each sample was applied onto a single gel and determined by the autoradiography. (C) Quantitative analysis of 50-kDa band detected in (B). The amounts of α-Gal A remaining in the vesicles were plotted. The radioactivity of each band for wild-type (closed circle) and R301Q (open circle) was quantified by a phosphoimage analyzer. The mean and standard deviation of three independent experiments are shown. (D) Degradation of R301Q in murine fibroblasts and the effect of lactacystin (LC) addition into culture medium. TMK2 cells were pulse-labeled and treated with 2 μM lactacystin as described in Materials and methods.

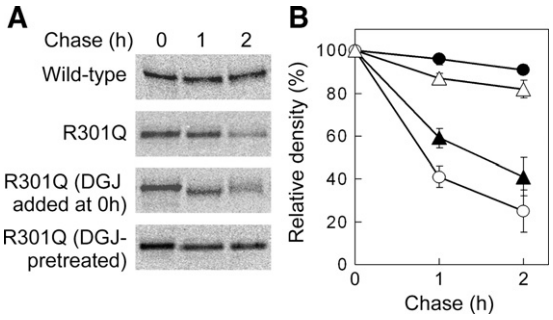

Fig. 2. Effect of DGJ on the degradation of R301Q in cells treated with brefeldin A. (A) Murine fibroblasts expressing wild-type and R301Q α-Gal A were labeled in the presence of 5 μg/ml brefeldin A with or without 20 μM DGJ. The treatment of brefeldin A was started 30 min before the radiolabeling period and continued until the end of the chase period. DGJ was applied in two ways (one way was adding DGJ at same time as brefeldin A-treatment, and the other was pretreatment starting from 12 h prior to radiolabeling). After 30-min radiolabeling and chase period, cell lysates were prepared and subjected to immunoprecipitation with an anti-α-Gal A antibody, followed by SDS-PAGE and fluorography. (B) Quantitative analysis of (A). The mean and standard deviation of three independent experiments are shown. (Closed circle), wild-type; (open circle), R301Q; (closed triangle), R301Q (DGJ added at 0 h); and (open triangle), R301Q (DGJ-pretreated).

2.6. Western blot analysis

Western blot analysis for the detection of α-Gal A protein was performed using polyclonal anti-α-Gal A antibody and HRP-conjugated anti-rabbit IgG antibody as described previously [18]. Cell homogenate containing approximately 30 μg protein was applied to a 10% polyacrylamide gel. After SDS-PAGE, proteins were electorophoretically transferred to a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). The membrane was blocked with 5% skim 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 rabbit polyclonal anti-α-Gal A antibody diluted in a milk/BLOT solution (1% skim milk in BLOT solution) for 1 h at room temperature with mild shaking. After washing with an excess amount of the milk/BLOT solution, the membrane was treated for 1 h at room temperature with HRP-conjugated anti-rabbit IgG antibody diluted in the milk/BLOT solution. After extensive washing with the milk/BLOT solution, protein bands were visualized with SuperSignal® Chemiluminescent Substrate (Pierce Biotechnology).

2.7. Immunoelectron microsopy

The COS-7 cells expressing wild-type or R301Q α-Gal A were cultured with or without 20 μM DGJ for 48 h after transfection, then fixed in 2% glutaraldehyde in 0.2 M Tris–HCl (pH 7.4) for 3 h; and further incubated in 1% osmium tetraoxide for 2 h, dehydrated in ethanol, and embedded in Epok 812. Immunoelectron microscopy was performed as described previously [21]. Thin sections were briefly microwaved in Target Retrieval Solution, pH 10 (DAKO, Carpinteria, CA), and then incubated for 30 min at room temperature with affinity purified anti-α-Gal A antibody diluted 1:50. After washing with 50 mM Tris–HCl (pH 7.5) containing 0.8% NaCl, 0.1% BSA, and 0.1% Tween 20, the ultrathin sections were incubated with gold-conjugated goat anti-rabbit IgG. After washing, the ultrathin sections were stained with uranyl acetate and lead citrate, and examined using a transmission electron microscope (JEM-1200EXII, JEOL, Japan).

3. Results

In order to examine the stability of R301Q in the ER, we established the *in vitro* translation and translocation system using rabbit reticulocyte lysates and canine pancreas RM. The α-Gal A was synthesized as a single product 46-kDa polypeptide by translation with reticulocyte lysates (Fig. 1A, lane 1). An approximate 50-kDa band appeared with the addition of RM (upper band in Fig. 1A, lane 2). Evidence of translocation into RM is recovery of material in this upper band in the vesicular precipitates (Fig. 1A, lanes 3 and 4), resistance to trypsin-digestion when it was not made permeable with Triton X-100 (Fig. 1A, lane 5) and disappearance following trypsin-digestion in the presence of Triton X-100 (Fig. 1A, lane 6). Two minor bands (approximately 48 kDa and 46 kDa) other than the 50-kDa band were observed in the incorporated products into RM. All bands incorporated into RM were sensitive to Endo H, which removes high-mannose type oligosaccharides from glycoproteins; and as a result, were shifted to an approximate 43-kDa band after Endo H treatment (Fig. 1A, lane 7). From the trypsin-treatment with or without Triton X-100, we demonstrated that this *in vitro* system is well functioning, and suggested that α-Gal A is properly translocated and processed as previously described for other proteins [22,23]. There was no difference between wild-type and R301Q α-Gal A on the profiles and in the efficiency of the translation, translocation, and glycosylation from the same amount of transcripts. The amount of 50-kDa polypeptide of wild-type and R301Q α-Gal A was 33% and 36%, respectively, of the total product. To study the stability of the protein product from an *in vitro* translation and translocation system, the microsomal vesicles containing R301Q or wild-type α-Gal A were pooled and further incubated. Two minor bands other than the 50-kDa band were decreased within 4-h chase in both wild-type and R301Q. Although the 50-kDa band was remained in wild-type during 4-h chase, the 50-kDa band of R301Q was decreased in this treatment (Fig. 1B and C). These data suggest that R301Q is unstable in the microsomal vesicles, and this instability may cause low enzyme activity in patients' cells. The addition of DGJ had no effect on the degradation of R301Q in this cell-free system (data not shown). To confirm further that R301Q was degraded by ERAD, the effect of lactacystin, which is an inhibitor for proteasome (ERAD) [24] on the degradation of R301Q in TMK2 cells was studied (Fig. 1D). Pulse-labeled R301Q was markedly degraded within a 2-h chase period, but it was blocked by the treatment with lactacystin.

To identify the cellular compartment in which the effect of DGJ on R301Q occurred, the pulse-chase experiment using TMK2 cells was carried out in the presence of brefeldin A, which is a transport inhibitor causing retention of newly synthesized membrane proteins and secretory proteins in the ER [25,26]. As shown in Fig. 2, R301Q pulse-labeled with [^{35}S]-Met/Cys was substantially reduced within a 2-h chase period in the presence of brefeldin A, whereas the wild-type α-Gal A was not changed during this time. The pulse-labeled R301Q in the cells, which were pretreated with DGJ for 12 h prior to the treatment of brefeldin A, was stable during this chase period. The simultaneous addition of DGJ with brefeldin A did not protect the degradation of R301Q at all, presumably due to the inhibitory effect of brefeldin A on the DGJ import into the ER. These data suggest that R301Q is degraded by ERAD and that DGJ acts in the ER to restore R301Q.

To elucidate the change in enzyme activity of R301Q in the ER, we used a transient expression system because stable transformants such as TMK2 cells are not suitable when we selectively determine the activity of newly synthesized protein. Wild-type or R301Q α-Gal A was

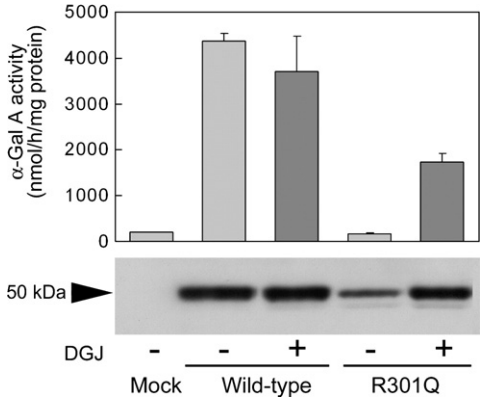

Fig. 3. Enzyme activity and the amount of R301Q in brefeldin A-treated COS-7 cells. COS-7 cells were transfected with pCXN2-GLA, pCXN2-GLA-R301Q, or pCXN2 vector [49] (mock transfection) and brefeldin A (5 μg/ml) was present in culture medium during 24–48 h after transfection. Before brefeldin A addition, at 6 h after transfection, some cells were treated with DGJ. At 48 h after transfection, cells were harvested and homogenized in water using a micro-homogenizer (Physcotron, Niti-on, Inc., Chiba, Japan). The supernatant collected after centrifugation of the homogenate (10,000 ×*g* for 5 min) was used in enzyme assays. The assay of α-Gal A activity and Western blot analysis with an anti-α-Gal A antibody was performed as described in Materials and methods.

transiently expressed in COS-7 cells and kept in their ER by treatment with brefeldin A for 24–48 h after transfection. In the expression system using COS-7 cells, recombinant α-Gal A was transiently synthesized after 24 h of transfection, and the enzyme activity and amount rapidly increased during this time. Therefore, newly synthesized enzymes were retained in the ER by the treatment with brefeldin A, and the effect of DGJ on the activity of newly synthesized proteins could be examined. The enzyme activity in COS-7 cells expressing R301Q (202 ± 12 nmol/h/mg protein) was almost the same level as mock transfected COS-7 cells (168 ± 5 nmol/h/mg protein) and was much lower than cells expressing wild-type α-Gal A (4380 ± 160 nmol/h/mg protein) (Fig. 3). The pretreatment of those cells with DGJ allowed a significant increase to be selectively observed in cells expressing R301Q (1740 ± 176 nmol/h/mg protein), which was approximately 50% of the level in cells expressing wild-type α-Gal A treated with DGJ and brefeldin A (3700 ± 765 nmol/h/mg protein). The enzyme amount detected by Western blot analysis was high in cells expressing wild-type α-Gal A and DGJ-treated cells expressing R301Q. These data indicated that the degradation of R301Q was related to its enzyme activity; and enzymes that lost their activity could be recognized by the ERAD in mammalian cells.

To verify the distribution of R301Q in transfected COS-7 cells, immunoelectron microscopic study was conducted with anti-α-Gal A antibody (Fig. 4). Although it is a little hard to distinguish ribosomes (20 nm) from gold particles (15 nm, clear dots) at lower magnification, it is much easier at higher magnification. After we applied the modified technique [21] to prepare the specimens, the COS-7 cells expressing R301Q revealed gold particles predominantly in the ER surrounding the nucleus (Fig. 4A–C). In contrast, gold particles were observed mainly in the lysosomes but could not be detected in the ER in cells expressing R301Q treated with DGJ for 48 h (Fig. 4D–F), and there was a similar pattern for cells expressing wild-type α-Gal A (data not shown). To determine the relative amount of R301Q localized in the lysosomes, we counted the whole number of gold particles in 50 independent cells and calculated a percentage of gold particles located in the lysosomes. Almost of all gold particles (represents α-Gal A) were detected in lysosomes and the ER, and the ratio of R301Q localized in lysosomes and the ER was 6:94 in the absence of DGJ, but it was changed to 79:21 in the presence of DGJ, and it became similar to that of wild-type α-Gal A (82:18), implying that R301Q rescued by DGJ in the ER was normally processed and sent to its final destination, the lysosomes.

4. Discussion

In this study, we established an *in vitro* translation and translocation assay for studying human α-Gal A and observed some characteristics of R301Q in the ER. The *in vitro* translated product (approximately 46-kDa) had a similar size as that predicted from the 429-amino acid precursor sequence of α-Gal A [27]. The translocated protein (50-kDa band) appeared when *in vitro* translation was performed in the presence of canine pancreas RM. This protein, recovered in RM sedimented by centrifugation, had a resistance to trypsin-digestion, and it was characterized as the precursor-form of α-Gal A translocated into RM. The size of precursor-form α-Gal A was identical to that observed in mammalian cells [12,28]. The Endo H-digestion of the 50-kDa protein strongly suggested that it was glycosylated. The difference in size between the 50-kDa precursor protein and digested product (43 kDa) was approximately 7 kDa, which is equal in size to 3 U of *N*-glycan (about 2.4 kDa). The product of our *in vitro* translation and translocation system may be fully glycosylated because 3 sites were glycosylated with a recombinant human α-Gal A expressed in mammalian cells [29]. Furthermore, the 3-kDa difference between the *in vitro* translated product (46-kDa) and Endo H-digested product (43-kDa) was equal to the size predicted from the signal sequence containing 31 amino acids [27]. Two minor bands (48-kDa and 46-kDa bands) translocated into RM may be 2 U and single unit, respectively, of *N*-glycan-binding products, and these minor bands of both wild-type and R301Q were unstable in the microsomal vesicles. We feel that all of the above data indicate that mutant α-Gal A, as well as wild-type protein, was normally translated, translocated, and glycosylated in our cell-free system.

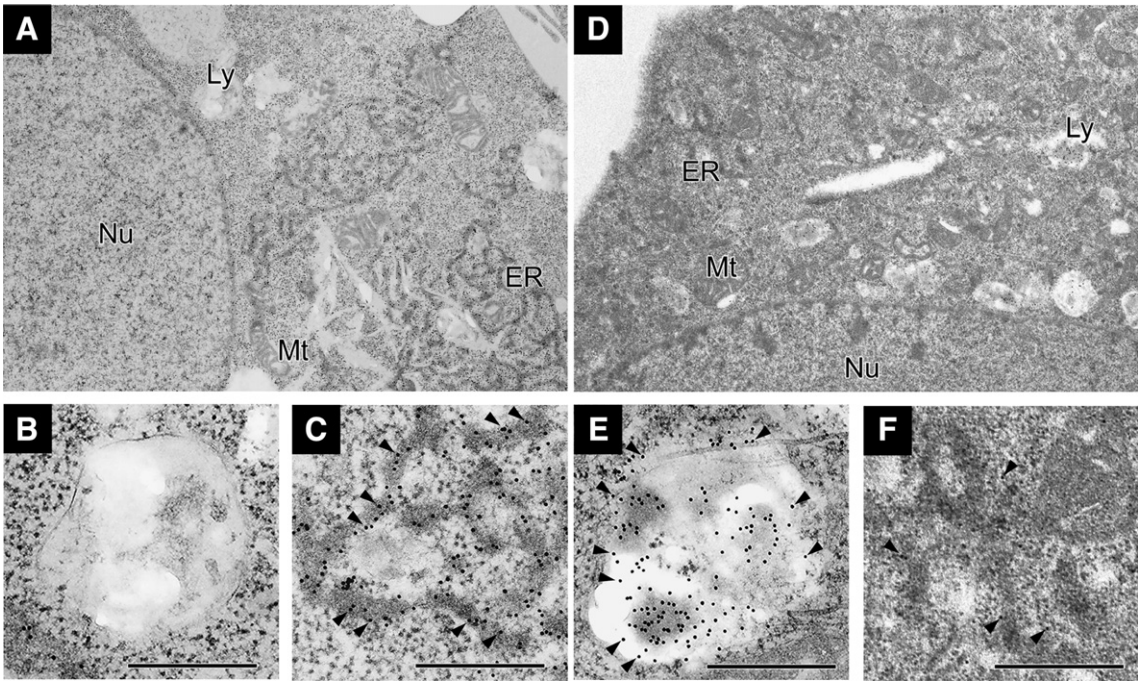

Fig. 4. Intracellular localization of R301Q. The intracellular localization of R301Q in COS-7 cells in the absence (A–C) or presence (D–F) of DGJ. COS-7 cells were transfected with pCXN2-GLA-R301Q and treated with 20 μM DGJ for 48 h, then cells were fixed and embedded as described in Materials and methods. The ultrathin sections were incubated with an anti-α-Gal A antibody followed by immunogold labeling. Each thin section was examined with a transmission electron microscope. (B) and (E) show the higher magnifications of the lysosome. (C) and (F) show the higher magnifications of the ER. Scale bars represent 500 nm. Nu, nucleus; Mt, mitochondria; Ly, lysosomes. Typical gold particles are pointed by arrowhead.

Although there was no difference between wild-type and R301Q α-Gal A in the process of translation, translocation and glycosylation, they could be distinguished on the basis of their stability. R301Q was rapidly degraded in microsomal vesicles (Fig. 1B), while α-Gal A was found to be stable. The stability of R301Q in the ER may be the crucial factor which causes its low activity in patients' cells.

The addition of DGJ in this cell-free system did not show any protective effect on this degradation, which may be due to the inaccessibility of DGJ to R301Q in this system. Although we do not have any data on the transport of DGJ, we predicted that DGJ would be taken up by cells through other iminosugar derivatives like 1-doxymannojirimycin (dMM) because of their structural similarity. Neefjes et al. [30] have reported that uptake of dMM by human erythroleukemic cells was not inhibited by glucose, and it (and other iminosugars) may not be carried into cells by hexose transporters [31]. They also discussed the presence of the translocation system for dMM in cells because passive diffusion of dMM cannot explain the difference of the K_i value (80 μM) of dMM for isolated mannosidase and the concentration (1 mM) of dMM necessary to inhibit mannosidase in cells. The presence of two distinct mannose transporters which are dMM-sensitive and insensitive had been reported in intestinal epithelial cells [32,33]. If dMM were incorporated into cells through glucose transporter GLUT4, it might be affected by the treatment with brefeldin A [34]. The data in our present study supported the presence of a translocation system for DGJ into the ER; pretreatment of DGJ prior to brefeldin A-treatment was necessary for showing the protective effect against R301Q degradation. We used a pulse-chase technique on TNK and TMK2 mouse fibroblasts cultured in the presence of brefeldin A (Fig. 2), so that newly synthesized enzymes were retained in the ER and their stability in the ER could be observed. The pulse-labeled R301Q quickly degraded within a 2-h chase period, but wild-type α-Gal A did not change. The pretreatment of DGJ for 12 h prior to brefeldin A-treatment was necessary for the protective effect of DGJ on the degradation of R301Q. Therefore, transport of DGJ into the ER must be required to have an effect on R301Q. DGJ protected against not only the degradation of R301Q, but also the loss of enzyme activity (Fig. 3).

In our previous paper [12], we described that majority of enzyme mutants, which were detected in Fabry patients who display residual activity, were thermolabile proteins in a solution at neutral pH, and degraded in ERAD because the amount of protein was increased by treatment with kifunensine, a selective inhibitor of ER α-mannosidase I and lactacystin (a proteasome inhibitor) [35]. In the present study, we confirmed the effect of lactacystin on R301Q-degradation by using R301Q-expressing murine fibroblasts (Fig. 1D). Its effect had previously been observed in some mutant α-Gal A's such as E66Q, F113L, N215S, M296I, and R301Q, which were transiently expressed in COS-7 cells [12].

Several chaperones such as BiP and calnexin are known to exist in the ER [36], and BiP might be a strong candidate as a molecular sensor, since it is associated with only R301Q but not wild-type proteins [37] or other misfolded proteins [38]. Recently, it has been postulated that ER degradation enhancing α-mannosidase-like protein (EDEM) or the EDEM family sequesters ERAD candidates released from calnexin, thereby interfering with the UDP-Glc:glycoprotein glucosyltransferase that reglucosylates misfolded glycoproteins released from the calnexin cycle [39,40]. Although the molecular mechanism of recognizing misfolded α-Gal A's to be degraded in the ER is still unclear, we may be able to shed some light on this area by using mutant enzyme R301Q as a good model of misfolded proteins.

The addition of DGJ changed the localization of R301Q from the ER to lysosomes (Fig. 4). Although Yam et al. [37] reported that rescued R301Q by DGJ-treatment was located in the lysosomes in fibroblasts established from transgenic mice [16] (as observed by immunoelectron microscopy), we further demonstrated its localization in lysosomes in COS-7 cells expressing R301Q in the presence of DGJ. COS-7 cells were established from monkey kidney cells [41], and the kidney is one of the target organs for the treatment of Fabry disease. In our present study, we demonstrated using immunoelectron microscopy that mutant α-Gal A (R301Q) was mainly located in the ER. These data strongly suggested that mutant α-Gal A was degraded by ERAD, and the enhancement of enzyme activity in ASSC therapy using DGJ was caused by restoration of R301Q in the ER and normalization of its movement to the lysosomes. It has been reported that lysosomal glycosphingolipids accumulation in fibroblasts from patients with Fabry disease was diminished by the treatment with DGJ [42], and the data demonstrated that the rescued mutant protein could work after reached to the lysosomes. Our therapeutic strategy may be useful for many diseases, and positive results using our concept of therapeutic strategy have been reported for Gaucher disease [43,44], G_{M1}-gangliosidosis [45], Tay–Sachs disease [46], retinitis pigmentosa 17 [47], and Pompe disease [48].

In our present study, we demonstrated that mutant α-Gal A (R301Q) was retained and degraded in the ER; DGJ acted as a folding template in the ER and rescued R301Q from the loss of enzyme activity and degradation. Although the details of the degradation process of R301Q in the ER still remain to be clarified, our *in vitro* translation and translocation technique and use of cells treated with brefeldin A will be useful tools for studying the degradation process of misfolded proteins.

Acknowledgements

We would like to thank Mr. Glen Hill for his critical reading of this manuscript, Dr. T. Shimada and Mr. H. Kawazato for technical help, and Dr. K. Kashima and Mr. K. Kuroki for editorial help. This work was supported in part by a research grant from the Ministry of Health, Labour and Welfare of Japan (S.I.).

References

[1] O.R. Brady, A.E. Gal, R.M. Bradley, E. Martensson, A.L. Warshaw, L. Laster, Enzymatic defect in Fabry's disease: ceramidetrihexosidase deficiency, N. Engl. J. Med. 276 (1967) 1163–1167.
[2] R.J. Desnick, Y.A. Ioannou, C.M. Eng, α-Galactosidase A deficiency: Fabry disease, in: C.R. Scriver, A.L. Beaudet, W.S. Sly, D. Valle (Eds.), The Metabolic and Molecular Bases of Inherited Disease, McGraw-Hill, New York, 2001, pp. 3733–3774.
[3] C.M. Eng, N. Guffon, W.R. Wilcox, D.P. Germain, P. Lee, S. Waldek, L. Caplan, G.E. Linthorst, R.J. Desnick, International Collaborative Fabry Disease Study Group, Safety and efficacy of recombinant human α-galactosidase A replacement therapy in Fabry's disease, N. Engl. J. Med. 345 (2001) 9–16.
[4] R.S. Schiffmann, J.B. Kopp, H.A. Austin, S. Sabnis, D.F. Moore, T. Wiebel, J.E. Balow, R.O. Brady, Enzyme replacement therapy in Fabry disease, JAMA 285 (2001) 2743–2749.
[5] M. Banikazemi, T. Ullman, R.J. Desnick, Gastrointestinal manifestations of Fabry disease: clinical response to enzyme replacement therapy, Mol. Genet. Metab. 85 (2005) 255–259.
[6] M.R. Bonquiorno, G. Pistone, M. Arico, Fabry disease: enzyme replacement therapy, J. Eur. Acad. Dermatol. Venereol. 17 (2003) 676–679.
[7] D. Tsambaos, E. Chroni, A. Manolis, A. Monastirli, E. Pasmatzi, T. Sakkis, P. Davlouros, D. Goumenos, A. Katrivanou, S. Georqiou, Enzyme replacement therapy in severe Fabry disease with renal failure: a 1-year follow up, Acta Derm.-Venereol. 84 (2004) 389–392.
[8] L. Spinelli, A. Pisani, M. Sabbatini, M. Petretta, M.V. Andreucci, D. Procaccini, N. Lo Surdo, S. Federico, B. Cianciaruso, Enzyme replacement therapy with agalsidase beta improves cardiac involvement in Fabry's disease, Clin. Genet. 66 (2004) 158–165.
[9] J.-Q. Fan, S. Ishii, N. Asano, Y. Suzuki, Accelerated transport and maturation of lysosomal α-galactosidase A in Fabry lymphoblasts by an enzyme inhibitor, Nat. Med. 5 (1999) 112–115.
[10] J.-Q. Fan, S. Ishii, Cell-based screening of active site specific chaperone for the treatment of Fabry disease, Methods Enzymol. 363 (2003) 412–420.
[11] J.-Q. Fan, S. Ishii, Active-site-specific chaperone therapy for Fabry disease: Yin and Yang of enzyme inhibitors, FEBS J. 274 (2007) 4962–4971.
[12] S. Ishii, H.-H. Chang, K. Kawasaki, K. Yasuda, H.-L. Wu, S.C. Garman, J.-Q. Fan, Mutant α-galactosidase A enzymes identified in Fabry patients with residual enzyme activity: biochemical characterization and restoration of normal intracellular processing by 1-deoxygalactonojirimycin, Biochem. J. 406 (2007) 285–295.
[13] J.-Q. Fan, A contradictory treatment for lysosomal storage disorders: inhibitors enhance mutant enzyme activity, Trends Pharmacol. Sci. 24 (2003) 355–360.
[14] J. Lippincott-Schwartz, L.C. Yuan, J.S. Bonifacino, R.D. Klausner, Rapid redistribution of Golgi proteins into the ER in cells treated with brefeldin A: evidence for membrane cycling from Golgi to ER, Cell 56 (1989) 801–813.

[15] J. Lippincott-Sdhwartz, L. Yuan, C. Tipper, M. Amherdt, L. Orci, R.D. Klausner, Brefeldin A's effects on endosomes, lysosomes and the TGN suggest a general mechanism for regulating organelle structure and membrane traffic, Cell 67 (1991) 601–616.
[16] S. Ishii, H. Yoshioka, K. Mannen, A.B. Kulkarni, J.-Q. Fan, Transgenic mouse expressing human mutant α-galactosidase A in an endogenous enzyme deficient background: a biochemical animal model for studying active-site specific chaperone therapy for Fabry disease, Biochim. Biophys. Acta 1690 (2004) 250–257.
[17] S. Ishii, R. Kase, T. Okumiya, H. Sakuraba, Y. Suzuki, Aggregation of the inactive form of human α-galactosidase in the endoplasmic reticulum, Biochem. Biophys. Res. Commun. 220 (1996) 812–815.
[18] S. Ishii, R. Kase, H. Sakuraba, S. Fujita, M. Sugimoto, K. Tomita, T. Semba, Y. Suzuki, Human α-galactosidase gene expression: significance of two peptide regions encoded by exons 1-2 and 6, Biochim. Biophys. Acta 1204 (1994) 265–270.
[19] P. Walter, G. Blobel, Preparation of microsomal membranes for cotranslational protein translocation, Methods Enzymol. 96 (1983) 84–93.
[20] R. Hamanaka, M.R. Smith, P.M. O'Connor, S. Maloid, K. Mihalic, J.L. Spivak, D.L. Longo, D.K. Ferris, Polo-like kinase is a cell cycle-regulated kinase activated during mitosis, J. Biol. Chem. 270 (1995) 21086–21091.
[21] S. Yano, K. Kashima, T. Daa, S. Urabe, K. Tsuji, I. Nakayama, S. Yokoyama, An antigen retrieval method using an alkaline solution allows immunoelectron microscopic identification of secretory granules in conventional epoxy-embedded tissue sections, J. Histochem. Cytochem. 51 (2003) 199–204.
[22] C.V. Nicchitta, G. Blobel, Lumenal proteins of the mammalian endoplasmic reticulum are required to complete protein translocation, Cell 73 (1993) 989–998.
[23] H. Yuki, R. Hamanaka, T. Shinohara, K. Sakai, M. Watanabe, A novel approach for N-glycosylation studies using detergent extracted microsomes, Mol. Cell. Biochem. 278 (2005) 157–163.
[24] G. Fenteany, R.F. Standaert, W.S. Lane, S. Choi, E.J. Corey, S.L. Schreiber, Inhibition of proteasome activities and subunit-specific amino-terminal threonine modification by lactacystin, Science 268 (1995) 726–731.
[25] T. Fujiwara, K. Oda, S. Yokota, A. Takatsuki, Y. Ikehara, Brefeldin A causes disassembly of the Golgi complex and accumulation of secretory proteins in the endoplasmic reticulum, J. Biol. Chem. 263 (1988) 18545–18552.
[26] J.B. Ulmer, G.E. Palade, Effects of Brefeldin A on the Golgi complex, endoplasmic reticulum and viral envelope glycoproteins in murine erythroleukemia cells, Eur. J. Cell Biol. 54 (1991) 38–54.
[27] D.F. Bishop, D.H. Calhoun, H.S. Bernstein, P. Hantzopoulos, M. Quinn, R.J. Desnick, Human α-galactosidase A: nucleotide sequence of a cDNA clone encoding the mature enzyme, Proc. Natl. Acad. Sci. U. S. A. 83 (1986) 4859–4863.
[28] P. Lemansky, D.F. Bishop, R.J. Desnick, A. Hasilik, K. von Figura, Synthesis and processing of α-galactosidase A in human fibroblasts. Evidence for different mutations in Fabry disease, J. Biol. Chem. 262 (1987) 2062–2065.
[29] Y.A. Ioannou, K.M. Zeidner, M.E. Grace, R.J. Desnick, Human α-galactosidase A: glycosylation site 3 is essential for enzyme solubility, Biochem. J. 332 (1998) 789–797.
[30] J.J. Neefjes, J. Lindhout, H.J.G. Broxterman, G.A. van der Marel, J.H. van Boom, H.L. Plegh, Non-carrier-mediated uptake of mannosidase I inhibitor 1-deoxymannnojirimycin by K562 erythroleukemic cells, J. Biol. Chem. 264 (1989) 10271–10275.
[31] G. Hanozet, H.P. Pircher, P. Vanni, B. Oesch, G. Semenza, An example of enzyme hysteresis. The slow and tight interaction of some fully competitive inhibitors with small intestinal sucrase, J. Biol. Chem. 256 (1981) 3703–3711.
[32] E. Ogier-Denis, G. Trugnan, C. Sapin, M. Aubery, P. Codogno, Dual effect of 1-deoxymannojirimycin on the mannose uptake and on the N-glycan processing of the human colon cancer cell line HT-29, J. Biol. Chem. 265 (1990) 5366–5369.
[33] E. Ogier-Denis, A. Blais, J.J. Houri, T. Voisin, G. Trugnan, P. Codogno, The emergence of a basolateral 1-deoxymannojirimycin-sensitive mannose carrier is a function of intestinal epithelial cell defferentiation, J. Biol. Chem. 269 (1994) 4285–4290.
[34] E. Ralston, T. Ploug, GLUT4 in cultured skeletal myotubes is segregated from the transferrin receptor and stored in vesicles associated with the TGN, J. Cell Sci. 109 (1996) 2967–2978.
[35] C.M. Cabral, Y. Liu, R.N. Sifers, Dissecting glycoprotein quality control in the secretory pathway, Trends Biochem. Sci. 26 (2001) 619–624.
[36] R. Sitia, I. Braakman, Quality control in the endoplasmic reticulum protein factory, Nature 426 (2003) 891–894.
[37] G.H.F. Yam, C. Zuber, J. Roth, A synthetic chaperone corrects the trafficking defect and disease phenotype in a protein misfolding disorder, FASEB J. 19 (2005) 12–18.
[38] S.D. Chessler, P.H. Byers, BiP binds type I procollagen pro alpha chains with mutations in the carboxyl-terminal propeptide synthesized by cells from patients with osteogenesis imperfecta, J. Biol. Chem. 268 (1993) 18226–18233.
[39] Y. Oda, N. Hosokawa, I. Wada, K. Nagata, EDEM as an acceptor of terminally misfolded glycoproteins released from calnexin, Science 299 (2003) 1394–1397.
[40] M. Molinari, V. Calanca, C. Galli, P. Lucca, P. Paganetti, Role of EDEM in the release of misfolded glycoproteins from the calnexin cycle, Science 299 (2003) 1397–1400.
[41] Y. Gluzman, SV40-transformed simian cells support the replication of early SV40 mutants, Cell 23 (1981) 175–182.
[42] G.H.F. Yam, N. Bosshard, C. Zuber, B. Steinmann, J. Roth, Pharmacological chaperone corrects lysosomal storage in Fabry disease caused by trafficking-incompetent variants, Am. J. Physiol., Cell Physiol. 290 (2006) C1076–C1082.
[43] A.R. Sawkar, W.C. Cheng, E. Beutler, C.H. Wong, W.E. Balch, J.W. Kelly, Chemical chaperones increase the cellular activity of N370S beta-glucosidase: a therapeutic strategy for Gaucher disease, Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 15428–15433.
[44] H.H. Cheng, N. Asano, S. Ishii, Y. Ichikawa, J.-Q. Fan, Hydrophilic iminosugar active-site-specific chaperones increase residual glucocerebrosidase activity in fibroblasts from Gaucher patients, FEBS J. 273 (2006) 4082–4092.
[45] J. Matsuda, O. Suzuki, A. Oshima, Y. Yamamoto, A. Noguchi, K. Takimoto, M. Itoh, Y. Matsuzaki, Y. Yasuda, S. Ogawa, Y. Sakata, E. Nanba, K. Higaki, Y. Ogawa, L. Tominaga, K. Ohno, H. Iwasaki, H. Watanabe, R.O. Brady, Y. Suzuki, Chemical chaperone therapy for brain pathology in G(M1)-gangliosidosis, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 15912–15917.
[46] M.B. Tropak, S.P. Reid, M. Guiral, S.G. Withers, D. Mahuran, Pharmacological enhancement of beta-hexosaminidase activity in fibroblasts from adult Tay-Sachs and Sandhoff patients, J. Biol. Chem. 279 (2004) 13478–13487.
[47] G. Bonapace, A. Waheed, G.N. Shah, W.S. Sly, Chemical chaperones protect from effects of apoptosis-inducing mutation in carbonic anhydrase IV identified in retinitis pigmentosa 17, Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 12300–12305.
[48] T. Okumiya, M.A. Kroos, L.V. Vliet, H. Takeuchi, A.T. Van der Ploeg, A.J. Reuser, Chemical chaperones improve transport and enhance stability of mutant α-glucosidases in glycogen storage disease type II, Mol. Genet. Metab. 90 (2007) 49–57.
[49] H. Niwa, K. Yamamura, J. Miyazaki, Efficient selection for high-expression transfectants with a novel eukaryotic vector, Gene 108 (1991) 193–200.