Stable expression of protective protein/cathepsin A–green fluorescent protein fusion genes in a fibroblastic cell line from a galactosialidosis patient

Model system for revealing the intracellular transport of normal and mutated lysosomal enzymes

Yasunori NAGANAWA*†, Kohji ITOH*, Michie SHIMMOTO*, Sachiko KAMEI†, Kyoko TAKIGUCHI‡, Hirofumi DOI‡ and Hitoshi SAKURABA*¹

*Department of Clinical Genetics, The Tokyo Metropolitan Institute of Medical Science, Honkomagome 3-18-22, Bunkyo-ku, Tokyo 113-8613, Japan, †School of Allied Health Sciences, Faculty of Medicine, Tokyo Medical and Dental University, Yushima 1-5-45, Bunkyo-ku, Tokyo 113-0034, Japan, and ‡Institute for Social Information Science, Fujitsu Laboratories Ltd., Nakase 1-9-3, Mihama-ku, Chiba 261-0023, Japan

Fibroblastic cell lines derived from a galactosialidosis patient, stably expressing the chimaeric green fluorescent protein variant (EGFP) gene fused to the wild-type and mutant human lysosomal protective protein/cathepsin A (PPCA) cDNA, were first established as a model system for revealing the sorting and processing of lysosomal enzymes and for investigating the molecular bases of their deficiencies. In the cell line expressing the wild-type PPCA–EGFP chimaera gene (EGFP–PPwild), an 81 kDa form (27 kDa EGFP fused to the C-terminus of the 54 kDa PPCA precursor) was produced, then processed into the mature 32/20 kDa two-chain form free of the EGFP domain. The intracellular cathepsin A, α -N-acetylneuraminidase and β -galactosidase activities, which are deficient in the parent fibroblastic cells, could also be significantly restored in the cells. In contrast with the uniform and strong fluorescence throughout the cytoplasm and nucleus in the mock-cell line expressing only EGFP cDNA, weak reticular and punctate fluorescence was distributed throughout the EGFP–PPwild cell line. Bafilomycin A₁, a potent inhibitor of vacuolar ATPase and intracellular acidification, induced the distribution of Golgi-like perinuclear fluorescence throughout the living and fixed cells, in which only the 81 kDa product was detected. After removal of the agent, time-dependent transport of the chimaeric protein from the Golgi apparatus to the prelysosomal structure in living cells was monitored with a confocal laser scanning microscope system. Leupeptin caused the distribution of lysosome-like granular fluorescence throughout the cytoplasm in the fixed cells, although it was hardly observed in living cells. The latter agent also dose-dependently induced an increase in the intracellular amount of the

81 kDa product containing the EGFP domain and inhibited the restoration of cathepsin A activity in the EGFP–PPwild cells after the removal of bafilomycin A₁. In parallel, both the mature two-chain form and PPCA function disappeared. These results suggested that the chimaera gene product was transported to acidic compartments (endosomes/lysosomes), where proteolytic processing of the PPCA precursor/zymogen, quenching of the fluorescence, and random degradation of the EGFP portion occurred. A cell line stably expressing a chimaeric gene with a mutant PPCA cDNA containing an A¹¹⁸⁴ → G (Y395C) mutation, commonly detected in Japanese severe early-infantile type of galactosialidosis patients, showed an endoplasmic reticulum (ER)-like reticular fluorescence pattern. The PPCA-immunoreactive gene product was hardly detected in this cell line. The mutant chimaeric product was suggested to be degraded rapidly in the ER before transport to post-ER compartments. A cell line expressing the chimaeric gene with a T⁷⁴⁶ → A (Y249N) PPCA mutation exhibited both ER-like reticular and granular fluorescence on the reticular structure that was stronger than that in the EGFP–PPwild cells. Some of them contained large fluorescent inclusion-body-like structures. The ineffectiveness of transport inhibitors in the distribution changes in the two mutant chimaeric proteins suggested that they were not delivered to acidic compartments. Therefore this expression system can possibly be applied to the direct analysis of the sorting defects of mutant gene products in living cells and will be useful for the molecular investigation of lysosomal diseases, including galactosialidosis.

Key words: bioimaging, lysosomal disease, mutant gene product.

INTRODUCTION

Lysosomal protective protein/cathepsin A (Cath A; EC 3.2.1.14) (PPCA) is a multifunctional glycoprotein; it associates with lyso-

somal α -N-acetylneuraminidase (Neur; EC 3.2.1.18) and lysosomal β -galactosidase (β -Gal; EC 3.2.1.23) to activate the former enzyme and to stabilize the latter enzyme against proteolytic degradation through the formation of a multi-enzymic

Abbreviations used: anti-complex, a rabbit polyclonal antibody against the human placental PPCA/ β -Gal complex; anti-PP20C12, rabbit polyclonal antibody against a synthetic C-terminal peptide (residues 441–452) of the 20 kDa subunit of the human mature PPCA; anti-PP32N12, rabbit polyclonal antibody against a synthetic N-terminal peptide (residues 1–12) of the 32 kDa subunit of the human mature PPCA; Cath A, cathepsin A; DMRIE-C, 1,2-dimyristyloxypropyl-3-dimethyl-hydroxyethylammonium bromide C; EGFP, GFP variant containing F64L and S65T substitutions; EGFP–PPwild, EGFP–PPY249N and EGFP–PPY395C, fibroblastic cell lines stably expressing the EGFP chimaeric gene fused with the wild-type, or mutant human PPCA cDNA with the Y249N or Y395C mutation respectively; ER, endoplasmic reticulum; FCS, fetal calf serum; β -Gal, lysosomal β -galactosidase; GFP, green fluorescent protein; G418, geneticin (a neomycin derivative); Neur, lysosomal α -N-acetylneuraminidase; PPCA, protective protein/cathepsin A; Z-Phe-Leu, N-benzoyloxycarbonyl-L-phenylalanyl-L-leucine.

¹ To whom correspondence should be addressed (e-mail sakuraba@rinshoken.or.jp).

complex (protective function) [1–3]; it also independently exhibits serine esterase (acid carboxypeptidase and neutral esterase/deamidase) activities, which modify *in vitro* the C-termini of a subset of neuropeptides, including tachykinins and endothelin 1 [4–6].

The enzyme is synthesized as a 54 kDa glycosylated precursor/zymogen [2]. The precursor undergoes dimerization at neutral pH and is then transported to an acidic subcellular compartment via the binding and dissociation of a mannose 6-phosphate receptor [2,7]. The zymogen is processed proteolytically into a catalytically active 32/20 kDa two-chain mature form during transport to lysosomes [2,5,7]. However, the physiological events during its biosynthesis and transport to lysosomes have not been fully elucidated.

Galactosialidosis is a human PPCA deficiency inherited as an autosomal recessive trait, associated with simultaneous decreases in two glycosidases, leading to the consequent storage of sialylated oligosaccharides in tissues and urine [1,8,9], as well as in serine esterase activity [10–12]. This disease shows quite heterogeneous clinical phenotypes in age of onset and severity [8,9]. Most patients develop loss of vision as an initial symptom at age 10–15 years, followed by neurological abnormalities such as action myoclonus, cerebellar ataxia, skeletal dysplasia, cherry-red spots, and angiokeratoma (juvenile/adult form). Patients with the most severe manifestations, occurring in early infancy, have fetal hydrops, oedema, marked ascites, skeletal dysplasia, cherry-red spots, renal dysfunction, visceromegaly and coarse facies (early infantile form). A small group of patients have a milder late infantile form, in which the symptoms develop at 12–24 months of age and then gradually progress with visceromegaly, dysostosis multiplex, heart involvement and no or only minor neurological abnormalities.

The PPCA gene mutations have been identified in galactosialidosis patients with various clinical phenotypes [13–15]. Most of the amino acid substitutions identified so far are characterized by their expression levels and functional defects. Clinical severity has also been demonstrated to depend on the combination of two mutant alleles encoding different PPCA variants [14,15]. Y395C and Y249N are known as common mutations causing the severe early infantile form and the mild late infantile form of galactosialidosis respectively. An expression study revealed that the Y395C mutant with no residual activities cannot be transported to lysosomes, whereas the Y249N mutant product with residual PPCA functions is partly transported [14,15].

In recent years, a green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* has been used as a reporter molecule for directly monitoring and revealing the patterns of protein localization, gene expression, the distribution and dynamics of intracellular organelles and the sorting of proteins *in vivo*, *in situ*, and in real time [16–18]. GFP variants with modified spectral properties and resistance to photobleaching have also been developed [19,20].

In the present study we attempted to establish a new expression system for a human PPCA–GFP chimaera gene to reveal the protein sorting in living cells and to clarify the details of the mechanism underlying the processing defect in galactosialidosis with the Y395C and Y249N mutations. This system could be applicable to the analysis of the sorting defects of other PPCA mutants.

MATERIALS AND METHODS

Materials

Geneticin (G418), a neomycin derivative, and 1,2-dimyristyl-oxypropyl-3-dimethyl-hydroxyethylammonium bromide C

(DMRIE-C) reagent for transfection were purchased from Gibco/BRL (Grand Island, NY, U.S.A.). Leupeptin was from the Peptide Institute (Osaka, Japan). Bafilomycin A₁ and *N*-benzyloxycarbonyl-L-phenylalanyl-L-leucine (Z-Phe-Leu) were from Sigma (St. Louis, MO, U.S.A.). 1,4-Diazabicyclo[2,2,2]octane was from Wako Pure Chemical Industries (Osaka, Japan). The restriction enzymes were from New England Biolabs (Beverly, MA, U.S.A.).

Antibodies

A polyclonal antibody against the high-molecular-mass PPCA/ β -Gal complex from human placenta (anti-complex) [21,22] was used to detect both the mature and precursor forms of PPCA and β -Gal. Two antibodies against a synthetic N-terminal peptide (residues 1–12) of the 32 kDa subunit (anti-PP32N12) and a C-terminal peptide (residues 441–452) of the 20 kDa subunit (anti-PP20C12) respectively of PPCA were used [23]. The antibodies recognized both the mature and precursor forms of the protein on immunoblotting. An IgG fraction was prepared from each antiserum, with a Protein A–Cellulofine column (Seikagaku Kogyo, Tokyo, Japan) [24]. An anti-GFP polyclonal antibody was purchased from Clontech (Palo Alto, CA, U.S.A.).

Construction of an expression vector for the PPCA–EGFP chimaeric protein

The pEGFP-N1 vector (EGFP is a GFP variant containing F64L and S65T mutations) was purchased from Clontech. Human PPCA cDNA [14] was used as a template for PCR with the following primers to create a *XhoI*–*EcoRI* restriction site and to eliminate the stop codon for PPCA: sense, 5'-TCTCTCGA-GAGGACGCGGGGAGCAGAGAT-3'; anti-sense, 5'-CAA-GAATTCGGTATGGCTGCTTGTTTCAGGA-3'. The amplified cDNA fragment was subcloned into the *XhoI*–*EcoRI* site of pEGFP-N1, generating pEGFP(PPwild). The mutant PPCA cDNA species with the Y249N and Y395C mutations respectively, each subcloned into the pCAGGS plasmid [14], were excised by digestion with *BstXI* and *XmnI*. The corresponding *BstXI*–*XmnI* regions in pEGFP(PPwild) were replaced by the PPCA mutants at the cognate sites. The resultant plasmids were designated pEGFP(PPY395C) and pEGFP(PPY249N) respectively.

Cell culture and transfection

A fibroblastic cell line derived from a galactosialidosis patient [14] was maintained in Ham's F-10 medium supplemented with 10% (v/v) fetal calf serum (FCS) and antibiotics.

Cells [(1–2) × 10⁵] were seeded on 60 mm dishes on the day before transfection. The transfection was conducted with DMRIE-C. A mixture comprising the plasmid DNA (5–10 μ g) and DMRIE-C (10 μ l) was added to cells cultured in FCS-free Ham's F-10 medium, followed by incubation for 5 h. After the incubation, the culture medium was replaced with Ham's F-10 containing 10% (v/v) FCS. After 96 h, the cultured medium was replaced with a selection medium [Ham's F-10 supplemented with 10% (v/v) FCS and 800 μ g/ml G418]. After 1 month, G418-resistant and fluorescence-positive colonies were isolated and routinely maintained in the selection medium.

In another experiment, the transformed cell lines were cultured in Ham's F-10 supplemented with 10% (v/v) FCS in the presence of 1 mM leupeptin for 4–6 days or 10 nM bafilomycin A₁ for 2 days.

Enzyme assays

Confluent cells were washed with PBS and harvested by scraping, then suspended in distilled water containing 0.1 mM leupeptin. A suspension was prepared by sonication and then centrifuged at 10000 *g* for 15 min at 4 °C. The resultant supernatant was designated the cell extract. The lysosomal enzyme activities, including those of Neur, β -Gal and β -hexosaminidase, were assayed fluorometrically with 4-methylumbelliferyl glycosides as substrates [25]. The Neur assay was performed immediately after the preparation of the cell suspension by pipetting at 4 °C. Acid carboxypeptidase (Cath A) activity was measured at pH 5.6, with Z-Phe-Leu as a substrate [26]. Protein determination was performed with a D_c assay kit (Bio-Rad, Richmond, CA, U.S.A.), with BSA as a standard.

Fluorescent microscopic analysis

For analysis of the fluorescence localization of PPCA–EGFP fusion proteins, the isolated cell lines were grown on eight-well plastic chamber slides (Lab-Tek; Nunc, Naperville, IL, U.S.A.). After 16 h, the cells were fixed with ice-cold 4% (w/v) para-formaldehyde/PBS for 1 h on ice and washed with PBS, then mounted with 55% (v/v) glycerol/PBS containing 3.8% (w/v) 1,4-diazabicyclo-[2,2,2]octane. The fixed cells were examined with a confocal fluorescence microscope system (MRC-600; Bio-Rad, Hemel Hempstead, Herts., U.K.) attached to a Nikon microscope, Optiphot 2 (Nikon, Tokyo, Japan).

For time-lapse studies, the living cell lines were placed in glass-bottomed culture dishes (P35GC-0-10-C-gm; MatTek Corp., Ashland, MA, U.S.A.), then cultured in medium containing 10% (v/v) FCS and 10 nM bafilomycin A₁ for 2–4 days. After washing of the cell surface with PBS three times, the culture medium was replaced with fresh medium containing 1 mM leupeptin but no bafilomycin A₁. The time-dependent change in the intracellular fluorescence distribution in the living cells at room temperature was examined with a confocal fluorescence microscope system (Fluoview; Olympus, Tokyo, Japan) with the use of an argon laser and filters for fluorescein (excitation 488 nm, emission 506 nm). Serial 0.6–0.9 μ m optical sections were acquired every 4 s at 30 min intervals for 5 h. Nomarski images were also obtained at the same intervals. Three-dimensional image reconstruction was performed with the software attached to the confocal microscope system.

SDS/PAGE and immunoblotting

Aliquots of cell extracts were subjected to SDS/PAGE by the method of Laemmli [27] on a 10–20% (w/v) gradient gel (Resep Gel SPA-191; Wakamori, Tokyo, Japan). Proteins were subjected to SDS/PAGE after reduction with 25 mM 2-mercaptoethanol, then detected by immunoblotting with anti-GFP, anti-complex, and anti-PP32N12 and PP20C12 as described previously [21–23,28], with the use of a Super signal ultra chemiluminescence kit (Pierce, Rockford, IL, U.S.A.). Prestained SDS/PAGE standards (Bio-Rad, Hercules, CA, U.S.A.) were used as molecular mass standard proteins: phosphorylase *b* (110 kDa), BSA (84 kDa), ovalbumin (50 kDa), carbonic anhydrase (33 kDa), soybean trypsin inhibitor (24 kDa) and lysozyme (16 kDa).

RESULTS

Expression of PPCA–EGFP chimaeric genes in galactosialidosis cells

Figure 1 shows schematic diagrams of the expression vector plasmids and the structures of the chimaeric genes. We used a

27 kDa EGFP as a reporter protein, i.e. a GFP variant with two mutations [Phe → Leu at residue 64 (F64L) and Ser → Thr at residue 65 (S65T)] [20], which makes the chromophore 35-fold more fluorescent. We generated PPCA–EGFP chimaera constructs by fusing the EGFP cDNA in frame to the last amino acid (Tyr, residue 452) of human PPCA via a 17-residue sequence insertion (Figure 1A). EGFP cDNA was also fused to mutant PPCA cDNA species that contained a Tyr → Cys substitution at position 395 (Y395C mutation) and a Tyr → Asn substitution at position 249 (Y249N mutation) [14] (Figure 1B).

Immunoblotting with a mixture of anti-PPCA antibodies revealed that the major specific bands corresponding to molecular masses of 81, 63, 32 and 20 kDa were observed for the fibroblastic cell line stably expressing the EGFP chimaeric gene fused with wild-type human PPCA cDNA (EGFP-PPwild cells) (Figure 2A, lane 2), although non-specific minor bands were also detected. The 32 and 20 kDa bands migrated to the same positions as observed for the cells expressing wild-type PPCA cDNA alone (Figure 2A, lane 5). However, the predicted 47 kDa band (the 27 kDa EGFP fused to the C-terminus of the 20 kDa PPCA subunit) was not detected. The 81 kDa protein was immunoreactive with anti-GFP (Figure 2B, lane 2). These results indicated that the chimaeric cDNA was transcribed to produce a 81 kDa precursor protein comprising the 27 kDa EGFP domain fused to the C-terminus of the 54 kDa PPCA precursor, and that it was then processed, probably via a 63 kDa intermediate form, into the 32/20 kDa two-chain form free of the EGFP domain. In contrast, few immunoreactive bands were detected for the fibroblastic cell line stably expressing the EGFP chimaeric gene fused with mutant human PPCA cDNA with the Y395C mutation (EGFP-PPY395C) (Figures 2A and 2B, lanes 4). For the fibroblastic cell line stably expressing the EGFP chimaeric gene fused with mutant human PPCA cDNA with the Y249N mutation (EGFP-PPY249N), a remarkable increase in the amount of the 81 kDa form was observed (Figures 2A and 2B, lanes 3). The 63 kDa band was also detected, but the mature 32/20 kDa form was hardly observed for this cell line. The mock cells, expressing EGFP cDNA alone, gave no immunoreactive bands with anti-PPCA (Figure 2A, lane 1).

We also measured several lysosomal enzyme activities in the isolated cell lines. Table 1 summarizes the results. In the parental or mock cells, the intracellular Cath A, Neur and β -Gal activities were deficient, as reported previously [14]. However, these enzyme activities were partly restored in the EGFP-PPwild cell line. The increase in Neur activity was more remarkable than those of Cath A and β -Gal. Lysosomal β -hexosaminidase activity, as a reference, did not differ between the EGFP-PPCA and mock cell lines. These results indicated that the expression of the chimaeric wild-type PPCA gene partly restored the normal function of PPCA in the parental cells from the galactosialidosis patient. In contrast, neither the EGFP-PPY395C cell line nor the EGFP-PPY249N cell line exhibited intracellular Cath A, Neur and β -Gal activities.

Intracellular fluorescence of PPCA–EGFP chimaeric proteins in galactosialidosis cells

As shown in Figure 3(A), there was uniform and strong fluorescence throughout the cytoplasm and nucleus in the mock cell line. In contrast, weak reticular fluorescence in the perinuclear region was observed in EGFP-PPwild cells (Figure 3B). Some punctate fluorescence was also detected in the perinuclear region (Figure 3B). EGFP-PPY395C cells exhibited endoplasmic reticulum (ER)-like reticular fluorescence (Figure 3D). In EGFP-PPY249N cells, stronger ER-like reticular fluorescence was

Figure 1 Construction of expression plasmids and structures of EGFP fusion proteins

(A) Schematic structure of the chimaeric gene expression vector encoding the full-length EGFP fused to the 3' end of wild-type PPCA for expression in mammalian cells. The chimaeric gene is transcribed from the human cytomegalovirus (CMV) immediate early promoter, followed by a simian virus 40 (SV40) small t intron and poly(A)⁺ signal. The stop codon for PPCA was eliminated by creating a *XhoI*–*EcoRI* restriction site, resulting in the insertion of 17 linker amino acid residues between PPCA and EGFP. **(B)** Expression plasmids for mutant PPCAs with the Y249N and Y395C mutations were generated by replacing the *Bst*XI–*Xmn*I restriction fragments of wild-type PPCA by those with the mutations.

Figure 2 Immunoblotting of chimaeric proteins expressed in stably transformed galactosialidosis cell lines

Cell extracts were prepared from the isolated cell lines as described in the Materials and methods section: mock expressing EGFPcDNA alone (lane 1), EGFP-PPwild (lane 2), EGFP-PPY249N (lane 3), EGFP-PPY395C (lane 4) or positive control for PPCA expressing wild-type PPCA cDNA alone (lane 5). The cell extracts were subjected to SDS/PAGE under reducing conditions, and then immunoblotting with anti-complex, anti-PP32N12 and anti-PP20C12 **(A)**, or with anti-GFP **(B)**. Immunoreactive proteins were detected by chemiluminescence as described in the Materials and methods section. The molecular masses of bands are indicated.

Table 1 Lysosomal enzyme activities in isolated galactosialidosis fibroblastic cell lines expressing the wild-type and mutant PPCA–EGFP chimaeric genes

Cells were cultured to subconfluence and harvested; lysosomal enzyme activities were then measured as described in the Materials and methods section. The substrates were 4-methylumbelliferyl derivatives for glycosidases, and Z-Phe-Leu for Cath A.

Plasmid	Mutation	Cath A ($\mu\text{mol/h}$ per mg of protein)	Neur (nmol/h per mg of protein)	β -Gal (nmol/h per mg of protein)	β -Hexosaminidase ($\mu\text{mol/h}$ per mg of protein)
None*	—	0.05	1.0	11	3.28
Mock*	Mock	0.08	0.9	9	3.82
EGFP-PPwild†	Normal	1.44 ± 1.12	32.7 ± 39.1	86 ± 53	4.38 ± 2.12
EGFP-PPY249N†	Y249N	0.03 ± 0.03	0.9 ± 1.0	17 ± 4	4.39 ± 1.56
EGFP-PPY395C†	Y395C	0.02 ± 0.02	1.0 ± 0.2	14 ± 7	4.44 ± 0.75
PPwild*	Normal	2.29	36.0	66	3.53
Normal fibroblast‡	—	4.07 ± 1.57	36.0 ± 9.5	479 ± 30	4.80 ± 1.77

* Values are means for two independent measurements.

† Values are means $\pm$ S.D. for three independent measurements.

‡ Normal levels of the enzymes in normal human fibroblasts ($n = 7$).

Figure 3 Fluorescence distribution in the transformed galactosialidosis cell lines expressing wild-type and mutant PPCA–EGFP chimaeric genes

Each transformed cell line, mock (A), EGFP-PPwild (B), EGFP-PPY249N (C) and EGFP-PPY395C (D), was seeded at subconfluence and, 24 h later, fixed, washed with PBS and mounted. The fixed cells were examined with a confocal laser scanning fluorescence microscope system (MRC-600; Bio-Rad) attached to an Optiphot 2 (Nikon). Scale bar, 25 μm .

observed than in EGFP-PPwild cells (Figure 3C). Furthermore, a larger inclusion-body-like fluorescence of the reticular structure was often observed in these cells (Figure 3C).

Effects of bafilomycin A₁ and leupeptin on the intracellular localization and processing of PPCA–EGFP chimaeric proteins

To examine the intracellular transport and processing of the wild-type and mutant PPCA–EGFP chimaeric proteins, we analysed the effects of bafilomycin A₁ and leupeptin. Bafilomycin

A₁, a potent inhibitor of vacuolar ATPase, prevents the transport of lysosomal enzymes from the Golgi apparatus to acidic compartments *in vivo* by inhibiting intracellular acidification [29,30]. Figures 4 and 5 show the results of immunoblotting and the intracellular fluorescence distribution respectively. Treatment of the EGFP-PPwild cells with 10 nM bafilomycin A₁ for 4 days caused a marked decrease in Cath A activity in the cells (Figure 4B, column 2), associated with the disappearance of the intracellular mature two-chain form of PPCA (Figure 4A, lane 2), although the reactivity of the anti-PP32N12 antibodies used here

Figure 4 Effects of bafilomycin A_1 and leupeptin on expression of the wild-type PPCA-EGFP chimaeric protein

EGFP-PPwild cells (10^6) were cultured in the absence (lane 1 and column 1) or presence of 10 nM bafilomycin A_1 for 4 days (lane 2 and column 2); the cultured medium was then replaced with fresh medium, followed by further incubation for 4 days in the absence (lane 3 and column 3) or presence of 0.1 mM leupeptin (lane 4 and column 4) or 1 mM leupeptin (lane 5 and column 5). Cell extracts were prepared after treatment with the agents, and then aliquots of the extracts were analysed by immunoblotting with anti-PPCA (**A**) and by measuring Cath A activity (**B**) as described in the Materials and methods section.

Figure 5 Effects of bafilomycin A_1 and leupeptin on the distribution of the wild-type and mutant PPCA-EGFP chimaeric proteins

The EGFP-PPwild (**A**), EGFP-PPY249N (**B**) and EGFP-PPY395C (**C**) cell lines were cultured in the presence of 10 nM bafilomycin A_1 for 4 days. After washing with PBS, the cultured medium was replaced with fresh medium containing 1 mM leupeptin, followed by further incubation for 4 days (**D**, **E** and **F** respectively). After treatment with the agents, the cells were fixed and examined with a confocal laser scanning fluorescence microscope system (MRC-600; Bio-Rad). Scale bar, 25 μ m.

Figure 6 Time-dependent translocation of the wild-type PPCA–EGFP chimaeric protein

EGFP-PPwild cells were cultured on a glass-bottomed dish in the presence of 10 nM bafilomycin A₁ for 2 days before microscopy. After washing with PBS, the culture medium was replaced with fresh medium containing 1 mM leupeptin without bafilomycin A₁; intracellular fluorescent and Nomarski images were then acquired for 4 s at 30 min intervals at room temperature. Fluorescence images at 30 min (A), 1 h (B), 1.5 h (C) and 2 h (D) are presented. Magnification $\times 1800$.

was very weak and the 32 kDa subunit could not be detected under these experimental conditions. Correspondingly, perinuclear fluorescence, characteristic of the Golgi apparatus, was evident in bafilomycin A₁-treated cells (Figure 5A). The effect of bafilomycin A₁ was reversible: after removal of the agent from the culture medium, the intracellular content of the mature PPCA as well as Cath A activity increased time-dependently in the absence of leupeptin (Figure 4A, lane 3; Figure 4B, column 3), associated with restoration of the same fluorescence pattern as that observed in Figure 3(B). These results indicated that the chimaeric gene products accumulated in the Golgi apparatus without being transported to acidic compartments in the presence of bafilomycin A₁. In contrast, the agent had no effect on the distribution of the mutant chimaeric products in the EGFP-PPY249N and EGFP-PPY395C cell lines (Figures 5B and 5C).

Because the EGFP domain of the chimaeric protein (81 kDa) was degraded in the EGFP-PPwild cells (Figure 2A, lane 2), we further examined the effect of leupeptin, a cysteine protease inhibitor. EGFP-PPwild cells pretreated with bafilomycin A₁ were also cultured in the presence of leupeptin after removal of the former agent. Leupeptin inhibited the restoration of the Cath A activity dose-dependently (Figure 4B, columns 4 and 5). Concomitantly, an increase in the amount of the 81 kDa protein containing the EGFP domain was observed, as well as a decrease in that of the two-chain form with a slight elevation of their molecular masses due to probable modification at their terminal sites by leupeptin-sensitive proteases (Figure 4A, lanes 4 and 5). Fine granular fluorescence throughout the cytoplasmic region, characteristic of lysosomes, was also observed in the fixed leupeptin-treated cells (Figure 5D). In contrast, the fluorescence patterns of the EGFP-PPY395C and EGFP-PPY249N cell lines were not influenced by the presence of leupeptin, although the intensity increased slightly (Figures 5E and 5F).

We also analysed the time-dependent changes in the fluorescence localization in the living cell lines with the combined use of bafilomycin A₁ and leupeptin. The perinuclear Golgi-like fluorescence pattern was confirmed in the EGFP-PPwild cells pretreated with bafilomycin A₁ (Figure 6A). After removal of the agent from the culture medium, the time-dependent shift of the intracellular fluorescence from the perinuclear Golgi apparatus to the cytoplasmic prelysosomal structure could be monitored over a few hours (Figures 6A to 6D). However, further incubation did not cause a fine granular lysosomal distribution even in the presence of leupeptin, which was observed

in the fixed leupeptin-treated cells (Figure 5D). Changes in the fluorescence distribution in the EGFP-PPY395C and EGFP-PPY249N cell lines were not observed under the same conditions (results not shown).

DISCUSSION

The GFP of *A. victoria* is a unique tool for monitoring gene expression, protein localization and intracellular transport, especially in living cells, including eukaryotic cells [16–18]. The advantages are that its simple recombinant expression yields a strong fluorescence signal without the need for additional factors and that GFP can be fused to resident proteins with no alteration in its fluorescence characteristics. Some chimaeric proteins including 27 kDa GFP also retain their intrinsic functions, and even the capacity for complex formation or interaction with other native proteins *in vivo* [18]. The development of GFP variants with modified spectral properties (such as EGFP and blue fluorescent protein) has promoted numerous applications in cell biology [19,20].

In the present study we tried to establish an expression system for a lysosomal enzyme (PPCA)–EGFP gene chimaera. As a result, a system that is applicable to the analysis of the intracellular distribution and transport of lysosomal enzyme gene products was found for the first time, although there was the limitation that EGFP is unstable and thus degraded in an acidic compartment, probably in lysosomes. Expression of the wild-type PPCA–EGFP chimaeric protein restored a significant fraction of the deficient enzyme activities and the mature form of PPCA in the fibroblastic cells derived from a patient with PPCA deficiency. These results indicated that fusion of EGFP to wild-type PPCA did not prevent the expression of normal protective and catalytic functions, or the routing to lysosomes of the latter enzyme. In addition, we succeeded in monitoring the time-dependent transport of the chimaeric gene product by the combined use of some inhibitors that influence intracellular protein transport or degradation. Bafilomycin A₁ inhibited both the transport of the chimaeric product from the Golgi apparatus to an acidic compartment and the appearance of the mature form of PPCA. The addition of leupeptin to the EGFP-PPwild cells inhibited the non-specific degradation of the EGFP domain of the chimaeric product and made it visible as lysosomal granular fluorescence in the fixed cells. It also prevented in a dose-dependent manner the conversion of the newly synthesized

PPCA precursor form into the mature two-chain form. These results suggest that the proteolytic processing of the wild-type PPCA precursor occurs in an acidic compartment and that some leupeptin-sensitive proteases are involved in the processing and activation of the PPCA precursor form.

We further analysed the intracellular distribution of PPCA mutants by using this system and demonstrated that the transport of chimaeric proteins was defective in different ways, depending on the kind of mutation. A previous transfection experiment revealed that the Y395C mutant gene is transcribed normally but that the translated precursor protein is degraded before transport to lysosomes [14]. Recently we performed immunochemical analyses, with anti-PP32N12 and anti-PP20C12, of cultured cells derived from early-infantile patients homozygous for the Y395C mutation. The mutant PPCA product was hardly detected in the cultured cells [28]. In the present study, the EGFP-PPY395C chimaeric gene was transcribed but the gene product was hardly detected on immunoblotting. ER-like reticular fluorescence due to the gene product was observed in the EGFP-PPY395C cell line. Bafilomycin A₁ also had no effect on the distribution of the chimaeric gene product. This suggests that the chimaeric product was translated and was then rapidly degraded before it reached the Golgi apparatus, presumably through the 'quality control' in the ER system.

We also demonstrated that transfection of Y249N cDNA into the galactosialidosis cell line partly restored the Cath A, Neur and β -Gal activities [14]. Pulse-chase analysis also revealed that a small amount of the expressed protein was mannose-6-phosphorylated, routed to lysosomes and then processed proteolytically into the mature two-chain form. However, the rest existed as the precursor form. Zhou et al. [15] also demonstrated that small amounts of residual Cath A activity and the mature two-chain PPCA product were observed in fibroblasts from late-infantile patients with the Y249N mutation. Our present results showed that most of the EGFP-PPY249N chimaeric product exhibited a non-lysosomal distribution, including in the ER and Golgi apparatus. We could barely detect residual Cath A activity or the two-chain form of PPCA with the present expression system because of the lower expression of the cDNA (one-tenth of that in the previous study) [14]. Therefore the intracellular fluorescence distribution is considered to be due to the mutant precursor protein in this assay system.

The three-dimensional structure of the PPCA dimer has been determined [31] and the effects of PPCA gene mutations on the structure have been simulated [32]. Most of the amino acid substitutions, including the Y395C mutation, identified in patients with severer clinical forms was suggested to alter the protein folding and stability of the PPCA variants drastically, leading to disruption of the protective and catalytic functions of PPCA. In contrast, a few, including the Y249N substitution, which were identified in more moderate clinical cases, were also suggested to have a milder effect on the PPCA structure, leaving some residual functions. This method is applicable to the study of post-translational processing of lysosomal enzymes. In addition, it would be interesting and useful to determine the correlation of natural and artificial amino acid substitutions in the structure with the transport of the mutant gene products present in living cells by using the recombinant GFP expression system.

This research was supported by grants from the Ministry of Education, Science and Culture of Japan, and the Suzuken Memorial Foundation.

REFERENCES

- d'Azzo, A., Hoogeveen, A. T., Reuser, A. J. J., Robinson, D. and Galjaard, H. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 4535–4539
- Galjart, N. J., Gillemans, N., Harris, A., van der Horst, G. T., Verheijen, F. W., Galjaard, H. and d'Azzo, A. (1988) *Cell* **54**, 755–764
- Bonten, E. J., van der Spoel, A., Fornerod, M., Grosveld, G. and d'Azzo, A. (1996) *Gene Dev.* **10**, 3156–3169
- Jackman, H. L., Tan, F., Tamei, H., Beurling-Harbury, C., Li, X.-Y., Skidgel, R. A. and Erdős, E. G. (1990) *J. Biol. Chem.* **265**, 11265–11272
- Galjart, N. J., Morreau, H., Willemsen, R., Bonten, E. J. and d'Azzo, A. (1991) *J. Biol. Chem.* **266**, 14754–14762
- Itoh, K., Takiyama, N., Kase, R., Kondoh, K., Sano, A., Oshima, A., Sakuraba, H. and Suzuki, Y. (1993) *J. Biol. Chem.* **268**, 1180–1186
- Bonten, E. J., Galjart, N. J., Willemsen, R., Usmany, M., Vlak, J. M. and d'Azzo, A. (1995) *J. Biol. Chem.* **270**, 26442–26445
- d'Azzo, A., Andria, G., Strisciuglio, P. and Galjaard, H. (1995) In *The Metabolic and Molecular Bases of Inherited Disease*, 7th edn. (Scriver, C. R., Beaudet, A. L., Sly, W. S. and Valle, D., eds.), pp. 2825–2837. McGraw-Hill, New York
- Suzuki, Y., Sakuraba, H., Yamanaka, T., Ko, Y.-M., Iimori, Y., Okamura, Y. and Hoogeveen, A. T. (1985) In *The Developing Brain and Its Disorders* (Arima, M., Suzuki, Y. and Yabuuchi, H., eds.), pp. 161–175. Karger, Basel
- Tranchemontagne, J., Michaud, L. and Potier, M. (1990) *Biochem. Biophys. Res. Commun.* **168**, 22–29
- Kase, R., Itoh, K., Takiyama, N., Oshima, A., Sakuraba, H. and Suzuki, Y. (1990) *Biochem. Biophys. Res. Commun.* **172**, 1175–1179
- Itoh, K., Kase, R., Shimamoto, M., Satake, A., Sakuraba, H. and Suzuki, Y. (1995) *J. Biol. Chem.* **270**, 515–518
- Shimamoto, M., Takano, T., Fukuhara, Y., Oshima, A., Sakuraba, H. and Suzuki, Y. (1990) *Proc. Jpn. Acad.* **66B**, 217–222
- Shimamoto, M., Fukuhara, Y., Itoh, K., Oshima, A., Sakuraba, H. and Suzuki, Y. (1993) *J. Clin. Invest.* **91**, 2393–2398
- Zhou, X.-Y., van der Spoel, A., Rottier, R., Hale, G., Willemsen, R., Berry, G. T., Strisciuglio, P., Andria, G. and d'Azzo, A. (1996) *Hum. Mol. Genet.* **5**, 1977–1987
- Chalfie, M., Tu, Y., Euskirchen, G., Ward, W. W. and Prasher, D. C. (1994) *Science* **263**, 802–805
- Ogawa, H., Inoue, S., Tsuji, F. I., Yasuda, K. and Umezono, K. (1995) *Proc. Natl. Acad. Sci. U.S.A.* **92**, 11899–11903
- Marshall, J., Molloy, R., Moss, G. W. J., Howe, J. R. and Hughes, T. E. (1995) *Neuron* **14**, 211–215
- Rizzuto, R., Brini, M., De Giorgi, F., Rossi, R., Heim, R., Tsien, R. Y. and Pozzan, T. (1996) *Curr. Biol.* **6**, 183–188
- Cormack, B. P., Valdivia, R. and Falkow, S. (1996) *Gene* **173**, 33–38
- Nanba, E., Tsuji, A., Omura, K. and Suzuki, Y. (1987) *Biochem. Biophys. Res. Commun.* **144**, 138–142
- Takiyama, N., Itoh, K., Shimamoto, M., Nishimoto, J., Inui, K., Sakuraba, H. and Suzuki, Y. (1997) *Brain Dev.* **19**, 126–130
- Satake, A., Itoh, K., Shimamoto, M., Saido, T. C., Sakuraba, H. and Suzuki, Y. (1994) *Biochem. Biophys. Res. Commun.* **205**, 38–43
- Ey, P. L., Prowse, S. L. and Jenkin, C. R. (1987) *Immunochemistry* **15**, 429–436
- Sakuraba, H., Aoyagi, T. and Suzuki, Y. (1982) *Clin. Chim. Acta* **125**, 275–283
- Itoh, K., Takiyama, N., Nagao, Y., Oshima, A., Sakuraba, H., Potier, M. and Suzuki, Y. (1991) *Jpn. J. Hum. Genet.* **36**, 169–175
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Itoh, K., Miha, N., Ohama, K., Mizoguchi, N., Sakura, N. and Sakuraba, H. (1997) *Clin. Chim. Acta* **266**, 75–82
- Yoshimori, T., Yamamoto, A., Moriyama, Y., Futai, M. and Tashiro, Y. (1991) *J. Biol. Chem.* **266**, 17707–17712
- Bowman, E. M., Siebers, A. and Altendorf, K. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 7972–7976
- Rudenko, G., Bonten, E., d'Azzo, A. and Hol, W. G. J. (1995) *Structure* **3**, 1249–1259
- Rudenko, G., Bonten, E., Hol, W. G. J. and d'Azzo, A. (1998) *Proc. Natl. Acad. Sci. U.S.A.* **95**, 621–625