A mutation in a mild form of galactosialidosis impairs dimerization of the protective protein and renders it unstable

Xiao Yan Zhou, Niels J. Galjart, Rob Willemsen, Nynke Gillemans, Hans Galjaard and Alessandra d'Azzo¹

Department of Cell Biology and Genetics, Erasmus University, PO Box 1738, 3000 DR Rotterdam, The Netherlands

¹To whom correspondence should be addressed

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The lysosomal disorder galactosialidosis is caused by deficiency of the protective protein in the absence of which the activities of the enzymes β -galactosidase and neuraminidase are reduced. Aside from its protective function towards the two glycosidases, this protein has cathepsin A-like activity. A point mutation in the protective protein gene, resulting in the substitution of Phe412 with Val in the gene product, was identified in two unrelated patients with the late infantile form of the disease. Expression in COS-1 cells of a protective protein cDNA with the base substitution resulted in the synthesis of a mutant protein that lacks cathepsin A-like activity. The newly made mutant precursor was shown to be partially retained in the endoplasmic reticulum. Only a fraction is transported to the lysosomes where it is degraded soon after proteolytic processing into the mature two-chain form. Since the mutant precursor, contrary to the wild type protein, does not form homodimers, the dimerization process might be a condition for the proper targeting and stable conformation of the protective protein. These results clarify the mechanism underlying the combined deficiency in these patients, and give new insight into the structure–function relationship of the wild type protein.

Key words: cathepsin A/dimerization/galactosialidosis/lysosomal protective protein/point mutation

Introduction

Galactosialidosis is an inherited metabolic storage disorder transmitted as an autosomal recessive trait (O'Brien, 1989). It is caused by mutation of the gene encoding the lysosomal protective protein (d'Azzo *et al.*, 1982) which normally associates with the enzymes β -D-galactosidase (EC 3.2.1.23) and N-acetyl- α -neuraminidase (sialidase, EC 3.2.1.18) and regulates their intralysosomal activity and stability (Verheijen *et al.*, 1982; Hoozeveen *et al.*, 1983; van der Horst *et al.*, 1989). Deficient or non-functional protective protein causes impaired activities of the two glycosidases and consequent storage of predominantly sialylated oligosaccharides in tissues and urine (Wenger *et al.*, 1978; Okada *et al.*, 1978; van Pelt *et al.*, 1988a,b, 1989).

Although the disorder is rare, several dozens of patients of different ethnic origin have been described (Pinsky *et al.*,

1974; Andria *et al.*, 1981; Loonen *et al.*, 1984; Suzuki *et al.*, 1984; Sewell *et al.*, 1987; Chitayat *et al.*, 1988). They are clinically heterogeneous, having either a very severe early onset form of the disease, mostly fatal at birth, or mild and slowly progressive late onset types. Patients with an early infantile phenotype have severe CNS involvement, macular cherry red spots, visceromegaly, skeletal abnormalities, renal dysfunction and coarse facies. Juvenile/adult forms, mainly of Japanese origin, are characterized by features like skeletal dysplasia, dysmorphism, macular cherry red spots, slowly progressive mental and motor deterioration and angiokeratoma. So far a defined, small group of patients represents the late infantile phenotype. These patients are all alive and have developed symptoms at 12–24 months of age with the main features being visceromegaly, dysostosis multiplex, heart involvement, but no mental retardation (Pinsky *et al.*, 1974; Andria *et al.*, 1978, 1981; Chitayat *et al.*, 1988; Strisciuglio *et al.*, 1990). This broad spectrum of clinical manifestations in distinct galactosialidosis phenotypes can only in part be correlated with differences in the level of expression of protective protein mRNA (Galjart *et al.*, 1988) and the amount and/or property of immunoprecipitated polypeptide (d'Azzo *et al.*, 1982; Palmeri *et al.*, 1986).

The human protective protein is synthesized as a 54 kDa precursor that is cleaved and modified in lysosomes into a mature 32/20 kDa two-chain product (d'Azzo *et al.*, 1982; Galjart *et al.*, 1988). Traces of 32 kDa polypeptide could be immunoprecipitated from fibroblasts of two late infantile galactosialidosis patients, but only when these cells were treated with the protease inhibitor leupeptin (Palmeri *et al.*, 1986; Strisciuglio *et al.*, 1988). These findings led to the supposition that the presence of a minimal amount of mature protective protein in these patients could account for their milder clinical course (Strisciuglio *et al.*, 1988).

The primary structures of human, mouse and chicken protective proteins have been found to be homologous to yeast and plant serine carboxypeptidases (Galjart *et al.*, 1988, 1990, 1991). This observation led to the discovery that the protective protein has cathepsin A-like activity (Galjart *et al.*, 1991). Clinically distinct galactosialidosis patients, reported to have reduced activity of a lysosomal carboxypeptidase called carb L (Tranchemontagne *et al.*, 1990; Kase *et al.*, 1990), are thus deficient in the cathepsin A-like enzyme (Galjart *et al.*, 1991). Although the physiological implications of these findings are as yet unclear, this lysosomal disorder should be viewed as the first one that is also associated with a protease deficiency. Jackman *et al.* (1990) have reported on a deamidase/carboxypeptidase enzyme purified from human platelets that enzymatically resembles cathepsin A and has sequence identity to the N-termini of the protective protein chains. Using protective protein mutants with targeted amino acid substitutions we have established that the protective and catalytic activities of the protein are distinct (Galjart *et al.*,

1991), and the latter can only be exerted after conversion of the inactive precursor (zymogen) into the mature and stable two-chain form (Galjart *et al.*, 1990).

We have identified different mutant alleles associated with the three clinical galactosialidosis phenotypes of mainly European patients. In the present study we focus on the mutation underlying two unrelated patients with the late infantile form of the disease. They both carry the same point mutation in the protective protein gene resulting in the substitution of Phe412 with Val in the gene product. The mutation was shown to impair the formation of protective protein precursor homodimers which in turn might be responsible for the observed partial retention of the mutant precursor polypeptide in the endoplasmic reticulum (ER). The fraction that does reach the lysosomes is catalytically inactive and undergoes rapid intralysosomal degradation.

Results

Point mutation identified in the protective protein/cathepsin A cDNA of two galactosialidosis patients

The first patient analyzed is the child of healthy unrelated Italian parents. His disorder was diagnosed at the age of two and his clinical and biochemical characteristics have been described earlier (Andria *et al.*, 1978, 1981; Palmeri *et al.*, 1986). In total RNA preparations from the patient's

fibroblasts a normal amount of 2 kb protective protein transcript was detected (Galjart *et al.*, 1988). In cultured fibroblasts this mRNA was translated into a 54 kDa mutated precursor that was only poorly, if at all, processed to the mature form (Palmeri *et al.*, 1986). Consequently, no cathepsin A-like activity was measured in cell lysates from the patient (Galjart *et al.*, 1991).

To identify the genetic lesion underlying this phenotype, we first analyzed the cDNA derived from the patient's mRNA. Four overlapping fragments, encompassing the entire coding region, were reverse transcribed, amplified by the polymerase chain reaction (PCR), cloned and sequenced on both strands. The only difference between normal and mutant nucleotide sequences was a single base substitution, T to G, at position 1324 of the normal cDNA, changing Phe412 (TTC) to Val (GTC). Ten subclones of the cDNA portion including the base substitution showed the same mutation. The results were somewhat surprising since the parents of this patient were reported to be non-consanguineous. However, direct sequencing of a 239 bp genomic region surrounding the mutation, amplified by PCR from total DNA of the patient and his parents, confirmed the cDNA data (Figure 1, upper panel). The patient is clearly homozygous for the T to G transversion, whereas both carriers are heterozygous for this mutation.

Since the T to G substitution generates a new *Acyl*I restriction site in the mutant DNA, this point mutation can

Fig 1. Above. Partial nucleotide sequence of the protective protein/cathepsin A gene from the Italian galactosialidosis patient and his parents. Genomic DNA was isolated from fibroblasts of a normal individual, the patient and his parents and subjected to asymmetric PCR in the region containing the mutation. The products were directly sequenced. A portion of the autoradiograph from the sequencing gels is shown. The T to G transversion is indicated in bold. Arrows point to the double band at one position that is present in the nucleotide sequences of both parents. 5' and 3' refer to the orientation of the protective protein cDNA (Galjart *et al.*, 1988). Below. *Acyl*I restriction enzyme assay for the T to G mutation. Genomic fragments, amplified from total DNA of the patient and his parents, were incubated with *Acyl*I at 37°C for 2 h. Restriction fragments were analyzed by electrophoresis on a 4% NuSieve agarose gel and stained with ethidium bromide. Molecular sizes are indicated at left.

be easily detected by digesting the amplified 239 bp genomic fragment with *AcyI*. As shown in Figure 1 (lower panel), the patient's DNA releases upon digestion two fragments of 159 bp and 80 bp (lane 4), instead of the uncut fragment present in normal DNA (lane 1). Amplified samples from both parents display heterozygous patterns (lanes 2 and 3). We used this assay to screen for the T to G transversion DNA samples of seven clinically different galactosialidosis patients. In only one other unrelated Canadian late infantile patient (Pinsky *et al.*, 1974) was the same mutation found (not shown). It was reported earlier that in cultured fibroblasts from this patient a small amount of protective protein precursor was synthesized that again failed to mature (Palmeri *et al.*, 1986). As deduced from the *AcyI* digestion pattern, this patient carries the T to G substitution on one allele, whereas the other allele has a normal sequence in that region. Direct sequencing of the corresponding genomic DNA fragment validated the compound heterozygosity. Only the product of the T → G mutant allele was seen in direct sequences of the patient's cDNA (not shown), suggesting that a second mutation in the other allele either impairs its transcription or gives rise to an unstable mRNA. Our findings indicating compound heterozygosity explain why a reduced amount of protective protein transcript is detected in total RNA preparations from the patient's fibroblasts.

Val412 mutation impairs the catheptic activity of the mutant protein and causes partial retention of its precursor in the ER

To assess the effect of the Phe412 to Val amino acid substitution on the biochemical behaviour of the protective protein/cathepsin A, the mutation was introduced into the normal cDNA (Hu54) by oligonucleotide mediated site-directed mutagenesis. Normal and mutant cDNAs were expressed in COS-1 cells under the control of the SV40 early promoter. As shown in Table I, the Phe412 to Val change renders the enzyme inactive since no increase in cathepsin A-like activity can be measured above endogenous COS-1 levels; in contrast a 10-fold higher activity is detected in cells expressing the wild type protein. In transfected cells labeled with either [³H]leucine (Figure 2, left panel) or with [³²P]phosphate (Figure 2, right panel) the mutant protective protein is immunoprecipitated as a 54 kDa phosphorylated precursor that does not appear to be processed and is secreted to a lesser extent than the wild type form. These results demonstrate that the site directed Val412 mutation reproduces the patient's phenotype in that it gives rise to a catalytically inactive protective protein/cathepsin A with impaired post-translational processing.

Table I. Cathepsin A-like activity in COS-1 cells transfected with wild type and mutant protective protein cDNAs

Transfected DNA	Activity (U/mg protein)
Hu54 (sense)	367
Hu54 Phe412 → Val	31
Hu54 (antisense)	34

COS-1 cells were transfected with pCDHu54 sense, pCDHu54 Phe412 → Val or pCDHu54 antisense. After 72 h cells were harvested by trypsinization and cathepsin A-like activity was measured in cell extracts using *N*-carbobenzoyl-L-phenylalanyl-L-alanine (Z-Phe-Ala) as a substrate. One unit of activity is defined as the activity that releases 1 nmol of alanine per min.

Using immuno-electron microscopy, we have studied the intracellular distribution of the Val412 protein overexpressed in COS-1 cells. As shown in Figure 3, the amount of gold particles present in the ER of cells expressing wild type and mutant protective proteins is similar (Figure 3A and E). However, a drastic reduction in labeling of the Golgi complex is found in cells expressing the Val412 mutant compared with those expressing the normal protein (Figure 3C and D). Labeling of lysosomes is detected in cells transfected with either of the two cDNA constructs, but again the overall number of grains is distinctly diminished (<10%) in lysosomes of cells transfected with the mutant cDNA (Figure 3B and E and Table II). It therefore appears that substitution of Phe412 with Val causes retention of the precursor polypeptide in an early biosynthetic compartment. However, this retention can only be partial since some molecules of the total mutant precursor pool are routed to the Golgi complex, undergo phosphorylation as well as secretion and are correctly compartmentalized in lysosomes.

Mutant precursor does not dimerize at neutral pH

It was previously reported that mature lysosomal cathepsin A, purified from different species, elutes on gel filtration as a dimer of ~95–100 kDa (Simmons and Walter, 1980; Jackman *et al.*, 1990). We have recently shown that the protective protein can also form homodimers at the precursor level and neutral pH (N.J.Galjaart and A.d'Azzo, submitted), suggesting this to be an endoplasmic reticulum/early Golgi event. We have now tested the effect of the Phe412 to Val change on the elution pattern of the mutant precursor after

Fig. 2. Immunoprecipitation of normal and mutant protective proteins in transfected COS-1 cells. COS-1 cells were transfected with pCDHu54 sense (wt, se), pCDHu54 antisense (wt, a) and pCDHu54 Phe412 to Val (FV, se). Two days after transfection newly synthesized proteins were labeled for an additional 12 h with [³H]leucine (left panel) or for 7 h with [³²P]phosphate (right panel). Labeled proteins from cells and media were immunoprecipitated, resolved on 12.5% SDS-polyacrylamide gels under reducing conditions, and visualized by fluorography (left) or by autoradiography (right). Molecular sizes of precursor and mature polypeptides are indicated. Exposure times were 5 days for left panel, 1 day for right panel.

Fig. 3. Ultrathin cryo-sections of COS-1 cells, transfected with pCDHu54 (A, B and C) or pCDHu54 Phe412 to Val (D and E) and labeled with anti-32 kDa antibodies and goat anti-rabbit IgG-gold. In A, B, and C extensive labeling of rough endoplasmic reticulum structures (R), Golgi complex (G) and a lysosome (L) is shown, but not of a mitochondrion (M). In contrast, a weakly labeled Golgi complex (G) is seen in D and E shows strong labeling of rough endoplasmic reticulum (R) and a few gold particles in a lysosome (L). Magnifications were 46 000 $\times$ for A, 58 000 $\times$ for B, 93 000 $\times$ for C, 64 000 $\times$ for D and 65 000 $\times$ for E.

gel filtration. COS-1 cells were transfected with either wild type or mutant protective protein cDNA constructs, and labeled with [3 H]leucine. Medium samples containing the secreted precursor forms, were concentrated, desalted and applied onto a Sephacryl S-200 column. Protective protein precursor was immunoprecipitated from each fraction using anti-54 kDa antibodies. As shown in Figure 4, ~50% of wild type precursor is resolved as a dimer of ~85 kDa, whereas the remaining ~50% forms aggregates of high molecular weight (upper panel). Multimerization seems to be concentration dependent (N.J.Galjart and A.d'Azzo, submitted). In contrast the majority of the mutant polypeptide is immunoprecipitated as a monomer of ~37 kDa and only a small fraction elutes as a multimer (Figure 4, lower panel). Both normal and mutant native precursors appear to be non-globular proteins since they elute later than expected for polypeptides of 54 kDa. These findings suggest a correlation between the absence of dimerization of the mutant proform and its retention in an early biosynthetic compartment.

Mutant 32/20 kDa two-chain product is rapidly degraded

To test whether the amino acid substitution would affect or mask the recognition site of the endoprotease that normally

Table II. Quantitative data on the immunolabeling of lysosomes in different COS-1 transfected cells.

Transfected DNA	Lysosomes counted	Gold particles ^a
Hu54 (sense)	30	125 (13)
Hu54 Phe412 to Val	30	7 (1)
Hu54 (anti)	55	1 (0.3)

^aThe values represent the average number of gold particles per lysosome. The standard error of the mean (SEM) is indicated between brackets.

converts the precursor into the two-chain form, the following experiment was performed. COS-1 cells, overexpressing either the normal or the mutant cDNA, secrete sufficient amounts of the corresponding 54 kDa precursors. These secreted forms were subjected to partial *in vitro* proteolysis using a fixed concentration of trypsin at increasing time points of incubation (Figure 5). After 2 min the normal precursor is already fully trimmed into 32 and 20 kDa subunits that remain rather stable during longer incubation times, though some degradation seems to occur after 30 min (Figure 5, left panel). The mutant precursor instead does undergo proteolytic cleavage but this is followed by rapid degradation of the 20 kDa subunit which is only barely visible after 2 min incubation. It is likely that this event renders the 32 kDa chain also unstable (Figure 5, right panel).

We next investigated whether the mutant protective protein retains the capacity to associate with β -galactosidase. For this purpose COS-1 cells were doubly transfected with constructs encoding either wild type protective protein and β -galactosidase, or Val412 protein and β -galactosidase. The physical association between either of the two protective proteins with β -galactosidase was determined by measuring co-precipitation of the latter enzyme with monospecific anti protective protein antibodies (anti-54). In both COS-1 cell lysates ~70% of β -galactosidase activity was co-precipitated, under conditions that brought down virtually all cathepsin A-like activity. The association was specific since in cells co-expressing the mutant protective protein and human lysosomal β -hexosaminidase the latter enzyme was not co-precipitated with the anti-54 antibodies. In addition, biosynthetic labeling studies have provided evidence that the interaction between the mutant protective protein and β -galactosidase can occur at precursor level (H.Morreau and A.d'Azzo, submitted for publication).

Taken together these results suggest that the Phe412 to

Fig. 4. Gel filtration analysis of normal and mutant protective protein precursors secreted by transfected COS-1 cells. Secreted radiolabeled proteins from COS-1 cells transfected with wild type or mutant (Phe412 to Val) cDNAs were concentrated, desalted and applied on a Sephacryl S-200 column. Precursors were immunoprecipitated, resolved and visualized as in Figure 2. The elution position of the separately applied globular protein markers is indicated above the fluorographs: ferritin (F), aldolase (A), BSA (B) and ovalbumin (O).

Fig. 5. Trypsin digest of normal and mutant protective protein precursors secreted by transfected COS-1 cells. Transfections were carried out with the pCD-construction described in Table I. Cells were labeled with [3 H]leucine 48 h after transfection. Aliquots of medium concentrates containing the secreted precursor polypeptides were incubated with 1 μ g of trypsin at 37°C for 0, 2, 5, 10 and 30 min respectively. Trypsin was inactivated by addition of 3 μ g trypsin inhibitor. Radiolabeled proteins were immunoprecipitated, resolved and visualized as in Figure 2. Lane 1, immunoprecipitation of intracellular protective protein from pCDHu54 transfected COS-1 cells. Lanes 2–6, trypsin digest of normal protective protein precursor. Lanes 7–12, trypsin digest of mutant precursor. Molecular sizes of precursor and mature polypeptides are indicated on the left. Exposure time for lanes 1–6 was 48 h, and for lanes 7–12 was 5 days.

Val substitution does not disturb the tertiary structure of the precursor polypeptide to the extent that it would completely inhibit its transport to lysosomes, endoproteolytic processing, and even association with the other two lysosomal enzymes. Once in lysosomes, however, the fast degradation of the mutant protein, initiated by endoproteolytic cleavage of the 54 kDa precursor, would explain the loss of protective function towards β -galactosidase and neuraminidase.

Discussion

Galactosialidosis patients with the late infantile form of the disease make up a defined subtype for the following reasons: they exhibit symptoms within the first two years of life, they have a slow and relatively mild progression of the disease, and, most important, the patients for whom a clinical update has been made (Chitayat *et al.*, 1988; Strisciuglio *et al.*, 1990; G.Andria and P.Strisciuglio, personal communication) do not show signs of mental retardation. The latter characteristic seems to be unique for this group and is in contrast with the severe neurological involvement observed in early infantile and even juvenile/adult cases. We were interested in identifying the genetic lesion(s) underlying this phenotype starting with the understanding of how a natural mutation in the gene impairs the gene product and at what subcellular level.

In this report we have described a point mutation in the protective protein gene present in two unrelated late infantile galactosialidosis patients of Italian and Canadian origin. The mutation consists of a T to G transversion at position 1324

of the human cDNA which results in the replacement of phenylalanine with valine at residue 412. The Italian patient is homozygous for this mutation whereas the Canadian patient is a compound heterozygote carrying the Val412 substitution together with an as yet unidentified lesion in the other allele. The unknown mutation apparently causes a significant decrease in the amount of protective protein mRNA suggesting a defect in transcription or mRNA stability. The T $\rightarrow$ G substitution in contrast does not interfere with the synthesis of an mRNA of correct size and quantity. In both patients' fibroblasts this transcript is translated into an abnormal precursor that is not or is hardly processed to the mature two-chain product and consequently lacks cathepsin A-like activity (Palmeri *et al.*, 1986; Galjart *et al.*, 1991).

Using site directed mutants with the Phe412 to Val change we have demonstrated that this mutation is the one responsible for the *in vivo* phenotype affecting post-translational modifications, intracellular transport and catalytic activity of the protective protein/cathepsin A. Overexpression of the mutant protein in COS-1 cells made it possible to follow its intracellular distribution for the first time. It became apparent that only a fraction of the newly synthesized precursor pool leaves the endoplasmic reticulum and gets compartmentalized. These molecules fold properly inasmuch as they acquire the mannose-6-phosphate marker, are routed to lysosomes and are also secreted. In addition, there is evidence that the amino acid change does not prevent the recognition of the mutant precursor by one of the other components of the complex, β -galactosidase. It is conceivable that *in vivo* a fraction of Val412 precursor that exits the ER is associated with β -galactosidase and reaches the lysosomes, where it gets rapidly degraded in its processed two-chain form. This event would in turn provoke the loss of protective function towards β -galactosidase and neuraminidase, although the mode and site of interaction of the latter with the other two components is until now unknown. Our results raise the possibility that the protective function of the protein, contrary to its catalytic activity, could be exerted already at precursor level. They also conform with the presence of residual mature protective protein in lysosomes of patients' fibroblasts (Palmeri *et al.*, 1986; Strisciuglio *et al.*, 1988).

The fate of the intralysosomal Val412 precursor and its retention in the ER could be attributed to a common faulty event, the lack of oligomerization. During the last few years it has become increasingly clear from a variety of systems that it is not only a protein's tertiary structure but also its oligomeric state that determines its intracellular transport, final targeting and stability. These early steps in protein biosynthesis often take place and are regulated in the ER (for review see Hurtley and Helenius, 1989). The protective protein precursor by itself appears to exist as an oligomer, probably a dimer. In this form proteins thought to be identical or very homologous to the protective protein are also found in their mature and active state (Simmons and Walter, 1980; Jackman *et al.*, 1990). The dimerization process could play a very important role in the correct targeting and stable conformation of the protective protein, since we find that the monomeric Val412 mutant is retarded in the ER. It is unclear at this time whether the rapid degradation of the mutant protein upon proteolytic cleavage is solely due to its monomeric state or also to its inability to stably assemble into a two-chain polypeptide. The effects of the impaired

dimerization of mutant protective protein resemble to some extent those caused by a number of natural mutations identified in the α -subunit of lysosomal β -hexosaminidase (Neufeld, 1989; Navon and Proia, 1991; Paw *et al.*, 1991).

It is noteworthy that phenylalanine at position 412 is highly conserved among protective proteins of different species (Galjart *et al.*, 1991). The mutation falls in a region of the 20 kDa subunit that is part of an internal repeat occurring once in each of the two chains and is characterized by recurring tryptophane residues that could be engaged in inter/intramolecular bonds. It has been postulated that initial folding of at least one domain of a subunit is required for correct oligomeric assembly (Hurtley and Helenius, 1989). The 'repeated' motif in the protective protein chains might expose surface features essential for the recognition and binding of monomeric molecules with each other. In this context the Phe412 to Val change could have dramatic structural consequences.

Finally it is important to keep in mind that the protective protein shows, at least *in vitro*, a cathepsin A-like activity towards a variety of bioactive peptides (Jackman *et al.*, 1990). This activity is distinct from its protective function toward β -galactosidase and neuraminidase and is deficient in different galactosialidosis patients. The consequences of the deficiency of the enzymatic activity on other metabolic pathways, could be clarified by the identification of the natural substrate(s) that are the target of this multifunctional protein *in vivo*.

Materials and methods

Cell culture

Human skin fibroblasts from normal individuals and the Canadian late infantile galactosialidosis patient (Pinsky *et al.*, 1974) were obtained from the European Cell Bank, Rotterdam (Dr W.J. Kleijer). Cells from the Italian late infantile galactosialidosis patient (Andria *et al.*, 1978) and both parents were provided by Dr G. Andria, Department of Pediatrics, University of Naples, Italy. Fibroblasts were maintained in Dulbecco's modified Eagle's medium—Ham's F10 medium (1:1 v/v) supplemented with antibiotics and 10% fetal bovine serum. COS-1 cells (Gluzman, 1981) were grown in the same medium, supplemented with 5% fetal bovine serum.

cDNA synthesis and cloning

For the synthesis and amplification of mutant cDNAs, four sets of oligonucleotide primers were synthesized on an Applied Biosystems 381A oligonucleotide synthesizer according to the sequence of the human cDNA, Hu54 (Galjart *et al.*, 1988). The four sense primers correspond to nucleotide positions 5–24, 367–383, 701–720 and 1051–1070 respectively; the four antisense primers to positions 388–407, 750–731, 1080–1099 and 1450–1469 respectively. Eight bases were added to the 5' end of each primer to generate *Eco*RI sites and facilitate ligation of amplified DNA into a plasmid. Total RNA was isolated by the method of Auffray and Rougeon (1980). Four overlapping cDNA fragments, encompassing the entire coding region, were synthesized by reverse PCR (Hermans *et al.*, 1988) and amplified on a Perkin–Elmer Cetus thermocycler programmed for 25 cycles. The fragments were digested with *Eco*RI and subcloned into pTZ18 or pTZ19. Nucleotide sequence analysis on both strands was performed using the dideoxy chain termination method adapted for double-stranded DNA (Murphy and Kavanagh, 1988).

Analysis of amplified genomic DNA

The 239 bp region of genomic DNA surrounding the mutation was PCR amplified according to Saiki *et al.* (1988). The two oligonucleotide primers used in the reaction are derived from intronic sequences surrounding exon XIV (N.J. Galjart and A.d'Azzo, in preparation) in the protective protein gene that contains the mutation. The sense oligonucleotide primer sequence is 5'-TCTTCTGGTGGGGCAGAT-3' (primer 1) and includes 2 bp of the exon; the antisense primer sequence is: 5'-CCATACAGGGCCAG-ATGGT-3' (primer 2) and is ~100 bp downstream of the exon. For direct DNA sequence analysis an aliquot of the amplified DNA sample was subjected to asymmetric PCR (Kadowaki *et al.*, 1990), using only primer

1 for another 35 cycles. The amplified DNAs were sequenced with primer 2. *Ac*VI digestion of PCR amplified genomic fragments was carried out using standard conditions (Sambrook *et al.*, 1989). The resulting fragments were analyzed on a 4% NuSieve agarose gel.

Site directed mutagenesis

In vitro mutagenesis of human protective protein cDNA was done as described by Higuchi *et al.* (1988), using the whole 1.8 kb protective protein cDNA as a template. After synthesis by PCR of a full length cDNA containing the T to G transversion, the entire fragment was verified by sequencing on both strands. The two oligonucleotides used for site directed mutagenesis are 5'-ATTGCCGGCTTCGTGAAGGAG-3' (sense) and 5'-CTCCTTCACGAAGCCGGAAT-3' (antisense).

Transfection of cDNA into COS-1 cells

Normal and mutant protective protein cDNAs were subcloned into a derivative of the mammalian expression vector pCD-X (Okayama and Berg, 1983) as described earlier (Galjart *et al.*, 1988). Transfection of COS-1 cells, metabolic labeling and preparation of cell extracts and media were carried out as reported earlier (Proia *et al.*, 1984; Galjart *et al.*, 1988). Cells were labeled with [³H]leucine (143 Ci/mmol; Amersham Corp.) or with [³²P]phosphate (carrier free, Amersham Corp.), for 12 h or 7 h, respectively. Labeled proteins were immunoprecipitated with anti-54 kDa antibodies that recognize precursor and mature protective protein (Galjart *et al.*, 1991). They were resolved on 12.5% SDS–polyacrylamide gels under reducing conditions and visualized by autoradiography ([³²P]labeled samples) or fluorography ([³H]labeled samples). Transfected COS-1 cells, lysed in double distilled water were assayed for cathepsin A activity using the N-blocked dipeptide Z-Phe-Ala (Bachem Feinchemikalien AG, Bubendorf, Switzerland), using protocols adapted from the methods of Taylor and Tappel (1973) and Roth (1971). Total protein concentration was measured using the method of Smith *et al.* (1985).

Immunoelectron microscopy

Transfected COS-1 cells were fixed in 0.1 M phosphate buffer pH 7.3, containing 1% acrolein and 0.4% glutaraldehyde. Further embedding in gelatin, preparation for ultracytometry and methods for immunoelectron microscopy were as reported earlier (Willemsen *et al.*, 1986). The antibodies against the 32 kDa denatured chain of human protective protein (anti-32 antibodies) have been described (Galjart *et al.*, 1988).

Gel filtration

Medium samples from transfected cells were collected, concentrated and desalted as described (Proia *et al.*, 1984). 50% of this material was diluted in 50 mM 2-[N-morpholino]ethane sulfonic acid (MES), pH 6.95, containing 100 mM NaCl, and applied onto a column (85 × 1.53 cm) of Sephacryl S-200 HR (Pharmacia) equilibrated in the same buffer. The whole procedure was performed at 4°C. After gel filtration radiolabeled proteins were immunoprecipitated using anti-54 antibodies and further processed as above. The column was separately run in the same buffer with the following set of globular protein markers (Pharmacia): ferritin (440 kDa), aldolase (158 kDa), BSA (67 kDa) and ovalbumin (43 kDa).

Limited proteolysis with trypsin

Transfections and metabolic labeling of transfected cells with [³H]leucine was performed as above, with the exception that fetal bovine serum was omitted from the labeling medium. Secreted proteins were concentrated 20-fold with (NH₄)₂SO₄, in the presence of 1 mg/ml BSA, and desalted. Aliquots of 90 μ l medium concentrate were diluted to 200 μ l volume with 20 mM sodium phosphate pH 6.8. A modified protocol of Frisch and Neufeld (1981) was used for trypsin digestion. One sample was left on ice, the others were incubated with 1 μ g of trypsin (Sigma) at 37°C for 0, 2, 5, 10 and 30 min, respectively. Reactions were stopped by addition of 3 μ g bovine pancreas trypsin inhibitor (Sigma). At $t = 0$ the inhibitor was added before the trypsin. Radiolabeled proteins were immunoprecipitated and further processed as described previously.

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