Human Lysosomal Protective Protein Has Cathepsin A-like Activity Distinct from Its Protective Function*

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The protective protein was first discovered because of its deficiency in the metabolic storage disorder galactosialidosis. It associates with lysosomal β-galactosidase and neuraminidase, toward which it exerts a protective function necessary for their stability and activity. Human and mouse protective proteins are homologous to yeast and plant serine carboxypeptidases. Here, we provide evidence that this protein has enzymatic activity similar to that of lysosomal cathepsin A: 1) overexpression of human and mouse protective proteins in COS-1 cells induces a 3–4-fold increase of cathepsin A-like activity; 2) this activity is reduced to ~1% in three galactosialidosis patients with different clinical phenotypes; 3) monospecific antibodies raised against human protective protein precipitate virtually all cathepsin A-like activity in normal human fibroblast extracts. Mutagenesis of the serine and histidine active site residues abolishes the enzymatic activity of the respective mutant protective proteins. These mutants, however, behave as the wild-type protein with regard to intracellular routing, processing, and secretion. In contrast, modification of the very conserved Cys60 residue interferes with the correct folding of the precursor polypeptide and, hence, its intracellular transport and processing. The secreted active site mutant precursors, endocytosed by galactosialidosis fibroblasts, restore β-galactosidase and neuraminidase activities as effectively as wild-type protective protein. These findings indicate that the catalytic activity and protective function of the protective protein are distinct.

Intralysosomal degradation is a composite process that is largely controlled by a battery of acidic hydrolases. The majority of these glycoproteins are synthesized on membrane-bound polysomes as high molecular weight precursors and routed to the lysosomes via a series of compartment-dependent posttranslational modifications. For the stepwise catabolism of different macromolecules to occur efficiently, a number of these hydrolases must work in concert and might, therefore, reside in a multienzyme complex. An example of such a complex could be the one consisting of lysosomal β-galactosidase (EC 3.2.1.23), N-acetyl-α-neuraminidase (sialidase, EC 3.2.1.18), and the protective protein (1–3). In human placenta (3), bovine testis (2), and porcine spleen and testis (4, 5) these three glycoproteins copurify through an affinity matrix for β-galactosidase.

The association of the protective protein with β-galactosidase and neuraminidase is essential for the stability and activity of these two glycosidases within the lysosomes (3, 6, 7). This is reflected by the existence of the metabolic storage disorder galactosialidosis (8), in which a primary defect of the protective protein results in a combined β-galactosidase/neuraminidase deficiency (1, 9). Among galactosialidosis patients distinct clinical phenotypes exist, ranging from severe early infantile forms in which visceromegaly with nephrotic syndrome, heart failure, and other abnormalities lead to early death or fetal hydrops, to milder late infantile and juvenile/adult variants (8, 10). Biochemical heterogeneity within these recognized phenotypes has also been observed (11, 12).

In human cultured fibroblasts the protective protein is synthesized as a precursor of 54 kDa which is proteolytically processed into a mature two-chain form of 32- and 20-kDa polypeptides linked together by disulfide bridges (1, 12). The predicted amino acid sequences of human as well as mouse protective proteins are homologous to yeast and plant serine carboxypeptidases (12, 13). Both protective proteins react with the serine protease inhibitor DFP, but only in their mature state (13). Together these findings allowed us to predict a similar carboxypeptidase activity for the protective protein that is apparently synthesized and transported to the lysosomes as a zymogen. Some of its characteristics correlate well with those of a previously identified carboxypeptidase, cathepsin A (EC 3.4.16.1). This enzyme has been partially purified from different sources (14) and was shown to exist in small and large aggregate forms (15). In the native small aggregate, subunits with molecular masses of 20, 23, and 55 kDa are present, of which the 25-kDa polypeptide reacts with DFP (16). Besides its carboxypeptidase activity, optimal at acidic pH, cathepsin A can also function as a peptidyl amidohydrolase (14, 17). Recently, a deamidase/carboxypeptidase purified from human platelets was shown to have sequence identity to the NH2 termini of the protective protein chains (18). Enzymatic characterization of this deamidase with a variety of substrates and inhibitors also suggested a similarity to cathepsin A.

Here we provide direct evidence that the protective protein maintains cathepsin A-like activity. Galactosialidosis is therefore the first example of a lysosomal storage disorder associated with a protease deficiency. We also demonstrate by site-

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directed mutagenesis of the human protective protein that its cathepsin A-like activity can be separated from its protective function toward β-galactosidase and neuraminidase.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Human skin fibroblasts from normal individuals, patients with the early infantile (19) and juvenile/adult (20) forms of galactosialidosis, and a Gna1-gangliosidosidase patient were obtained from the European Cell Bank, Rotterdam (Dr. W. J. Kleijer). Cells from the late infantile galactosialidosis patient (21) and both parents were provided by Dr. G. Andria, Dept. 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 16% fetal bovine serum. COS-1 cells (22) were grown in the same medium, supplemented with 5% fetal bovine serum.

**Enzyme Assays**—For enzyme activity assays and immunotitration experiments cells were harvested by trypsin treatment and homogenized in double-distilled water. When necessary, cell lysates were subsequently diluted in 20 mM sodium phosphate, pH 6.8, containing 1 mg/ml bovine serum albumin. Cathepsin A activity was measured in cell homogenates using a modification of the method of Taylor and Tappel (23). Briefly, 5 μl (2-10 μg of protein) of cell homogenates were incubated for 30 min at 37°C in 100 μl of 50 mM MES, pH 6.5, 1 mM EDTA, in the absence or presence of 1.5 mM blocked dipeptides Z-Phe-Ala, Z-Phe-Leu, or Z-Glu-Tyr (Bachem). Reactions were stopped by addition of an equal volume of 10% (v/v) trichloroacetic acid. Precipitates were removed by centrifugation, and a fraction (5%) of the supernatants was taken to measure the concentration of released tyrosine by the fluorometric method outlined by Roth (24). The activities of β-galactosidase, neuraminidase, and β-hexosaminidase were measured with artificial 4-methylumbelliferyl substrates (25). Total protein concentrations were determined as described previously (26).

**Antibodies and Immunotitration of Cathepsin A-like Activity**—We have previously described the preparation of antibodies raised against a denatured form of the 32-kDa subunit of human protective protein (12). These “anti-32” antibodies recognize under reducing and denaturing conditions the 54-kDa precursor form as well as the 32-kDa mature component of the protective protein. To obtain a monospecific antiserum that immunoprecipitates human protective protein under native conditions, the latter was overexpressed in Spodoptera frugiperda (Sf9) insect cells, that had been infected with recombinant baculovirus containing human protective protein cDNA. Protective protein was purified from the culture medium of infected cells using a chromatographic A-Sephasose column (Pharmacia), as described earlier (2). “Anti-54” antibodies were raised in rabbits against this purified protein preparation. An IgG fraction (2.2 mg of protein/ml) was prepared from anti-54 antiserum using a protein A-Sepharose column (Pharmacia).

Immunotitration of cathepsin A with anti-54 antibodies was performed essentially as described before (3). Formalin-fixed Staphylococcus aureus cells (Immunoprecipitin, Bethesda Research Laboratories) were added to the samples to remove antigen-antibody complexes.

**Isolation of cDNA Clones and DNA Sequence Analysis**—A chicken embryo lgt11 cDNA library (Clontech, Palo Alto, CA) (27) consisting of 1 x 10⁶ independent clones, was plated out as described before (13) and screened using the heterologous human protective protein cDNA, Hu54, as a probe, 1/20 (28). The longest cDNA insert was subcloned into pTZ18 and 19 (Pharmacia) (29) and sequenced on both strands (30). Comparison to the human sequence showed that the chicken cDNA lacks the ATG start codon and part of the signal peptide. All DNA fragments resulting from polymerase chain reactions amplification were verified by sequencing as described above. The oligonucleotides needed for site-directed mutagenesis were synthesized on an Applied Biosystems 381A oligonucleotide synthesizer.

Human/chicken cDNA, HCh1, was made by exchange of 5’-end chicken with human cDNA sequences at a conserved PstI restriction enzyme site. All constructs were cloned into a derivative of the mammalian expression vector pCD-X (34) as described previously (12).

**Transfection in COS-1 Cells**—COS-1 cells were seeded out in 30-mm dishes 1-2 days prior to transfection and grown to 30% confluency. Transfection in COS-1 cells, metabolic labeling of transfected cells, and preparation of cell extracts and media were carried out as described before (12, 35). Cells were labeled with [3H]leucine (143 Ci/mmol, Amersham Corp.). Immunoprecipitation of radiolabeled proteins from cell lysates and media was performed using anti-32 or anti-54 antibodies, as reported (36). Radioactive proteins were resolved on 12.5% polyacrylamide gels under reducing and denaturing conditions and visualized by fluorography of gels impregnated with Amplify (Amersham Corp.). For the DF-binding assay and direct measurement of cathepsin A activity, COS-1 cells were transfected with various pCD constructs and maintained afterwards for 72 h in normal culture medium. Subsequently, cells were harvested by treatment with trypsin. Cell lysates were either incubated with [3H]DFP (Du Pont-New England Nuclear, 3.0 Ci/mmol) or used as such for detection of cathepsin A activity as described above.

**Uptake Studies in Human Fibroblasts**—COS-1 cell-derived protective protein precursors were obtained from the medium of unlabeled COS-1 cells, transfected in 100-mm Petri dishes. Media were concentrated as described previously (35) and half of the concentrated material was added to the medium of recipient early infantile galactosialidosis fibroblasts (12). After 5 days of uptake the medium was replaced with fresh medium containing the other half of concentrated material. 2 days later cells were harvested by trypsin treatment, and cell lysates were partly used for enzyme activity assays. The remainder of these homogenates was diluted 7-fold in 10 mM sodium phosphate buffer, pH 6.0, containing 100 mM NaCl and 1 mg/ml bovine serum albumin. After centrifugation to remove insoluble material the cell lysates were divided into aliquots of 25 μl each, incubated for 1.5 h with 1.5 μl of preimmune serum, anti-54 antibodies, or antihuman β-galactosidase antibodies. Immunoprecipitation, extensively washed in the aforementioned buffer, was subsequently added to the samples, and after 30 min antibody-antigen complexes were removed by centrifugation. All steps were performed on ice or at 4°C. The supernatants were assayed for β-galactosidase activity.

**Indirect Immunofluorescence**—COS-1 cells, transfected with selected pCD constructs, were treated mildly with trypsin 48 h after transfection and subsequently resedated at low density on coverslips. 16 h later cells were fixed and incubated with anti-52 antibodies and in a second step with goat anti-rabbit IgG conjugated with fluorescein (36).

**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 the methods for immunoelectron microscopy were as reported earlier (37).

**RESULTS**

**Evidence That the Protective Protein Is Similar to Cathepsin A**—We first ascertained whether the protective protein maintains carboxypeptidase activity aside from its protective function. The choice of the synthetic substrate to use in the assay was dictated by the similarity of the protein to cathepsin A (14-18). The latter hydrolyzes preferentially at acidic pH acylated dipeptides having a hydrophobic residue in the pe-
multitame (P1) position (38). Of these N-blocked dipeptides Z-Phe-Ala was reported to be the most specific substrate for cathepsin A (38).

In total cell homogenates from human cultured fibroblasts we have measured the hydrolysis of Z-Phe-Ala as well as Z-Glu-Tyr and Z-Phe-Leu. The rate of hydrolysis is maximal for Z-Phe-Ala (Table I), 3-fold lower for Z-Phe-Leu (normal fibroblasts: 1, 163 milliunits/mg protein; 2, 93 milliunits/mg protein) and barely detectable for Z-Glu-Tyr (not shown). In order to prove that the protective protein is the enzyme responsible for the cleavage of Z-Phe-Ala, we raised monospecific polyclonal antibodies in rabbits against a human native protective protein preparation (anti-54 antibodies). As shown in Fig. 1, virtually all carboxypeptidase activity toward this substrate is precipitated at increasing antibody concentrations. Since the purified preparation used for immunization of the rabbits was obtained from the culture medium of Sf9 insect cells infected with a recombinant baculovirus expression vector (39), it is unlikely that proteins of human origin, other than the protective protein, are directly precipitated by the antibodies. From these results we conclude that lysosomal protective protein has a substrate specificity overlapping with that of cathepsin A. We have also tested whether β-galactosidase activity is coprecipitated with cathepsin A by virtue of the association of these two proteins. Indeed, about one-third of total β-galactosidase activity is brought down at maximal antibody concentration. The values for β-hexosaminidase, measured in the fibroblast homogenates as a reference enzyme, remained unchanged throughout the experiment.

**Table I**

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<td>Juvenile/adult galactosidosis</td>
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<td>Parents late infantile patient</td>
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* One milliunit is defined as the enzyme activity that releases 1 nmol of alanine per min.

![Graph](attachment:image.png)

*Fig. 1. Immunotitration of cathepsin A-like activity. Increasing amounts of an IgG antibody fraction, raised against human native protective protein precursor, were added to a cell extract of normal human fibroblasts. Antibody-antigen complexes were removed by addition of *S. aureus* cells and the remaining carboxypeptidase (CP) activity toward the acylated dipeptide Z-Phe-Ala was measured in the supernatant.*

Carboxypeptidase Deficiency in Galactosialidosis Fibroblasts—The same dipeptide, Z-Phe-Ala, was used as substrate to measure cathepsin A-like activity in fibroblast homogenates from normal individuals, a GMI-1 gangliosidosis patient with an isolated β-galactosidase deficiency, different galactosialidosis patients, and carriers (Table I). In contrast to normal as well as GMI-1 gangliosidosis fibroblasts the galactosialidosis cell strains tested have minute activities toward the substrate. Clear heterozygote values are measured in the carrier samples. The normal hydrolysis of Z-Phe-Ala measured in GMI-1 gangliosidosis cell extract indicates that an isolated β-galactosidase deficiency does not influence the carboxypeptidase activity of the protective protein.

Analysis of Conserved Domains in Protective Proteins of Different Species—Amino acid sequence comparison with other well defined serine carboxypeptidases (40–42) revealed that the protective protein/cathepsin A is a member of this family of enzymes (12, 13). Similarly, comparison of the primary structures of protective proteins from different species could disclose domains in the human protein important for its association with β-galactosidase/neuraminidase and, hence, for its protective function. However, the previously characterized mouse protective protein appeared to be almost identical to its human counterpart (13). We therefore isolated the cDNA encoding chicken protective protein. Its predicted amino acid sequence is shown in Fig. 2, aligned with those of the human and mouse proteins. The chicken sequence lacks the first methionine residue and part of the signal peptide.

Identity between the different proteins is 67% (chicken/human), 66% (chicken/mouse), and 87% (mouse/human). The serine, histidine, and aspartic acid residues that are known to form the catalytic triad of serine carboxypeptidases (43) are found in the chicken protective protein/cathepsin A at positions 150, 431, and 375, respectively. Ser<sup>150</sup> and His<sup>431</sup> are included in two of the three highly conserved regions (boxed in Fig. 2) in this family of enzymes. Remarkably, however, the chicken enzyme has a glycine for alanine substitution at position 152 that occurs within the Gly-Glu-Ser-Tyr-Ala-Gly domain, containing the active site serine. All three protective proteins have 9 conserved cysteine residues, probably crucial for their tertiary structure as well as function. Both chicken and mouse homologues have two additional cysteines, one on each subunit of their respective two-chain forms, but at different positions.

Additional essential residues and domains emerge from the sequence alignment. Amino acids surrounding the two proteolytic cleavage sites (Fig. 2, vertical arrows) are largely identical. An internal repeating motif (underlined in Fig. 2), characterized by 2 recurring Trp residues 16 amino acids apart, is present in each subunit of all three protective proteins, suggesting an ancient intragenic duplication. Notably, this “repeat” within the 32-kDa polypeptide includes the 10-amino acid domain (residues 53–62, boxed in Fig. 2), conserved in all serine carboxypeptidases. Four potential N-linked glycosylation sites are found in the chicken protective protein (Fig. 2, hatched boxes), two of which are in identical positions in the three sequences.

**Mutagenesis of Human and Chicken Protective Proteins**—To investigate whether the cathepsin A-like activity of the protective protein is essential for the activation and stabilization of β-galactosidase and neuraminidase we used a genetic approach. As summarized in Fig. 3, mutant and hybrid protective proteins were obtained by site-directed mutagenesis of wild-type cDNAs, encoding either the human or chicken forms. The first series of mutants (Fig. 3, upper bar) carried single amino acid substitutions in the human protein. Of the
catalytic triad, Ser150 to Ala150 amino acid substitution (Fig. 3, middle bars).

The human-chicken hybrid construct, HCh1 (Fig. 3, lower bar), was made to identify determinants on the human protective protein, important for its association to β-galactosidase/neuraminidase. Furthermore, the replacement of chicken with human 5' end cDNA sequences provided HCh1 with the correct translation initiation codon. As a result, the NH2 terminus of the HCh1 hybrid precursor contains 60 amino acids of human origin.

**Transient Expression of Mutant and Hybrid Proteins**

*in COS-1 Cells*—To follow the intracellular transport and processing of mutant and hybrid protective proteins, their corresponding cDNAs were subcloned into a derivative of the expression vector pCD-X and transfected into COS-1 cells. Human and mouse protective protein constructs (pCDHu54 and pCDMo54) were included in the experiments as controls. Transiently expressed proteins were detected 2 days after transfection by metabolic labeling with [3H]leucine followed by immunoprecipitation from cell lysates and media (Fig. 4). Immunoprecipitations were carried out using two different antibodies: those raised against the human 54-kDa precursor as well as the mature 32-kDa protein carrying the Ser150 to Ala150 amino acid substitution (Fig. 3, middle bars).

FIG. 2. Alignment of predicted amino acid sequences of human, mouse, and chicken protective proteins. Amino acid sequences of previously characterized human (HU) and mouse (MO) protective proteins are shown aligned with the chicken (CH) homologue. Residues on the fourth line (consensus sequence) denote identity at those positions in the three protective proteins. The three domains, characteristic of serine carboxypeptidases, and the aspartic acid residue, conserved throughout this family of enzymes, are boxed. Potential N-linked glycosylation sites are indicated with hatched boxes. Vertical arrows denote proteolytic cleavage sites. The internal repeating motif in 32- and 20-kDa subunits is underlined. Numbers on the left refer to positions of the amino acids within the sequences.

**Fig. 3. In vitro mutagenized and hybrid protective proteins.** The preproform of human protective protein is represented by the upper bar. Hatched part, signal sequence; stippled part, 32-kDa subunit; cross-hatched part, 20-kDa subunit. Single amino acid substitutions are indicated. The three middle bars represent the deletion mutants. In the 32SA(Δ20) mutant the Ser150 to Ala150 substitution is also present. The 20(Δ32) mutant is tagged with the human signal sequence (hatched part). The lower bar represents the human/chicken hybrid protein, having the signal sequence (hatched part) and the most N-terminal 60 amino acids (stippled part) of the human protective protein.

The catalytic triad, Ser150, was replaced by alanine and His429 was mutated into glutamine, in order to abolish cathepsin A-like activity. The third point mutation was introduced at residue Cys66 to study the effect of this alteration on the correct folding, transport, and processing of the protective protein. The mutants 32(Δ20) and 20(Δ32) were deleted of either of the two subunits in order to check their reciprocal influence within the two-chain form on the cathepsin A and/or protective activities. They encode truncated 32- and 20-kDa polypeptides, respectively. The latter was tagged with the human signal sequence to allow its translocation into the endoplasmic reticulum (ER). The 32SA(Δ20) mutant encodes a truncated 32-kDa protein carrying the Ser150 to Ala150 amino acid substitution (Fig. 3, middle bars).

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FIG. 4 shows that singly transfected pCD constructs direct the synthesis of mutated or hybrid protective proteins that are stable under the conditions used. The SA150 and HQ429 mutant precursors behave as wild-type protective protein in that they undergo normal proteolytic processing and the unprocessed precursors are secreted into the medium (Fig. 4, lanes 1, 3, and 4). In contrast, the cysteine to threonine substitution apparently interferes with the maturation and secretion of the precursor molecule (Fig. 4, lane 2). However, it is also possible that an aberrantly folded mature mutant protein evades recognition by the anti-32 antibodies.
This hypothesis was supported by the observation that assembly of a truncated 34-kDa polypeptide, carrying the Ser\textsuperscript{150} to Ala\textsuperscript{150} active site mutation, with the 20-kDa polypeptide does not lead to reduced immunoprecipitable material (Fig. 4, lanes 8 and 9). The results further indicate that association of the different subunits does not induce their secretion.

The HChl hybrid precursor is about 4 kDa larger than the human proform (Fig. 4, lanes 1 and 10). This is due to the presence of two extra sugar chains in the chicken protective protein, since tunicamycin treatment prior to and during labeling leads to the synthesis of precursor molecules of identical size (not shown). The hybrid precursor is secreted into the culture medium, but no mature form can be precipitated intracellularly (Fig. 4, lane 10). A likely explanation is that proteolytic processing to the mature hybrid two-chain protein does occur but this form is not brought down by the antibodies under the experimental conditions used.

**Localization of Mutant and Hybrid Protective Proteins**—Given the differential behavior of mutant and hybrid protective proteins in transfected COS-1 cells, we analyzed their subcellular distribution by indirect immunofluorescence and immunogold labeling techniques. At light microscopy a typical lysosomal labeling pattern and a diffuse staining of the perinuclear region are observed in cells expressing the wild-type human, the HChl hybrid, and the SA\textsuperscript{150} mutant protective proteins (Fig. 5, A–C). The HQ\textsuperscript{129} mutant protein behaves similarly (not shown). In contrast, the CT\textsuperscript{400} precursor as well as the deletion mutants all seem to accumulate in the perinuclear region (Fig. 5, D–F).

For a more refined localization, ultrathin sections of transfected cells were probed with anti-32 antibodies followed by...
goat anti-(rabbit IgG)-gold and analyzed by electron microscopy. As shown in Fig. 6, overexpressed wild-type protective protein is compartmentalized in lysosomes and is detected in large amounts in the Golgi complex and rough ER (Fig. 6, A and B). A similar pattern is seen in cells transfected with the HChl hybrid protein, the SA<sup>150</sup> mutant (Fig. 6, C and D) and the HQ<sup>229</sup> protein (not shown). Immunogold labeling is restricted exclusively to the rough ER in cells expressing either the CT<sup>66</sup> mutant or the truncated subunits (not shown). COS-1 cells transfected with an antisense cDNA were used to estimate background labeling due to endogenous protective protein. The number of gold particles in lysosomes, Golgi complex, and rough ER was on average one or two. All together these results identify two types of modified protective proteins: those whose intracellular transport and processing overlap with wild-type protective protein (SA<sup>150</sup>, HQ<sup>229</sup>, HChl) and those that accumulate in the ER and are neither processed nor secreted (CT<sup>66</sup>, deletion mutants).

**Protective Protein Active Site Mutants Lack Cathepsin A-like Activity**—The similar characteristics observed thus far for the active site mutants with respect to wild-type protective protein imply that their tertiary structures are not grossly modified by the amino acid substitutions. We next ascertained whether cathepsin A-like activity was measurable in cells expressing these two mutant proteins compared to cells transfected with Hu54, Mo54, HChl, and 32(A20) constructs. Two independent assays were used. First, COS-1 cell extracts were incubated with [H]DFP, followed by immunoprecipitation with anti-54 antibodies (Fig. 7, upper panel). As we have shown before, human and mouse protective proteins are able to react with the inhibitor after proteolytic cleavage of their zymogens (Fig. 7, lanes 1 and 2). Only the large subunit, carrying the serine active site, is detectable. The mouse form is slightly bigger in size and reacts poorly with the antibodies. In contrast, neither the SA<sup>150</sup> containing a modified active site serine, nor the HQ<sup>229</sup> mutants show any binding capacity (Fig. 7, lanes 4 and 5). Likewise, the 32(A20) deletion mutant, missing the 20-kDa subunit, does not react with the inhibitor (Fig. 7, lane 6). Mature HChl hybrid molecules, if present, again are not immunoprecipitable (Fig. 7, lane 3). Cells transfected with an antisense wild-type construct were included in the experiment as estimate of the level of endogenous COS-1

![Fig. 6. Cryosections of COS-1 cells, transfected with normal human (A and B), HChl hybrid (C) or SA<sup>150</sup> mutant (D) protective proteins and labeled with anti-32 antibodies and goat anti-(rabbit IgG)-gold.](image)

A, shows extensive labeling of the Golgi complex (G) and lysosomes (L), in cells expressing the normal human protein. A low magnification of the perinuclear region is shown in B with extensive labeling of rough endoplasmic reticulum structures (R), but not of a mitochondrion (M). An identical labeling pattern is observed in cells expressing the HChl hybrid or SA<sup>150</sup> mutant protective proteins. Correct lysosomal targeting of these modified proteins is shown in (C) and (D). Bars, 0.1 μm.
protective protein (Fig. 7, lane 7). Since the SA<sup>50</sup> and HQ<sup>429</sup> mutants resemble most the wild-type protective protein, it is conceivable that all antibodies are efficiently competed out by unlabeled molecules only in these cell extracts. This explains the lack of signal in lanes 4 and 5 compared to lane 7.

The results obtained with DFP inhibitor are well supported by direct measurements of Z-Phe-Ala hydrolysis in lysates of cells transfected independently with the same set of constructs (Fig. 7, lower panel). Equal increase in cathepsin A-like activity above endogenous COS-1 levels is measured in the different cell extracts, using Z-Phe-Ala as substrate (lower panel). Hatched vertical bars represent rates of hydrolysis. One unit is defined as the enzyme activity that releases 1 μmol of alanine per min.

Confirmation that the SA<sup>156</sup> and HQ<sup>429</sup> mutants exert their protective function via physical association with β-galactosidase was obtained by examining the coprecipitation of this enzyme with different endocytosed protective proteins. Galactosialidosis cell lysates used to measure correction of β-galactosidase/neuraminidase activities were incubated with anti-54 antibodies and, as control, with preimmune serum or anti-native human β-galactosidase antibodies. Fig. 8 shows that 22, 25, and 34% of β-galactosidase activity is coprecipitated with anti-54 antibodies in cells that have taken up the wild-type human, the SA<sup>156</sup> and the HQ<sup>429</sup> mutant protective proteins, respectively. A comparable percentage of activity is coprecipitated in a normal human fibroblast homogenate. These results demonstrate that a proportion of active β-galactosidase is indeed associated with the SA<sup>156</sup> and HQ<sup>429</sup> mutant proteins, and that the enzyme has equal affinity for the active site mutants and wild-type protective protein. Furthermore, the values are specific since β-galactosidase activity is either not at all or to a lesser extent coprecipitated in cells treated with the HCh1 hybrid or mouse protective proteins.

### Table II

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<th>Cell strain</th>
<th>Addition of protective proteins</th>
<th>Activity</th>
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One milliunit is defined as the enzyme activity that releases 1 nmol of 4-methylumbelliferone per min.
sialidosis fibroblasts after uptake of various cell homogenates from a GM1-gangliosidosis patient were treated in the protein at acid pH cleaves the acylated dipeptides Z-Phe-Ala, and Z-Phe-Leu with clear preference for the first product. We now directly demonstrate that the protective protein precursors (abbreviated as in Fig. 4).

Fig. 8. Precipitation of β-galactosidase activity in galactosialidosis fibroblasts after uptake of various COS-1 cell-derived protective protein precursors. Early infantile galactosialidosis (E.I.GS) cell homogenates from the uptake experiment described in Table II were each divided in three aliquots and incubated with either preimmune serum, the anti-54 antibodies, or anti-native human β-galactosidase antibodies. As controls, normal human fibroblasts and cells from a GM1-gangliosidosis patient were treated in the same manner. After precipitation of antibody-antigen complexes, the remaining β-galactosidase activity was measured in the supernatants (vertical bars). The value obtained in preimmune serum treated cells after endocytosis of wild-type human protective protein is taken as 100% activity. Except for normal and GM1-gangliosidosis fibroblasts, all β-galactosidase activities are expressed as a percentage of this value. Cell homogenates to the right of the arrow represent E.I.GS fibroblasts that have taken up the different COS-1 cell-derived protective protein precursors (abbreviated as in Fig. 4).

respectively. This, in turn, is not surprising if we take into account the lower affinity of anti-54 antibodies for chicken and mouse mature protective proteins. On the other hand, a complex formed by human β-galactosidase and protective proteins from other species may be more susceptible to dissociation. In human fibroblasts neuraminidase is inactivated upon dilution or freeze/thawing, therefore its coprecipitation with the protective protein could not be examined.

DISCUSSION

The primary structure of human protective protein has suggested a putative carboxypeptidase activity by virtue of its homology with yeast carboxypeptidase Y and the KEX1 gene product. We now directly demonstrate that the protective protein at acid pH cleaves the acylated dipeptides Z-Phe-Ala, Z-Phe-Phe, and Z-Phe-Leu with clear preference for the first named substrate. This chymotrypsin-like activity closely resembles that of lysozymal cathepsin A (14). Several lines of evidence confirm this similarity: 1) monospecific antibodies against native human protective protein precipitate virtually all carboxypeptidase activity toward Z-Phe-Ala; 2) overexpression of protective protein in COS-1 cells leads to increased cathepsin A-like activity; 3) cells from a galactosialidosis patient deficient in protective protein mRNA have less than 1% residual cathepsin A-like activity.

Considering the highly specific binding of the protein to lysosomal β-galactosidase and neuraminidase, it was reasonable to assume that terminal processing of these two enzymes would be the principal role of the carboxypeptidase. Our genetic analysis, however, provides evidence that the catalytic and protective functions of the protective protein are distinct, since loss of its cathepsin A activity does not influence its ability to stabilize and activate the other two enzymes. These separable functions could relate to the existence of free and associated pools of protective protein and β-galactosidase in human tissues. A number of indications support this notion. Preliminary studies by gel filtration suggest that precursor and mature protective protein/cathepsin A can form homodimers of ~95 kDa free of β-galactosidase/neuraminidase. Conversely, the immunotitration experiments presented here have shown that not all β-galactosidase activity is coprecipitated with cathepsin A using anti-54 antibodies. Earlier data agree with these results since a fraction of β-galactosidase was found unassociated in crude glycoprotein preparations of human platelets (3).

The reason for maintenance of these different pools of enzymes could be the need to catabolize a broad spectrum of substrates in different metabolic pathways. A recent report by Jackman et al. (18) emphasizes this hypothesis. These authors, in an effort to characterize a deamidase released from human platelets, came to the unexpected finding that their purified enzyme is probably identical to the protective protein. They further demonstrate that in vitro this platelet enzyme has deamidase as well as carboxypeptidase activity on biologically important peptides, like substance P, bradykinin, angiotensin I, and oxytocin. The deamidase activity is optimal at neutral pH, whereas the carboxypeptidase works best at pH 5.5. The purified two-chain enzyme forms homodimers of 95 kDa at this pH (18). In view of the characteristics of the enzyme, they also came to the conclusion that it is similar to cathepsin A. We can deduce from our mutagenesis studies that the deamidase activity of the protective protein is also separable from its protective function. This does not exclude, however, that in lysosomes the cathepsin A/deamidase works in cooperation with β-galactosidase and neuraminidase. For example, an exopeptidase might be required after endoproteolytic cleavage of glycoprotein substrates, to trigger the efficient hydrolysis of their sugar side chains by the associated glycosidases. On the other hand, complex formation may modulate cathepsin A/deamidase activity. A better understanding of the functions of the protective protein requires the identification of substrates that are targets of the enzyme in vivo. It is noteworthy that protective protein mRNA expression is high in mouse kidney, brain, and placenta (13), suggesting the need of a cathepsin A/deamidase activity in these tissues, e.g. for the inactivation of bioactive peptides such as oxytocin and kinins.

Extended knowledge of the protective protein could arise from the analysis of individual galactosialidosis patients, done in light of the results reported here. These patients have so far been identified and diagnosed on the basis of their reduced β-galactosidase/neuraminidase activities. Only recently, a carboxypeptidase deficiency was reported for the first time in three late infantile/juvenile patients (44), although the less specific Z-Phe-Leu substrate was used in these studies. The ability to directly detect residual cathepsin A activity in patients will allow the identification of individuals having an isolated cathepsin A/deamidase deficiency but normal protective protein function. The creation of animal models having targeted cathepsin A/deamidase active site mutations could prove instructive in this context.

Except for the active site mutants the other modified human protective proteins are all retained in the ER. In the case of the mutant precursor with a cysteine to threonine substitution at position 60 this is likely due to improper folding of the precursor polypeptide (45). This cysteine residue is embedded within the 10-residue motif that is most conserved among all carboxypeptidases and must be important for their three-dimensional structures (46). Moreover, this domain in the protective proteins is part of an internal repeat occurring three times, e.g. in light of the results reported here. These patients have so far been identified and diagnosed on the basis of their reduced β-galactosidase/neuraminidase activities. Only recently, a carboxypeptidase deficiency was reported for the first time in three late infantile/juvenile patients (44), although the less specific Z-Phe-Leu substrate was used in these studies. The ability to directly detect residual cathepsin A activity in patients will allow the identification of individuals having an isolated cathepsin A/deamidase deficiency but normal protective protein function. The creation of animal models having targeted cathepsin A/deamidase active site mutations could prove instructive in this context.

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or intermolecular hydrophobic interactions. As deduced from the cotransfection experiments, the truncated 32/20 and 32SA/20 polypeptides can spontaneously associate in the ER, but are subsequently retained. This could imply that a single chain precursor is essential for correct transfer of the protective protein to the Golgi complex. Alternatively, aberrant assembly of the two chains could also cause retention, although we have indications that coexpression of the separate subunits in insect cells leads to a 3-4-fold increase in cathepsin A activity. The possibility that formation of an active dimer in the ER has a deleterious effect on de novo synthesized proteins awaits further investigations.

The crystallization of wheat zeine carboxypeptidase II has recently revealed remarkable structural homology of this enzyme to zinc carboxypeptidase A (43). It was speculated that these two proteases share a common ancestor, perhaps a binding protein that had divergently acquired greater catalytic activity by two different mechanisms. In this scenario binding to other proteins comes before catalytic activity. The protective protein is about 30% identical to wheat serine carboxypeptidase II. Interesting questions that arise are those of how the catalytic/protective activities of this pleiotropic member of the serine carboxypeptidase family have evolved and what came first.

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