Purification and Characterization of Human Lysosomal Protective Protein Expressed in Stably Transformed Chinese Hamster Ovary Cells*

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Chinese hamster ovary cells were transfected with a recombinant DNA containing the entire coding sequence of human lysosomal protective protein cDNA under the control of mouse metallothionein I promoter. Neomycin and methotrexate-resistant stably transformed cell lines expressing this protein were isolated. Immunoprecipitation of the product with anti serum against human placental protective protein-β-galactosidase complex revealed a 52-kDa protective protein precursor, which was then processed to mature form, a heterodimer of 32- and 20-kDa polypeptides. The precursor secreted in the culture medium was taken up by the mannose 6-phosphate receptor system and restored acid carboxypeptidase activity and neuraminidase activity in galactosialidosis fibroblasts. The expressed protein showed a granular pattern in intracellular distribution, was fractionated at the density of lysosomes, and had serine esterase activities; acid carboxypeptidase activity at pH 5.6, esterase at pH 7.0, and carboxy-terminal deamidase activity at pH 7.0. They were inhibited simultaneously by phenylmethylsulfonyl fluoride; Z-PCK, chloromethyl ketone derivative of N-benzyloxycarbonyl-L-phenylalanyl-L-leucine; N-benzoyloxycarbonyl-1-phenylalanine chloromethyl ketone, or iodoacetamide. The acid carboxypeptidase activity of the purified monomeric protein (13). We demonstrated simultaneous deficiency of neuraminidase (5-7) and β-galactosidase (EC 3.2.1.23) and neuraminidase activities (11, 12). On the other hand, an enzyme with deamidase activity, esterase, acid carboxy peptidase, and cathespin A-like activities was purified from human platelets, which modified the carboxyl termini of tachykinins (13) and endothelin 1 (14). The sequences of the N-terminal 25 amino acids in each of its two polypeptide chains were identical with those deduced for two polypeptides of the mature form protective protein (13). We demonstrated simultaneous deficiency of activities in galactosialidosis fibroblasts (15). A significant increase of cathepsin A-like activity was confirmed in COS-1 cells transiently expressing the cDNA (16).

In this study, we established a stably transformed Chinese hamster ovary (CHO) cell line overexpressing human protective protein and multifunctional serine esterase (acid carboxypeptidase, esterase, and neuraminidase) activities. The gene product was then purified and characterized. Its activity was stabilized by saposins (sphingolipid activator proteins).

EXPERIMENTAL PROCEDURES

Materials—G418, a neomycin derivative, was purchased from Gibco, methotrexate (MTX), N-benzoyloxycarbonyl-L-phenylalanine (Z-Ph-Leu), benzoyloxycarbonyl ethyl ester, sodium p-mannose 6-phosphate (Man-6-P), phenylmethylsulfonyl fluoride (PMSF), N-benzoyloxycarbonyl-L-phenylalanine chloromethyl ketone (CHF), aspartic acid, and skeletal dysplasia (1, 3).

This glycophosphoprotein is processed in human fibroblasts from a precursor to a mature form, a heterodimer of two polypeptides held together by disulfide bonds (2, 4), and aggregates with β-galactosidase to form a high molecular weight complex also involving neuraminidase (5-7). The precursor protein secreted from normal fibroblasts treated with ammonium chloride is taken up by galactosialidosis fibroblasts via the mannose 6-phosphate receptor system and restores β-galactosidase and neuraminidase activities (1, 2).

A cDNA encoding this protein was cloned by Galjart et al. (8), and the deduced amino acid sequence was found to share homology to yeast carboxypeptidase Y and KEX1 gene product (8, 9) as well as plant carboxypeptidase (10). In fact, acid carboxypeptidase activity, with optimal pH at 5.5, is deficient in fibroblasts and lymphoblastoid cells from galactosialidosis patients (11, 12). On the other hand, an enzyme with deamidase activity, esterase, acid carboxy peptidase, and cathespin A-like activities was purified from human platelets, which modified the carboxyl termini of tachykinins (13) and endothelin 1 (14). The sequences of the N-terminal 25 amino acids in each of its two polypeptide chains were identical with those deduced for two polypeptides of the mature form protective protein (13). We demonstrated simultaneous deficiency of their activities in galactosialidosis fibroblasts (15). A significant increase of cathepsin A-like activity was confirmed in COS-1 cells transiently expressing the cDNA (16).

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Galactosialidosis is a hereditary metabolic disease caused by a genetic defect of a glycoprotein in the lysosome (protective protein), resulting in a marked decrease of β-galactosidase activity and neuraminidase activities in galactosialidosis fibroblasts. The expressed protein showed a granular pattern in intracellular distribution, was fractionated at the density of lysosomes, and had serine esterase activities; acid carboxypeptidase activity at pH 5.6, esterase at pH 7.0, and carboxy-terminal deamidase activity at pH 7.0. They were inhibited simultaneously by phenylmethylsulfonyl fluoride, N-benzoyloxycarbonyl-1-phenylalanine chloromethyl ketone, or iodoacetamide. The acid carboxypeptidase activity of the purified monomeric mature protective protein was labile in vitro under the acidic condition. Saposins (sphingolipid activator proteins) stabilized the activity at micromolar level concentrations.

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(ZPCK), iodocetamide, CHAPS, and n-octyl $b$-d-glucopyranoside (n-octyl glucoside) from Sigma; restriction enzymes and T4 DNA ligase from Nippon Gene (Toyama, Japan); p- Ala-Met-enkephalinamide (DME-NH$_2$), leupeptin, pepstatin A, and phosphoramidom from Peptide Institute (Osaka, Japan); and Triton X-100 from NakalaiTesque (Kyoto, Japan). $b$-Galactosidase complex antiserum ($b$-Gal MNT) was kindly supplied by Dr. K. Inui (Osaka University, Osaka, Japan).

Cell Culture—Skin fibroblasts were obtained from patients with late onset galactosidosis. The diagnosis of the disease was established by clinical manifestations and enzyme assays (3). CHO cells and skin fibroblasts were routinely maintained in Ham's F-10 medium supplemented with 10% fetal calf serum (FCS). For enzyme purification, the transformed cells were cultured for a week in a roller bottle (Falcon 303.5, Becton Dickinson Labware, NJ; surface area 1750 cm$^2$) with FCS-free GTC medium (Wako Pure Chemicals, Osaka, Japan; 300 ml/bottle) in the presence of 4 $\mu$M MTX.

Enzyme Assays—$b$-Galactosidase, neuraminidase, and $b$-hexosaminidase activities were assayed fluorometrically (17). Serine esterase activities of protective protein were measured as described previously (15) using Z-Phe-Leu for acid carboxypeptidase, benzoyltyrosine ethyl ester for esterase, and DME-NH$_2$ for deamidase as substrates. Protein determination was performed by the method of Bradford (18) using bovine serum albumin as standard.

For characterization of acid carboxypeptidase activity, some protease inhibitors or active site reagents were added to the assay mixture in 20 mM sodium acetate (pH 5.6). They did not interfere with determination of liberated L-leucine. 

Expression Vector—The expression vector pMSXND (19) was kindly provided by Drs. J. W. Kyle and W. S. Sly (St. Louis University School of Medicine, St. Louis, MO). The cDNA for human lysosomal protective protein cloned in our laboratory (20) was subcloned into the XhoI site of pMSXND, which also contained the genes for both G418 resistance and mouse dihydrofolate reductase, and designated as pMSXND(P111).

Transfection of CHO Cells and Selection of Stably Transformed Cells—The procedures were performed as described by Kyle et al. (19) with minor modifications. CHO cells (1-2 $\times$ 10$^5$ cells) were seeded on 60-mm dishes 18 h prior to the addition of DNA. Transfection was carried out using a lipofection reagent (Berthold Research Laboratories) (21) containing 20 $\mu$g of pMSXND or pMSXND(P111). The lipofection reagent-plasmid DNA complex was left on the cells in the FCS-free medium for 15 h, and then an equal volume of the medium containing 20% FCS was added. After 48 h in culture, the cells on each 60-mm dish were trypsinized and split 1:5 into two 100-mm dishes and a selection mixture (Ham's F-10 with 10% FCS and 400 $\mu$g/ml G418) was added. After 18 days, G418-resistant colonies were isolated and further cultured in the first amplification medium (Ham's F-10 with 10% dialyzed FCS and 0.2 $\mu$M MTX). The MTX concentration was increased four times every 4 days up to 4 $\mu$M. The selected cell lines were subcloned, and the buffer composition was changed three times, harvested, and homogenized in 0.2 ml of distilled water by sonication for 5 s. Enzyme activities and protein concentration in the homogenate were measured as described above.

Immunotitration of Protective Protein—The transformed cells (1-2 $\times$ 10$^6$) harvested from 20 roller bottle cultures were homogenized in distilled water containing 0.25 M leupeptin and 1 mM EDTA by sonication for 10 s and then centrifuged at 100,000 $\times$ g for 1 h at 4°C. Each 25 $\mu$l of the supernatant was incubated with various dosages of serum IgG in 20 mM sodium phosphate (pH 6.0) (final volume, 50 $\mu$l) and left for 3 h at 4°C. The IgG was prepared from the $b$-galactosidase complex antisera or from normal rabbit serum by ammonium sulfate precipitation (60% saturation) and 5-fold dialysis in 0.1 M sodium acetate buffer, pH 5.6. The mixture was diluted 1:8 with water (pH 4.0). Mainly acid carboxypeptidase activity was monitored for protective protein, but deamidase activities were also assayed. The reactions with enzyme activities were stopped and stored at -80°C. The combined supernatant was concentrated to 1 ml, and the buffer concentration was further diluted two times with water.

Uptake of Protective Protein Precursor in Galactosidosis Fibroblasts—The preparation described above was thawed and sterilized by passing through a 0.22-nm membrane filter (Mirex GV, Millipore, Tokyo, Japan). Galactosialidosis fibroblasts were seeded on 35-mm dishes in subconfluency and cultured in 2 ml of regular F-10. After a 3-day incubation, the culture medium was replaced with fresh medium.

Purification of Mature Protective Protein—One-ninth volume of 0.25 M imidazole HCl buffer (pH 7.4) was added to the cell extract prepared as described above, and then the suspension (12 ml) was loaded (120 ml/h) on a chromatofocusing column (Pharmacia PBE94, 0.9 x 30 cm) preequilibrated with 25 mM imidazole HCl buffer (pH 7.4). The column was washed with 2.5 volumes of the buffer, and the enzyme was eluted (12 ml/h) with Polybuffer 74 (Pharmacia) that had been diluted 1:8 with water (pH 4.0). Mainly acid carboxypeptidase activity was monitored for protective protein, but deamidase and esterase activities were also assayed. The fractions with enzyme activities were pooled and stored at -80°C. Any remaining activity was assayed.

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Finally, the fraction was concentrated to 1 ml, and 1 ml of 4 M ammonium sulfate was added. The suspension was loaded (15 ml/h) on a DEAE-cellulose DE52 column (Amicon Corp., 10 cm x 35 cm; Tosoh, Tokyo, Japan) preequilibrated with 20 mM sodium phosphate (pH 6.3) containing 2 M ammonium sulfate and washed with the same buffer. After the absorbance of the effluent at 280 nm was

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decreased to the base line, protein was eluted with a pH gradient (6.3-8.0)/ammonium sulfate gradient (2.0-0 M) made by the starting buffer and 20 mM sodium phosphate buffer (pH 8.0). The fractions with enzyme activities were pooled, and 0.01 volume of 10% CHAPS was added to each fraction.

The buffer composition was then changed to 20 mM sodium acetate buffer (pH 4.8) containing 2 mM CHAPS with Centriprep 30. The fraction was concentrated to 1 ml and applied to a Mono S column (HR5/5, Pharmacia) preequilibrated with the same buffer and eluted with a sodium chloride gradient (0-0.3 M) at room temperature. The fractions with enzyme activities were pooled and stored at -80 °C.

Purity of the preparation was monitored by SDS-PAGE on a 10-20% gradient gel. Proteins were visualized with a silver stain kit (Wako Pure Chemicals) or by immunostaining. SDS-PAGE gel was electroblotted to a nitrocellulose membrane (BA 85, Schleicher and Schuell) (25) and probed with a 1:500 dilution of the β-galactosidase complex antiserum. Detection of immunoreactive bands was performed with a blotting detection kit for rabbit antibodies (RPN 23, Amersham).

Gel Filtration—The molecular mass of purified protective protein was determined by gel filtration according to the method of Siegel and Monty (26) on a TSK gel G3000SWXL column (0.78 × 30 cm) preequilibrated and eluted with 20 mM sodium acetate buffer, pH 5.6, containing 0.1 M NaCl and 2 mM CHAPS at a flow rate of 0.5 ml/min. The protein preparation was applied in a volume of 25 μl, and 0.5-ml fractions were collected.

Characterization of Acid Carboxypeptidase Activity—The stability of acid carboxypeptidase activity in the purified mature protective protein was examined in the Ether-5PW preparation of 37 °C, either at pH 7.2 in 40 mM sodium acetate or at pH 7.2 in 40 mM sodium phosphate (total volume, 50 μl; activity, 30 nmol/h for each experiment). In some experiments, saposin, control proteins (lysozyme, bovine serum albumin), or laboratory detergent was added. Prosaposin was purified from human milk (27), and saposins A, B, C, and D were purified from human Gaucher disease spleen (28, 29).

RESULTS

Expression of Protective Protein in Transformed CHO Cells—Twenty-four G418-resistant cell lines were selected after transfection with the full-length protective protein cDNA. Immunoprecipitation revealed 66-, 52-, 32-, and 20-kDa polypeptides expressed by each transformed cell line (Fig. 1, lanes 1, 5, 7, and 9). They were not detected in mock transfected cells (lanes 3 and 4). After a chase of 6 h, their intensities decreased (lanes 2, 6, 8, and 10). Immunoreactive bands were not detected in the culture medium (data not shown).

Intracellular Distribution of Expressed Protective Protein—Indirect immunofluorescence staining was performed with the β-galactosidase complex antiserum for subcellular localization of expressed protective protein. A granular pattern was observed (Fig. 2). Mock transfected cells reacted poorly with the antiserum (data not shown).

Restoration of Enzyme Activities in Galactosialidosis Fibroblasts by Protective Protein Precursor—β-Galactosidase and neuraminidase activities were restored in galactosialidosis fibroblasts after the preparation of protective protein precursor from the transformed CHO cells was added in the culture medium (Table I). Acid carboxypeptidase activity was also increased. These changes were not observed when Man-6-P was added in the culture medium. We concluded that functionally active protective protein was expressed in the cells and secreted in the culture medium in the presence of ammonium chloride.

Expression of Serine Esterase Activities—Serine esterase activities were increased in the stably transfected cells; acid carboxypeptidase 7-fold, esterase 2-fold, and deamidase 1.4-fold, as compared with mock transfected cells (Fig. 3). The basal levels of the endogenous activities of parental CHO cells were different among the three enzyme activities, and therefore, the calculated amounts of increase were variable.

Fig. 1. Immunoprecipitation of human protective protein expressed in stably transfomed CHO cells. CHO cells were transfected with pMSXND (lanes 1, 2, and 5-10) or with pMSXND vector alone (lanes 3 and 4) and selected for resistance to G418 and MTX as described under "Experimental Procedures." The isolated clones were labeled with [35S]methionine/[35S]cysteine mixture for 16 h. The cells were then harvested (odd lanes) or chased for 6 h in the medium containing unlabeled methionine and cysteine (even lanes). The labeled proteins were immunoprecipitated from cells using protective protein-β-galactosidase complex antisera. Proteins were separated by SDS-PAGE under the reducing condition and visualized by fluorography. Prestained SDS-PAGE standards (Bio-Rad) were used as molecular mass standard proteins: phosphorlyase 6, 110 kDa; bovine serum albumin, 84 kDa; ovalbumin, 50 kDa; carbonic anhydrase, 33 kDa; soybean trypsin inhibitor, 24 kDa; and lysozyme, 16 kDa.

Fig. 2. Immunocytochemical distribution of protective protein expressed in transformed CHO cells. They were seeded in subconfluent and, 18 h later, fixed and incubated with protective protein-β-galactosidase antisera. Bar, 10 μm.

Immunotitration of Acid Carboxypeptidase Activity—Both β-galactosidase and acid carboxypeptidase activities in the transformed cells were dose dependently immunoprecipitated together, by the β-galactosidase complex antisera, by 50 and 70%, respectively, with 10 μl of the antisera (Fig. 4). Neither of them was precipitated by control serum. This result indicated the presence of acid carboxypeptidase activity in the β-galactosidase-protective protein complex. Endogenous CHO β-galactosidase was less efficiently precipitated probably due to low cross-immunoreactivity with human antibody.

Subcellular Distribution of Acid Carboxypeptidase Activity—Half of the acid carboxypeptidase activity in the postmitochondrial fraction was found in the heavy fraction (density, 1.06 g/ml) and another half in the light fraction (density, 1.04 g/ml) (Fig. 5). β-Hexosaminidase and β-galactosidase were recovered as lysosomal marker enzymes in the light fraction. This result indicates that half of the expressed protein was localized in the lysosome; the remainder stayed in heavy density granules and was not associated with the β-galactosidase molecule.

Effects of Protease Inhibitors on Acid Carboxypeptidase Ac-
TABLE I

Restoration of β-galactosidase, neuraminidase, and acid carboxypeptidase activities in galactosialidosis fibroblasts after addition of protective protein precursor

The precursor fraction prepared as described under "Experimental Procedures" was added to the culture medium of galactosialidosis fibroblasts. After 3 days, cells were harvested, and enzyme activities were measured. Substrates are 4-methylumbelliferyl derivatives for neuraminidase, β-galactosidase, and β-hexosaminidase and Z-Phe-Leu for acid carboxypeptidase. Enzyme activity is expressed as nmol/h/mg of protein (acid carboxypeptidase, neuraminidase, β-galactosidase) or as μmol/h/mg of protein (β-hexosaminidase) (mean ± S.D., four experiments).

| Addition                  | Carboxypeptidase (μmol/h/mg) | Neuraminidase (μmol/h/mg) | β-Galactosidase (μmol/h/mg) | β-Hexosaminidase (μmol/h/mg) |
|---------------------------|------------------------------|---------------------------|----------------------------|-----------------------------|
| Galactosialidosis*        |                              |                           |                            |                             |
| Precursor protein         | 1078 ± 64                    | 55.7 ± 7.31               | 119 ± 3.72                 | 2.98 ± 0.07                 |
| Precursor + Man-6-P       | 355 ± 44                     | 10.0 ± 7.45               | 22.1 ± 7.30                | 3.35 ± 0.25                 |
| No addition               | 334 ± 32                     | 2.5 ± 0.6                 | 14.8 ± 0.70                | 3.66 ± 0.27                 |
| Normal                    | 4069 ± 1574                  | 36.0 ± 0.95               | 479 ± 30.0                 | 3.24 ± 0.79                 |

*Enzyme activities in galactosialidosis fibroblasts with or without addition of precursor protective protein (100 μl) and/or Man-6-P (5 mM).

**Normal levels of the enzymes in normal human fibroblasts (n = 9).**

![Fig. 3. Serine esterase (acid carboxypeptidase, esterase, deamidase) activities in transformed CHO cells expressing protective protein.](image1)

![Fig. 4. Immunotitration of β-galactosidase and acid carboxypeptidase activities in transformed CHO cells.](image2)

![Fig. 5. Subcellular fractionation of acid carboxypeptidase activity by Percoll density gradient centrifugation.](image3)

![Table II. Inhibition of acid carboxypeptidase activity](table2)

**Activity**—The effects of protease inhibitors and active site reagents were examined on the acid carboxypeptidase activity in the extract from transformed cells. It was almost completely inhibited by PMSF (active site serine reagent) at 5 mM and partly by ZPCK (histidine reagent; 60% inhibition at 20 mM) or iodoacetamide (thiol reagent; 60% inhibition at 10 mM). Low molecular weight protease inhibitors of microbrial origin (30), such as leupeptin, pepstatin A, or phosphoramidon, did not affect the activity at pH 5.6 (Table II).

**Purification of Mature Protective Protein Monitored by Serine Esterase Activities**—Table III summarizes the purification of the enzyme. The total acid carboxypeptidase activity in the extract from 1-2 x 10^6 transformed cells was 742 μmol/h/mg of protein. About 51% of the initial acid carboxypeptidase activity was recovered in the two peaks of chromatofocusing (pH 5.6 and 5.3-4.3). The first peak contained 3.6% of the β-
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TABLE III

Purification of mature protective protein

| Procedure          | Volume / Protein | Total activity / Specific activity / Yield / Purification |
|--------------------|------------------|----------------------------------------------------------|
|                    | ml / mg          | a / b / c / μmol/h / μmol/h/mg protein / a / b / c / % / -fold |
| 100,000 × g supernatant Chromatofocusing | 12 / 63 | 747 / 1783 / 5.34 | 11.9 / 28.3 / 0.085 / 100 / 100 / 100 / 1 / 1 |
| Peak I            | 25 / 3.1         | 110 / 585 / 1.31 | 36.0 / 189 / 0.427 / 15.0 / 32.8 / 24.5 / 3.0 / 6.7 / 5.0 |
| Peak II           | 95 / 3.6         | 270 / 999 / 2.01 | 7.6 / 278 / 0.567 / 36.4 / 56.0 / 37.7 / 6.4 / 9.8 / 6.7 |
| Ether-5PW         | 3.5 / 0.13       | 94.8 / 402 / 0.70 | 754 / 3091 / 6.22 / 14.0 / 22.5 / 13.0 / 64 / 109 / 73 |
| Mono S            | 2.5 / 0.023      | 61.8 / 176 / 0.47 | 2687 / 7640 / 20.4 / 8.27 / 9.9 / 8.8 / 226 / 270 / 240 |

Fig. 6. SDS-PAGE and immunoblotting of purified protective protein from transformed CHO cells. The enzyme preparation (about 0.2 μg) was electrophoresed on a 10–20% gradient polyacrylamide gel in the presence of SDS after reduction with 2-mercaptoethanol. Proteins were visualized by silver staining (panel A) or by immunostaining (panel B) as described under “Experimental Procedures.” Prestained SDS-PAGE standards as described above (panel B) or biotinylated SDS-PAGE standards (Bio-Rad) (panel A) were used as molecular mass standards: rabbit muscle phosphorylase b, 97.4 kDa; bovine serum albumin, 66.2 kDa; hen egg white ovalbumin, 45 kDa; bovine carbonic anhydrase B, 31 kDa; soybean trypsin inhibitor, 21.5 kDa; and hen egg white lysozyme, 14.4 kDa.

galactosidase activity in the starting material and the second peak 24.3%.

The first peak with relatively high specific activity of acid carboxypeptidase and low β-galactosidase activity was concentrated and was applied to hydrophobic chromatography and then to Mono S. Initially, the enzyme activity disappeared almost completely after the second step (Ether-5PW); it was later found that the enzyme was unstable and lost its activity simply by standing at 4 °C for several hours. Detergents were found to maintain the enzyme activity. We therefore added 2 mM CHAPS for subsequent procedures. The recovery of the enzyme activity was 8%, and the degree of purification was about 230-fold. Esterase and deamidase activities were copurified with acid carboxypeptidase activity.

The resultant fraction contained two polypeptides of 32 and 20 kDa in SDS-PAGE under the reducing condition (Fig. 6A). They were immunostained by the β-galactosidase complex antisera (Fig. 6B). The 20-kDa polypeptide was more intensely stained because of a higher content of IgG against it in the antiserum. β-Galactosidase or neuroaminidase activity was hardly detected in this preparation. The molecular mass of purified enzyme was estimated to be approximately 52.5 kDa by gel filtration at pH 5.6 (Fig. 7). It was probably a monomeric mature protective protein. The value did not change significantly when estimated at pH 6.8 (data not shown).

Stability of Acid Carboxypeptidase Activity—The purified protective protein lost its acid carboxypeptidase activity in the eluate of hydrophobic chromatography after incubation at 37 °C for 1 h, either at pH 4.4 or at pH 7.2 (Fig. 8). This inactivation was almost completely protected by 2 mM CHAPS under the acidic condition but not at neutral pH. Other detergents also showed the same effect as CHAPS: Triton X-100 at 0.02–0.2 mM and n-octyl glucoside at 2–10 mM (data not shown).

We then surveyed the effects of saposins as natural detergents of lipid substrates or as acid hydrolase activators. As shown in Fig. 9, each molecular species of saposins A–D showed stabilizing effects on the enzyme activity under the acidic condition. No effect was observed at neutral pH. The half-maximal effective dose was 3.6 μg/ml (360 nM) for saposin A, 1.4 μg/ml (140 nM) for saposin B, 4.9 μg/ml (490 nM) for saposin C, and 5.4 μg/ml (540 nM) for saposin D. Saposin B was slightly more effective than the others. Their precursor prosaposin as well as lysozyme did not have remarkable effects on the enzyme activity. Bovine serum albumin also showed the same effect, but the half-maximal dose was much higher as compared with those for saposins.
pressed serine esterase activities assayed as acid carboxypeptidase activity of protective protein was synthesized as a 52-kDa precursor, which was processed to a mature heterodimer of 32- and 20-kDa polypeptides. Immunofluorescence staining of the transformed cells showed a granular pattern characteristic of lysosomes. The precursor form of this protein, secreted in the culture medium in the presence of alkalizing agent, was taken up via the Man-6-P receptor pathway and restored enzyme activities in galactosialidosis fibroblasts. These transformed cells expressed serine esterase activities assayed as acid carboxypeptidase, esterase, and deamidase. About half of the acid carboxypeptidase activity was recovered in the same fraction as other lysosomal enzymes by subcellular fractionation. This result indicates that the expressed protective protein was transported to lysosomes.

We reported that galactosialidosis cells are deficient in serine esterase activities as well as β-galactosidase and neuraminidase activities (12, 15). In this study, we further demonstrated that the mature form of protective protein was multifunctional serine esterase activities in a single molecule on the basis of the following observations: 1) simultaneous increase of protective effects and enzyme activities; 2) immunoprecipitation of acid carboxypeptidase activity by an antiserum against protective protein-β-galactosidase complex; 3) the same sensitivity to three enzyme inhibitors acting on different sites of the protein (11, 12), PMSF as serine reagent, ZPCK as histidine reagent, and iodoacetamide as a cysteine reagent; 4) the presence of neutral esterase and deamidase activities in a purified protective protein preparation; 5) the purified protein was recovered as a heterodimeric form composed of two smaller polypeptides (32 and 20 kDa) that were detected by an antiserum against the protective protein-β-galactosidase complex.

Protective protein interacts with β-galactosidase and neuraminidase in the lysosome to form a high molecular weight aggregate (5-7), and an inactive form of neuraminidase protein was activated by association with β-galactosidase and protective protein (31). In our present study, overexpressed human protective protein in transformed CHO cells was purified as a monomeric mature form dissociated from the complex with β-galactosidase and neuraminidase. The monomeric enzyme was labile at acidic pH. Jackman et al. (13) reported that purified human platelet deamidase, probably identical with mature protective protein, has a homodimeric structure in gel filtration at pH 6.0. These results suggest that serine esterase activity of protective protein is also stabilized by self-dimerization or by aggregate formation with other proteins in lysosomes. The interaction may be physiologically important for mutual stabilization of the three functional proteins in the cell.

Recently, Zhou et al. (32) demonstrated that the protective protein precursor overexpressed in COS cells exists as a homodimer at neutral pH. They also identified a point mutation in the protective protein gene from a patient with late infantile galactosialidosis, causing a substitution of Phe-412 with Val in the gene product. The expressed mutant precursor protein was partially retained in the endoplasmic reticulum and did not form the homodimeric structure. The dimerization process might be a condition for the proper targeting from the endoplasmic reticulum to endosome or prelysosome and for stable conformation of the protein (32). It is possible that a specific mutation of protective protein gene causing a defect in aggregate formation leads to inactivation of serine esterase function, even if the enzyme activities are not lost by the mutation itself, in some cases of galactosialidosis.

In this study, the enzymatic instability of dissociated mature protective protein was restored by experimental detergents or saposins in vitro. Saposins are heat-stable proteins of low molecular mass (8-13 kDa) necessary for hydrolysis of sphingolipids by lysosomal hydrolases (33). They are produced by a specific proteolytic processing of a 70-kDa precursor protein (saposin) consisting of four structurally similar domains (34-36). Each of them (saposins A-D) is approximately 800 amino acids long, with six similarly spaced cysteine residues, a glycosylation site, and specifically located proline residues. Saposin B binds and solubilizes sphingolipid substrates (sulfatide, ganglioside GM1, and globotriaosylceramide) (37-39). Saposin C associates with glucosylceramidase or galactosylceramidase and activates the enzyme (28, 40, 41).
Saposin D has been reported to act as a sphingomyelinase activator (29). The modes of intracellular processing are different among various tissues, and their intermediate forms of proteolytic conversion have been detected (28).

This is the first demonstration of the stabilizing effect of saposins on protective protein with serine esterase activities. Further analysis of intermolecular assembly between protective and β-galactosidase proteins will provide us with a clue to the molecular mechanism of multienzymic complex formation in the lysosome.

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