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DISTRIBUTION OF LYSOSOMAL PROTECTIVE PROTEIN IN HUMAN TISSUES

Akira Satake[1]* , Kohji Itoh[1], Michie Shimmoto[1], Takaomi C.Saido[2], Hitoshi Sakuraba[1], and Yoshiyuki Suzuki[1]

Departments of [1]Clinical Genetics and [2] Molecular Biology
The Tokyo Metropolitan Institute of Medical Science,
Honkomagome, Bunkyo-ku, Tokyo 113, Japan

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We raised two polyclonal antibodies against synthetic oligopeptides comprising amino acid sequences in the human lysosomal protective protein. The first antibody recognized the 54-kDa precursor and the N-terminal sequence of the 32-kDa mature protein subunit, and the second one recognized the precursor and the C-terminal sequence of the 20-kDa subunit. In normal fibroblasts, mature protective protein was detected on immunoblotting with these antibodies. Considerable amounts of mature protective protein also were detected in kidney, lung, liver, and spleen, but not in brain from a patient with Gaucher disease. Neither the precursor nor the mature protective protein was detected in cultured fibroblasts, liver or cerebrum from a galactosialidosis patient with protective protein deficiency.

Protective protein is a lysosomal glycoprotein that forms a high-molecular-weight complex with lysosomal β-galactosidase (EC 3.2.2.23) and neuraminidase (EC 3.2.1.18). In this complex, β-galactosidase is stabilized and neuraminidase is activated. A genetic defect in the protective protein results in a combined deficiency of both enzymes, galactosialidosis (1,2).

This protein also has several enzyme activities itself, such as those of acid carboxypeptidase, deamidase and esterase (3). These enzyme activities are lost in fibroblasts from galactosialidosis patients (4), but their pathophysiological significance is not known at present. The carboxypeptidase activity of the protective protein is distinct from its protective function (5). The protective protein is therefore considered to possess two or more functional domains.

In normal fibroblasts, the protective protein is synthesized as a 54-kDa precursor that is enzymatically inactive, and then processed to a heterodimer of 32-kDa and 20-kDa subunits that are held together by disulfide bridges. In this study, we raised two polyclonal antibodies against synthetic peptides, each comprising a part of one of the subunits constituting the protective protein. They were used for further characterization of the protective protein in human fibroblasts and for a survey of tissue-specific expression in human autopsy specimens.

*Corresponding author. Fax:81-3823-6008.

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MATERIALS AND METHODS

Preparation of cell and tissue extracts

Skin fibroblasts from a normal subject and two galactosialidosis patients (type II) (6) were cultured in Ham's F-10 medium supplemented with 10% fetal calf serum (FCS) and antibiotics. The diagnosis of galactosialidosis was established on the basis of clinical findings and the results of enzyme assays. The genotype was Y395C/SpDEx7 for patient 1 (6; case 5), and SpDEx7/SpDEx7 for patient 2 (7; case 4). Chinese hamster ovary (CHO) cells were stably transformed to express human protective protein (3). The cells were maintained in Ham's F-10 medium supplemented with 4% FCS and 20μM methotrexate (MTX).

The cells grown to confluency were washed three times with phosphate-buffered saline (PBS), harvested by scraping, and then suspended in distilled water. The suspension was homogenized by sonication, and centrifuged at 12,000 x g for 5 min at 4°C. The supernatant was stored as the cell extract at -20°C before use.

Tissues from a Gaucher disease patient and a type II galactosialidosis patient (8) were obtained at autopsy and stored at -80°C. The tissues were homogenized in 20 mM phosphate buffer (pH 6.0), containing 0.1 M NaCl, 5 mM EDTA and 1 mM leupeptin, and each homogenate was centrifuged at 12,000 x g for 15 min at 4°C. The supernatant was used for the carboxypeptidase assay and protein determination, an aliquot being stored at -20°C before immunoblotting.

Preparation of the protective protein precursor

The transformed CHO cells described above were washed three times with PBS, and cultured further in Ham's F-10 medium supplemented with 10 mM NH_4Cl and 20μM MTX. After three days' culture, the culture medium was collected and concentrated to 1 ml with Centriprep-30 (Amicon, Beverly, MA). The concentrated medium was diluted with 9 ml of PBS, and concentrated again. This procedure was repeated three times. The final concentrate (1 ml), a precursor-rich solution, was stored at -20°C before use.

Tryptic digestion of the protective protein precursor

The concentrated precursor solution was incubated at 37°C for 30 min with an excess amount of trypsin. The tryptic digestion was stopped with leupeptin (final concentration 5 mM), or by boiling with sodium dodecyl sulfate (SDS)-sample buffer for immunoblotting.

Preparation of synthetic peptides and their antibodies

We designed two synthetic oligopeptides. The first peptide was a 13mer peptide corresponding to the 12 amino acid residues at the N-terminus of the 32-kDa subunit of the protective protein (PP32N12). Cysteine was added to the C-terminus for linking to a carrier protein. The second peptide consisted of cysteine plus the 12 amino acid residues at the C-terminus of the 20-kDa subunit (PP20C12). They were prepared at Takara Shuzo Co. (Kyoto, Japan), and were covalently conjugated to keyhole limpet hemocyanin (KLH; Calbiochem, La Jolla, CA) via a heterobifunctional cross-linker, m-maleimidobenzoyl-N-hydroxysuccinimide ester (Pierce, Rockford, IL), by the method of Lerner et al. (9). New Zealand white rabbits were immunized by subcutaneous injection of the peptide-KLH conjugate (0.5 mg peptide equivalent) in Freund's complete adjuvant. Two weeks later, the conjugate (0.25 mg peptide equivalent) in Freund's incomplete adjuvant was injected intramuscularly. Booster immunizations were performed with the same dose of the conjugate at 2-week intervals. Antiserum was obtained 2 weeks after the last injection. The IgG fraction of the antiserum was prepared with a protein A-Cellulofine (Seikagaku Co., Tokyo, Japan) gel column (10).

SDS-Polyacrylamide gel electrophoresis (PAGE) and immunoblotting

An aliquot of the precursor-rich solution or its tryptic product was subjected to SDS-PAGE on a 10-20% gradient gel (11). Proteins were visualized by immunoblotting with either of the antisera (200-fold dilution) or with anti-high-molecular-weight complex (anti-HMW) antiserum (12). Immunostaining was performed as described previously (13). Prestained SDS-PAGE standards (Bio-Rad, Hercules, CA) were used as molecular mass standards.

Immunoprecipitation

The normal fibroblast extract (25 μl) was incubated at 4°C for 1 h with 5-20 μl of the IgG fraction of the anti-PP32N12, anti-PP20C12, or anti-HMW antiserum, incubated with protein A-

Cellulofine at 4°C for 1 h, and centrifuged at 12,000 x g for 5 min. The supernatant was used for the assay of carboxypeptidase activity.

Enzyme assays

Carboxypeptidase activity was measured with *N*-benzyloxycarbonyl-L-phenylalanyl-L-leucine as a substrate (4). β-Galactosidase and neuraminidase activities were assayed fluorometrically as described previously (14). Protein was determined with the DC Protein Assay Kit (Bio-Rad).

RESULTS

Characterization of the antibodies

For normal fibroblasts, two bands corresponding to the two mature protective protein subunits, 32 kDa and 20 kDa, were detected on immunoblotting with the anti-HMW antiserum. In addition, the 32-kDa band was detected with the anti-PP32N12 antiserum, and the 20-kDa band with the anti-PP20C12 antiserum (Fig. 1). Neither of them was detected in the two strains of galactosialidosis fibroblasts.

With the precursor-rich solution, a 54-kDa band was visualized with the anti-HMW antiserum, anti-PP32N12 antiserum, and anti-PP20C12 antiserum, but not with pre-immune serum (Fig. 2). Carboxypeptidase activity was low in this preparation. The 54-kDa band disappeared on tryptic digestion, and three new bands were detected; 34 kDa and 32 kDa with the anti-PP32N12 antiserum, and 20 kDa with the anti-PP20C12 antiserum. The enzyme activity increased (Fig. 2). They were also detected with the anti-HMW antiserum.

Carboxypeptidase activity in normal fibroblasts decreased to 5% by immunoprecipitation with 20 μl of anti-HMW IgG (data not shown). Neither anti-PP32N12 IgG nor anti-PP20C12 IgG precipitated the enzyme activity under the experimental conditions in this study.

Protective protein in human tissues

Immunoblotting of human tissue homogenates from a Gaucher disease patient revealed various amounts of the mature protective protein (Fig. 3). Both anti-PP32N12- and anti-PP20C12-reactive proteins were visualized in kidney, lung, liver and spleen, in decreasing order of amount. A faint band was detected for cerebellum, but not for cerebrum. The 54-kDa precursor

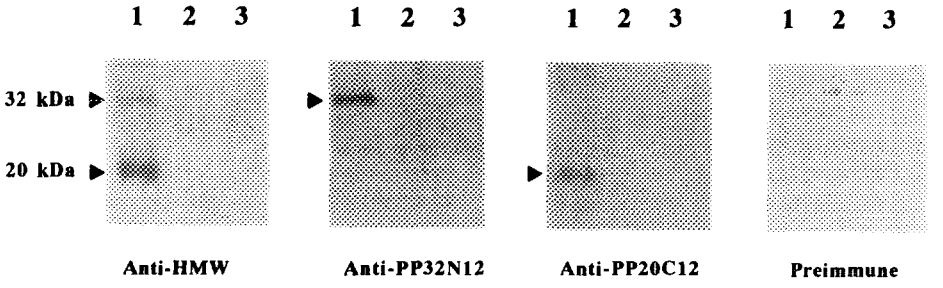

Fig. 1. SDS-PAGE and immunoblotting of the human fibroblast protective protein.
Extracts of normal human fibroblasts were subjected to electrophoresis on a 10-20% gradient gel in the presence of SDS after reduction with 2-mercaptoethanol and immunostained. Lane 1, normal subject; lane 2, galactosialidosis patient 1; lane 3, galactosialidosis patient 2.

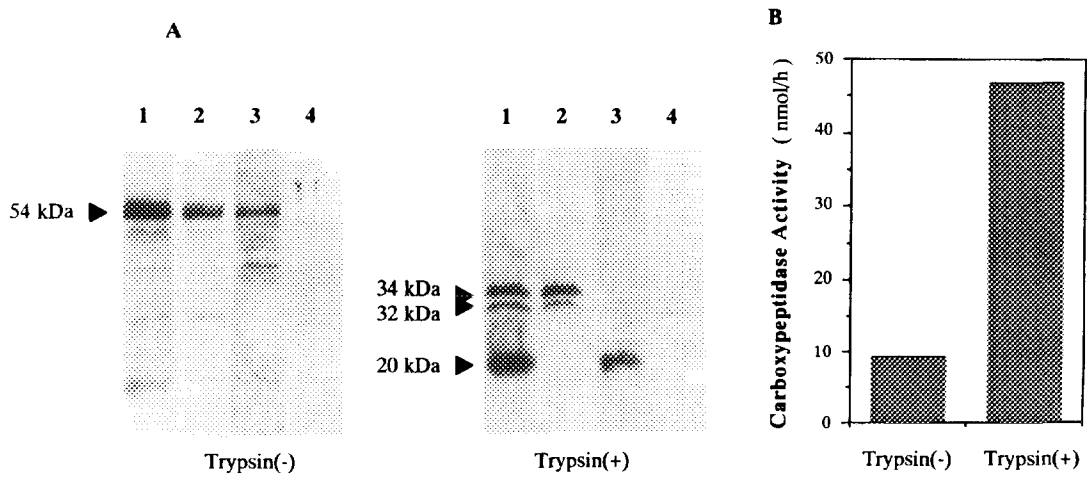

Fig. 2. Tryptic digestion of the protective protein precursor.
An aliquot of the precursor-rich solution was incubated at 37°C for 30 min with an excess amount of trypsin. A: Immunoblotting. Lane 1, staining with anti-HMW antiserum; lane 2, staining with anti-PP32N12 antiserum; lane 3, staining with anti-PP20C12 antiserum. B: Carboxypeptidase activity.

protein was not observed in any of the tissues examined in this study. The amount estimated by immunoblotting was roughly proportional to that of the carboxypeptidase activity in each tissue. A low but significant activity was detected in cerebrum. We could not detect any protective protein band in cerebrum or liver from a galactosialidosis patient, and carboxypeptidase activity was extremely low.

DISCUSSION

In this study, we raised specific antibodies against synthetic oligopeptides of two regions of the protective protein. Both of them reacted with two polypeptides constituting the mature protective protein as well as the precursor protein under the experimental conditions in this study. The antibody against oligopeptide PP32N12 recognized the N-terminal sequence of the precursor (or that of the 32-kDa mature protein subunit), and that against oligopeptide PP20C12 recognized the C-terminal sequence of the precursor (or that of the 20-kDa subunit). However, they did not precipitate the mature protective protein. Probably only the denatured protein is antigenic to them. We have no molecular information about the 34-kDa protein detected on immunoblotting. It may have been a tryptic product of two different digestion sites.

In human fibroblasts, most of the carboxypeptidase activity is derived from protective protein, judging from our previous study; it was almost completely deficient in galactosialidosis cells (15). Further evidence was provided by the present study, demonstrating the enzyme activity proportional to the amount of the enzyme protein detected by immunoblotting.

The tissue distribution of the human protective protein has not been known. We therefore studied several tissues using these specific antibodies by immunoblotting. In human tissues, this protein was identified as the mature form, no precursor protein being detected. The amount

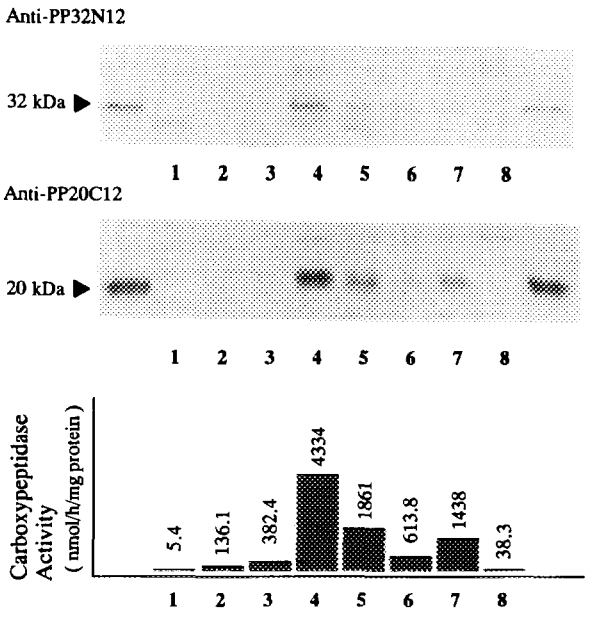

Fig. 3. Immunoblotting and carboxypeptidase activity of the protective protein in human tissues.
The tissue extract (40 μg protein) was used for immunoblotting and enzyme assay. Lane 1, galactosialidosis cerebrum; lane 2, Gaucher cerebrum; lane 3, Gaucher cerebellum; lane 4, Gaucher kidney; lane 5, Gaucher lung; lane 6, Gaucher spleen; lane 7, Gaucher liver; lane 8, galactosialidosis liver. Both ends: Normal fibroblasts as positive markers.

observed for each tissue was variable; it was highest in kidney, and then in lung, liver and spleen, in decreasing order. Neither the precursor nor the mature protective protein was detectable in the cells or tissues from galactosialidosis patients. Tissue-specific expression of protective protein mRNA has been reported in mice; a high expression was observed in kidney and placenta (16). We examined rat organs for carboxypeptidase activity, and the distribution was similar to that for human tissues; kidney, lung, and liver showed relatively high activity, and cerebrum and cerebellum showed less activity (Satake, A., et al., unpublished data).

Recently, a protease from rat kidney was demonstrated to degrade endothelin-1, a potent vasoconstrictive peptide, and it was found to be related to human protective protein (17). Moreover, it has been known that protective protein purified from human platelets inactivates endothelin-1 by removing Trp at its C-terminus (18). Kidney is a possible candidate tissue for endothelin-1 degradation by the protective protein.

The protective protein is a multifunctional protein exhibiting intralysosomal activity toward other lysosomal enzymes, and with catalytic activities toward various other bioactive peptides. It may also serve as an extra-lysosomal regulatory protein with tissue specificity. The antibodies developed in this report will be useful for further analysis of this unique, multifunctional protein under physiological and pathological conditions.

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