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Rapid report

Palmitoyl-protein thioesterase deficiency in fibroblasts of individuals with infantile neuronal ceroid lipofuscinosis and I-cell disease

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Abstract

Mutations in the gene encoding a recently described lysosomal enzyme, palmitoyl-protein thioesterase (PPT), have recently been shown to result in the neurodegenerative disorder, infantile neuronal ceroid lipofuscinosis (INCL). Reduced palmitoyl-protein thioesterase enzyme has been demonstrated previously in INCL brain and immortalized lymphoblasts. In the current paper, we demonstrate that: (1) PPT can be detected by immunoblotting and enzyme activity assays in normal human skin fibroblasts; (2) INCL fibroblasts are deficient in PPT activity; (3) I-cell disease fibroblasts show markedly reduced intracellular levels of PPT but markedly increased levels of PPT in cell culture medium. These data establish that PPT is transported to lysosomes via the lysosomal enzyme:lysosomal enzyme receptor phosphomannosyl recognition system under normal physiological conditions and provide the basis for a useful clinical assay for INCL.

Keywords: Lysosomal storage disorder; Infantile neuronal ceroid lipofuscinosis; I-cell disease; Fibroblast

Palmitoyl-protein thioesterase (PPT) was recently shown to be the defective enzyme responsible for the neurodegenerative disorder of children, infantile neuronal ceroid lipofuscinosis (INCL) [1]. This lysosomal storage disorder has an unusually high incidence in Finland (1:20 000) [2] and is characterized by global developmental delays, early vision loss, seizures, sleep disturbances, an isoelectric EEG by 3 years of age, and death between 8–14 years [3]. A homozygous mutation at nucleotide 364, leading to an arginine to tryptophan substitution near the putative active site of the enzyme, accounts for the vast majority of Finnish cases [1]. The diagnosis is currently made on the basis of clinical findings and light and electron microscopic analysis of tissue obtained from skin or rectal mucosal biopsy [4]. A characteristic granular autofluorescent storage material is found in brain, skin, chorionic villi and other tissues [5].

PPT has been recently shown to be a lysosomal enzyme, thereby confirming the classification of INCL, based on histologic criteria, as a lysosomal storage disease [6–8]. In vitro, PPT hydrolyzes long-chain fatty acids from modified cysteine residues in proteins [9,10]. It is likely that PPT functions in the cell to metabolize *S*-acyl proteins in the lysosome, as protein-derived [^{35}S]cysteine-labeled lipid thioesters

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accumulate in INCL cells [11]. Severely reduced PPT activity has been demonstrated in brain tissue from INCL patients as compared to normal controls (< 0.1 vs. 12.0 pmol/min/mg of soluble brain protein), whereas lymphoblasts showed about 40% of normal activity (0.37 vs. 0.89 pmol/min/mg of crude cell protein) [1]. Lysosomal PPT activity is absent from INCL lymphoblasts; the remaining low level of PPT activity in these cells resides in non-lysosomal subcellular fractions [12].

When recombinant PPT is overexpressed in a transient transfection system, the enzyme is phosphorylated on mannose residues and transported to lysosomes through the mannose 6-phosphate receptor pathway [6]. This provides strong, yet indirect, evidence that PPT is directed to lysosomes through this pathway under physiological conditions. To examine the targeting of PPT under conditions of normal expression, we examined PPT activity and immunoreactivity in fibroblasts of patients with I(inclusion)-cell disease, a disorder of lysosomal enzyme targeting through the mannose 6-phosphate receptor pathway. Patients with this disease have a defect in UDP-*N*-acetylglucosamine:glycoprotein *N*-acetylglucosaminylphosphotransferase, the enzyme responsible for adding *N*-acetylglucosamine 1-phosphate to specific mannose residues of lysosomal enzymes [13]. Subsequent post-translational enzymatic processing results in the removal of the *N*-acetylglucosamine residues, resulting in the exposure of mannose 6-phosphate moieties on the lysosomal enzymes. These mannose 6-phosphate moieties, in turn, enable an interaction between the newly synthesized lysosomal enzymes and their receptor, resulting in the intracellular transport of these enzymes to lysosomes. Fibroblasts from I-cell disease patients synthesize lysosomal enzymes that lack the mannose 6-phosphate recognition signal and are therefore not routed to lysosomes in these cells [14]. Rather, the lysosomal enzymes are secreted and found at elevated levels in the culture medium and body fluids [15].

In the current study, we analyzed two independent INCL fibroblast cultures for PPT enzyme activity and immunoreactivity, and also surveyed fibroblasts derived from a panel of patients with a variety of lysosomal storage diseases, including I-cell disease. We demonstrate that PPT is undetectable by immunoblotting and shows severely reduced activity in fibroblasts from two unrelated INCL patients. We also show that PPT is undetectable in fibroblasts from a patient with I-cell disease but that the enzyme appears at elevated levels in the culture medium, indicating that PPT, like many but not all lysosomal enzymes, is transported to lysosomes after its synthesis via a phosphomannosyl receptor mechanism.

Fibroblast cultures were derived from patients with lysosomal storage disorders at the Shriver Center (Waltham, MA). INCL fibroblasts were obtained from two unrelated U.S. patients without known Finnish ancestry. One of these cultures was obtained through Coriell (formerly, ATCC, NIGMS Mutant Cell Repository No. GM09405). Cells were maintained in monolayer culture in Dulbecco's modified Eagles' medium (high glucose) supplemented with 15% (v/v) heat-inactivated fetal bovine serum, 1% L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B. Whole cell lysates were prepared from confluent dishes of cells by harvesting cells in a buffer containing 50 mM Tris-HCl, pH 7.0, 150 mM NaCl, 2 mM EDTA, 1 μg/ml pepstatin A, and 2 μg/ml leupeptin and sonicating briefly on ice.

Whole cell lysates were analyzed by immunoblotting using anti-human PPT antibodies that were raised in rabbits using full-length recombinant human PPT as antigen in a manner similar to that previously described for the preparation of anti-bovine PPT antibodies [6]. An IgG fraction was prepared from preimmune and immune sera by protein A Sepharose CL-4B chromatography (Pharmacia Biotech Inc.). Crude cell lysates (25 μg of total protein) were electrophoresed on 12% SDS-polyacrylamide gels, transferred to nitrocellulose, and analyzed by immunoblotting using IgG purified anti-human PPT antibodies (1.5 μg/ml) as described previously [6].

To analyze PPT protein in the medium from fibroblast cultures, a partial purification was carried out to enrich for PPT prior to immunoblotting. Culture medium (4 ml) was harvested from confluent dishes of cells and adjusted to 2.5 M NaCl, 30 mM Tris-HCl pH 7.0, and 0.2 mM phenylmethanesulfonyl fluoride. Samples were incubated with 400 μl of a 50:50 slurry of phenyl Sepharose CL-4B (Pharmacia Biotech) in 2.5 M NaCl for 3 h on a laboratory rotator at 4°C. The resin-containing samples were poured into 10 ml Poly-Prep chromatography columns

(Bio-Rad Laboratories) and allowed to drain by gravity. The resin was washed with 10 ml high salt buffer (2.5 M NaCl, 30 mM Tris-HCl pH 7.0) and 40 ml low salt buffer (5 mM Tris-HCl pH 7.0). PPT was eluted from the resin with 2.5 ml elution buffer (75% ethylene glycol, 25% 5mM Tris-HCl pH 7.0 (v/v)). Samples were diluted with 4 volumes 50 mM Tris-HCl, pH 7.0, 150 mM NaCl, 2 mM EDTA, 0.2% *n*-octylglucoside and concentrated to 70 μl using Centricon-30 concentrators (Amicon). Samples (7 μl) were subjected to electrophoresis on a 12% polyacrylamide gel, transferred to nitrocellulose, and analyzed by immunoblotting as described for the crude cell lysates.

Fig. 1A shows an immunoblot of fibroblast whole-cell extracts from patients with a variety of lysosomal storage disorders. In normal fibroblasts (lane 1), PPT appears as a doublet at molecular masses of 36.5 K and 34 K. I-cell disease fibroblasts (lane 2) show no detectable PPT. Since PPT has been shown to be a lysosomal enzyme, this result is consistent with PPT lacking the mannose 6-phosphate recognition signal, with subsequent failure to target PPT to lysosomes in these cells. Patients with galactosialidosis (lane 3) show a PPT doublet with abnormally slow electrophoretic migration. These patients have a defect in oligosaccharide processing due to a deficiency in the lysosomal protective protein [16] that results in a secondary deficiency of lysosomal β-galactosidase and neuraminidase activities [17], and abnormal electrophoretic migration of a number of lysosomal proteins. Fibroblasts from a U.S. INCL patient (lane 9) showed an absence of detectable immunoreactivity. Fibroblasts from a second INCL patient yielded the same results (data not shown). PPT immunoreactivity was normal in fibroblasts from patients with other lysosomal storage diseases (lanes 4–8).

Fig. 1B shows an analysis of PPT in the culture medium of the same fibroblast lines shown in Fig. 1A. PPT was readily and reproducibly detected in culture medium of fibroblasts from the I-cell disease patient (lane 2). The secreted PPT appeared as a doublet of molecular masses of 39 K and 37 K. The slower migration of secreted forms of PPT is consistent with further oligosaccharide processing of PPT in the Golgi after failure to phosphorylate mannose residues [18]. Traces of PPT immunoreactivity were

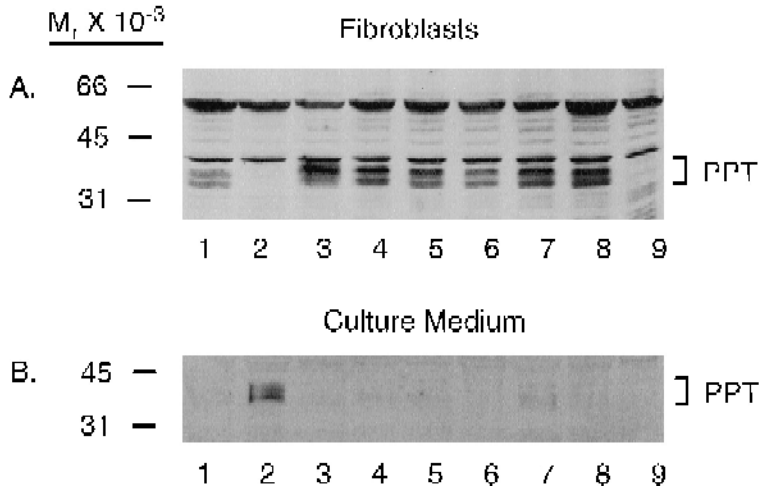

Fig. 1. Immunoblot analysis of PPT in fibroblasts and culture medium. A. Whole cell extracts were subjected to electrophoresis in 12% SDS-polyacrylamide gels and transferred to nitrocellulose filters. Immunoreactive bands were visualized by incubation with polyclonal rabbit anti-human PPT antibodies and enhanced chemiluminescence. PPT appears as a doublet of molecular masses 34 K and 36.5 K. Bands observed at 40 K and 58 K were also seen with preimmune IgG (data not shown). B. Fibroblast culture medium was treated as described in the text and subjected to electrophoresis and immunoblotting. PPT from the culture medium appears as a doublet of molecular masses 37 K and 39 K. Lane 1: normal fibroblasts. Lane 2: I-cell disease. Lane 3: galactosialidosis. Lane 4: Sandhoff disease. Lane 5: metachromatic leukodystrophy. Lane 6: Hurler disease. Lane 7: Krabbe disease. Lane 8: Pompe disease. Lane 9: INCL. This experiment was performed three times with similar results.

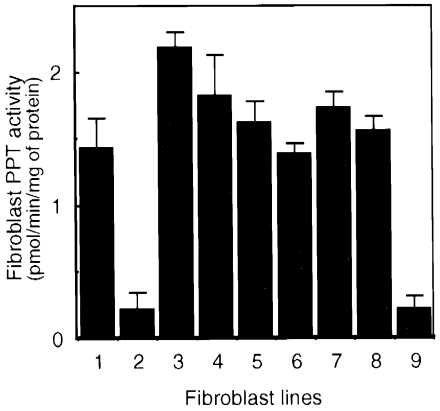

Fig. 2. PPT activity is decreased in fibroblasts from INCL and I-cell disease patients, but not in fibroblasts from patients with other lysosomal storage disorders. Whole cell extracts were prepared and assayed for PPT activity as described in the text. Lanes are exactly as described in Fig. 1. Values represent the mean ± standard error for three determinations performed on each cell line.

occasionally found in culture medium from some fibroblast samples (see, for instance, lane 7) but these findings were inconsistent.

We were unable to detect PPT in *serum* from I-cell patients by immunoblotting or enzyme assay. The explanation for this may be related to a marked inhibition of PPT activity observed when purified recombinant PPT is added to normal serum (data not shown).

Fig. 2 shows an analysis of PPT enzyme activity in whole cell lysates from the fibroblasts shown in Fig. 1. The assay measures the removal of [^{3}H]palmitate from a palmitoylated protein ([^{3}H]palmitate-labeled H-Ras) [9]. Cell extracts were preincubated in phenylmethanesulfonyl fluoride (PMSF) to inhibit background PMSF-sensitive thioesterase activity; PPT activity has previously been shown to be resistant to PMSF [9]. In keeping with the results of Fig. 1, PPT activity is significantly reduced in cells from patients with I-cell disease (lane 2) and INCL (lane 9) reflecting the absence of PPT enzyme activity. Fibroblasts from the second INCL patient also showed negligible activity (data not shown). There were normal levels of PPT activity in cells from patients with other lysosomal storage diseases (lanes 3–8). Cells from the patient with galactosialidosis (lane 3) show normal PPT activity, indicating that the abnormal processing of the oligosaccharides on PPT in these cells did not affect the catalytic activity of PPT measured in the in vitro assay.

In conclusion, the data presented here indicate that PPT can be demonstrated by both enzyme activity and immunoassays in normal cultured skin fibroblasts. In addition, PPT deficiency can be demonstrated in fibroblast cell lines from patients with INCL. In the two particular INCL lines reported here, PPT was undetectable both by activity and immunoblotting assays. These assays may have advantages over electron microscopic assays for INCL and should facilitate the identification of other PPT deficient patients having phenotypes different from classic INCL.

In addition, the data presented here provide direct support that PPT is routed to lysosomes via a mannose 6-phosphate receptor pathway, in a physiologic setting not involving overexpression of the protein in a heterologous cell line.

It is possible that PPT deficiency contributes, at least in part, to the accumulation of storage material in I-cell disease as well as in INCL. Further characterization of the storage material in both lysosomal storage diseases may help to address this possibility.

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