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Biochemistry

Lipid thioesters derived from acylated proteins accumulate in infantile neuronal ceroid lipofuscinosis: Correction of the defect in lymphoblasts by recombinant palmitoyl-protein thioesterase

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ABSTRACT **Palmitoyl-protein thioesterase is a lysosomal long-chain fatty acyl hydrolase that removes fatty acyl groups from modified cysteine residues in proteins. Mutations in palmitoyl-protein thioesterase were recently found to cause the neurodegenerative disorder infantile neuronal ceroid lipofuscinosis, a disease characterized by accumulation of amorphous granular deposits in cortical neurons, leading to blindness, seizures, and brain death by the age of three. In the current study, we demonstrate that [^{35}S]cysteine-labeled lipid thioesters accumulate in immortalized lymphoblasts of patients with infantile neuronal ceroid lipofuscinosis. The accumulation in cultured cells is reversed by the addition of recombinant palmitoyl-protein thioesterase that is competent for lysosomal uptake through the mannose-6-phosphate receptor. The [^{35}S]cysteine-labeled lipids are substrates for palmitoyl-protein thioesterase *in vitro*, and their formation requires prior protein synthesis. These data support a role for palmitoyl-protein thioesterase in the lysosomal degradation of S-acylated proteins and define a major new pathway for the catabolism of acylated proteins in the lysosome.**

The neuronal ceroid lipofuscinoses represent a group of progressive encephalopathies of children with a global incidence of 1:12,500 (1). The disease is divided into at least three autosomal recessive subtypes, assigned to different chromosomal regions, each with a characteristic age of onset. The infantile form, linked to chromosome 1p32 (2), is characterized by early visual loss and progressive mental deterioration, resulting in a flat electroencephalogram by age three; death occurs at 8–11 years. A characteristic finely granular autofluorescent sudanophilic storage material is found throughout the brain and other tissues. Interest in the disease has come from its relatively high economic and human costs (3) and as a model for accelerated aging in human brain (4).

The neuronal ceroid lipofuscinoses are not well understood at the biochemical level (5). Progress has been hampered by the lack of specific biochemical or genetic markers, causing uncertainties in classification and diagnosis. In addition, tissues available for analysis are usually at relatively late stages of the disease process, when secondary changes (such as marked neuronal loss) confuse interpretation of biochemical data. Analysis of the storage material has not been particularly revealing, showing a mixture of protein, lipid, and carbohydrate (6), not unlike the composition of bulk membranes. In infantile neuronal ceroid lipofuscinosis (INCL), saposins appear to be the predominant protein (6), but the significance of this observation is unclear at present because saposins may accumulate in a number of diverse storage diseases (7).

Recently, a mutation in the gene encoding the enzyme palmitoyl-protein thioesterase was shown to be responsible for the disorder in a group of patients in Finland (8). Palmitoyl-protein thioesterase is a lysosomal enzyme (9), one of the three or four most abundant lysosomal enzymes in brain (10). It was originally purified as a soluble enzymatic activity that removes palmitate from fatty S-acylated proteins and palmitoyl CoA (11, 12), and we have postulated that it functions to remove fatty acids from modified proteins and/or peptides in the lysosome. In most S-acylated proteins, the fatty acid (usually palmitate) is found in thioester linkage to one or more cysteine residues that are located in close proximity to the inner surface of the plasma membrane (13). Therefore, the inability to degrade the thioester linkage would be expected to lead to the accumulation of *S*-acylcysteine or *S*-acylcysteine-containing peptides.

In the current study, we examined the metabolism of [^{35}S]cysteine-labeled lipid thioesters in immortalized lymphoblastoid cells derived from Finnish INCL patients (or from normal controls). We labeled cells with [^{35}S]cysteine, treated them with chloroform/methanol to extract the labeled lipids, and analyzed the extracted lipids by thin-layer chromatography and fluorography. We found striking accumulation of five or more distinct [^{35}S]cysteine-labeled lipids that behave chemically as acyl thioesters, that are substrates for palmitoyl-protein thioesterase *in vitro*, and that disappear after incubation of INCL cells with conditioned COS cell medium that contains recombinant palmitoyl-protein thioesterase. Furthermore, the accumulation of these [^{35}S]cysteine-labeled thioesters was blocked by cycloheximide, indicating that prior incorporation of the [^{35}S]cysteine into protein is required for accumulation of the abnormal thioesters. These results provide strong evidence that palmitoyl-protein thioesterase is required for the efficient lysosomal degradation of thioesters derived from S-acylated proteins.

METHODS

Materials. Normal human Epstein–Barr virus (EBV)-transformed B-lymphoblastoid cell lines were provided by Adi F. Gazdar (University of Texas Southwestern Medical Center). EBV-transformed B-lymphoblastoid cell lines derived from Finnish INCL patients were provided by Leena Peltonen (National Public Health Institute, Helsinki). The Finnish mutation (Arg122Trp) leads to production of an inactive enzyme that is mislocalized to the endoplasmic reticulum (8) and rapidly degraded, as brain tissue and lymphoblasts from these patients contain little or no immunoreactive palmitoyl-protein thioesterase (L.A.V. and S.L.H., unpublished observations). EBV-transformed B-lymphoblastoid cell lines derived from patients with other neurological disorders were provided by Michael J. Bennett (University of Texas Southwestern Medical Center). L-[^{35}S]Cysteine (1030 Ci/mmol; 1

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Abbreviations: INCL, infantile neuronal ceroid lipofuscinosis; EBV, Epstein–Barr virus.

Ci = 37 GBq) was from ICN. Cycloheximide and hydroxylamine were obtained from Sigma. Other materials were obtained from previously described sources (11).

[^{35}S]Cysteine Labeling. Immortalized lymphoblastoid cell lines were maintained in 75-cm^2 culture flasks in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and amphotericin B (0.25 μg/ml). For metabolic labeling studies, 25 ml of logarithmic-phase cells (approximately 7.5 × 10^5 cells per ml) were centrifuged for 5 min at 220 × *g*, washed twice with prewarmed cysteine- and serum-free medium, and then resuspended in 0.5 ml of cysteine-free medium in a 25-cm^2 culture flask (final concentration, 2–4 × 10^7 cells per ml). The cells were incubated for 30 min at 37°C, and 0.5 ml of medium containing [^{35}S]cysteine (100 μCi/ml) was added, and incubations continued for up to 6 h. In pulse–chase experiments, the cells were washed three times in 5 ml of culture medium containing L-cysteine (325 μg/ml) and the incubation continued for various times up to 5 h. The cells were washed twice in 1 ml of ice-cold phosphate-buffered saline (PBS) and pelleted at 12,000 rpm in a microcentrifuge for 15 sec at 4°C. In one experiment, cycloheximide (50 μg/ml) was present for 30 min prior to and during the labeling period to block protein synthesis.

Lipid Extracts. Labeled cell pellets were resuspended in 100 μl of ice-cold PBS. An aliquot (600 μl) of chloroform/methanol, 1:1 (vol/vol), was added, and the mixtures were vortex mixed for 20 sec and centrifuged at 2400 rpm in a variable speed microcentrifuge for 5 min at 4°C. The organic (lower) phase was carefully withdrawn and washed once with one-sixth volume of ice-cold PBS, diluted with an equal volume of benzene, and brought to dryness under a stream of nitrogen. The residue was dissolved in 1:1 chloroform/methanol and adjusted to achieve a final concentration of 500 cpm/μl (in 100–200 μl) for further analysis. INCL cells yielded approximately 10^4 cpm of [^{35}S]cysteine-labeled lipids per 10^6 cells.

Thin-Layer Chromatography. Aliquots (20 μl) of [^{35}S]cysteine-labeled lipids were applied to high-performance thin-layer Kieselgel 60 F254 plates (10 × 10 cm) (Merck). The plates were developed using chloroform/methanol/water, 65:25:4 (vol/vol), for 7.5 cm (about 25 min), air-dried at room temperature, sprayed with EN^{3}HANCE spray (DuPont/NEN), dried 15 min, and processed for fluorography. In a typical experiment, 5000–10,000 cpm was loaded per lane, and exposure times were from 1 to 2 days. All experiments shown were repeated one or more times with similar results.

Enzyme Replacement. Conditioned COS cell medium containing recombinant human palmitoyl-protein thioesterase was prepared by transient transfection using the plasmid pCMV5-hPPT, which contains the entire coding region of human palmitoyl-protein thioesterase [prepared by polymerase chain reaction amplification of pSV-PPT (8) using the primers 5′-CAAGAATTCGTGGTGACACAGCGAAGATGGCGT-3′ and 5′-TCTAAGCTTGGGGCTCCCTGAGCTCTATTGTGA-3′ and insertion of the amplified product into the *Eco*RI–*Hin*dIII sites of pCMV5 (14)]. Simian COS-1 cells were transfected with pCMV5-hPPT using the Lipofectin method (9), and conditioned medium was collected 72 h after transfection, frozen under liquid nitrogen, and stored at −85°C until further use. [^{35}S]Cysteine pulse–chase labeling was subsequently performed in the presence of COS cell-conditioned medium containing human palmitoyl-protein thioesterase (500 ng/ml) (or conditioned medium harvested from cells transfected with pCMV5 containing no insert) and the lipids were analyzed as described above. In one experiment, human palmitoyl-protein thioesterase-containing conditioned medium was incubated with the cells for 40 h prior to labeling as indicated in the figure legends.

Thioesterase Assays. [^{35}S]Cysteine-labeled lipid extracts (20,000 cpm) were dried under nitrogen in glass tubes and resuspended with vigorous mixing in 50 μl of 50 mM Tris·HCl (pH 7.0) containing 50 mM NaCl, 1 mM EDTA, and 1 μg of recombinant bovine palmitoyl-protein thioesterase (12). Reactions were carried out at 37°C and stopped by addition of 600 μl of chloroform/methanol/benzene, 1:1:2 (vol/vol). Final mixtures were evaporated to dryness under nitrogen, and dissolved in 60 μl of chloroform/methanol, 1:1 (vol/vol), and a portion (15 μl) was analyzed by thin-layer chromatography and fluorography.

Hydroxylamine Treatment. [^{35}S]Cysteine-labeled lipid extracts (40,000 cpm) were dried under nitrogen in glass tubes and resuspended with vigorous mixing in 50 μl of 1 M hydroxylamine (pH 8.0) or 1 M Tris·HCl (pH 8.0), incubated at ambient temperature for 1 h, and extracted into 300 μl of chloroform/methanol, 1:1 (vol/vol). The phases were separated by brief centrifugation and the lower (organic) phase was processed for thin-layer chromatography as described above.

Other Methods. SDS/PAGE was performed in 12% vertical slab gels. Fluorography was performed by incubating Coomassie brilliant blue gels with Entensify (DuPont) according to the directions supplied by the manufacturer. Treated gels were dried and exposed directly to Kodak XAR-5 film at −70°C.

RESULTS

Fig. 1 shows a fluorograph of [^{35}S]cysteine-labeled lipids extracted from lymphoblasts derived from Finnish INCL patients (and normal controls) and separated by thin-layer chromatography. At least five labeled bands were found to be present in the extracts from INCL cells derived from five patients (Fig. 1*A*, lanes 2, 4, 6, 8, and 10); these bands were not present in five normal controls (lanes 1, 3, 5, 7, and 9). Furthermore, these bands were not present in lipids extracted from two patients with juvenile NCL (Fig. 1*B*, lanes 5 and 6), two patients with Kuf disease (adult NCL) (lane 7 and data not shown), and a patient with late infantile NCL (lane 8). Interestingly, one non-Finnish patient who carried the clinical diagnosis of INCL (lane 4) did not show the INCL-specific bands; this patient's lymphoblasts had normal palmitoyl-protein thioesterase enzyme activity (data not shown) and so presumably has another defect that does not involve palmitoyl-protein thioesterase.

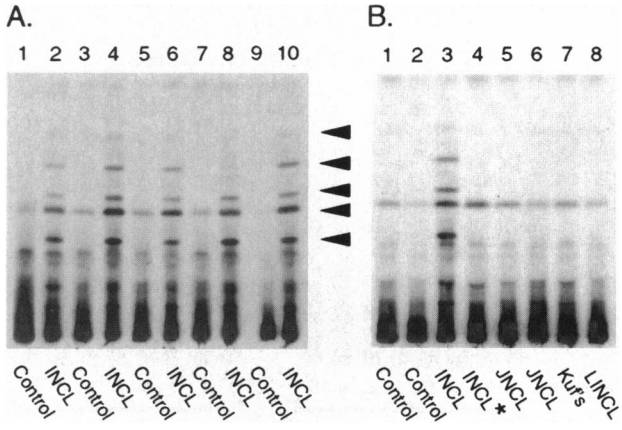

FIG. 1. [^{35}S]Cysteine-labeled lipids from 10 control and INCL lymphoblastoid cell lines (*A*) and from lymphoblasts from patients with related neurodegenerative disorders (*B*). EBV-transformed lymphoblastoid cells were cultured in the presence of [^{35}S]cysteine for 3 h, lipids were extracted into chloroform/methanol, and the organic phase was analyzed by high-performance thin-layer chromatography and fluorography. Arrowheads denote the INCL-specific bands. One band (denoted by the fourth arrow from the top) consists of two overlapping bands, one INCL-specific and one nonspecific, that are resolved by two-dimensional thin-layer chromatography (data not shown).

Of note, a fibroblast line from a patient with INCL did not show INCL-specific bands under the same conditions as shown in Fig. 1. The basis for the cell-type specificity of the phenomenon is unclear at present but may reflect more rapid membrane synthesis and turnover in the more rapidly dividing lymphoblastoid cells.

Fig. 2*A* shows the time course of labeling of these INCL-specific [^{35}S]cysteine-labeled lipids. The accumulation in the INCL cells is detectable at 1 h and has reached a plateau by 4–6 h. The INCL-specific bands are not seen in the control cells even after 6 h of labeling (Fig. 2*A*, lanes 1–4). To estimate the rate of turnover of the [^{35}S]cysteine-labeled lipids in INCL cells, a pulse–chase experiment was performed. In Fig. 2*B*, INCL cells were labeled for 3 h, then washed, and harvested at various times after the addition of unlabeled cysteine. The INCL-specific bands were stable for at least 5 h (Fig. 2*B*, lanes 1–4). To determine whether the accumulation of the [^{35}S]cysteine-labeled lipids was reversible, we added recombinant human palmitoyl-protein thioesterase to the culture medium during the chase period (Fig. 2*B*, lanes 5–8). In this case, the INCL-specific bands decreased over time (Fig. 2*B*, lanes 5–8), and the pattern of labeled lipids resembled that of the normal control after the 5-h chase period. Of note, the source of the recombinant human palmitoyl-protein thioesterase in this experiment was conditioned medium from pCMV5-hPPT-transfected COS cells. These cells secrete human palmitoyl-protein thioesterase that contains mannose-6-phosphate residues that support uptake of the enzyme into lysosomes through the mannose-6-phosphate receptor pathway (9). Conditioned medium from COS cells transfected with pCMV5 containing no insert had no effect (Fig. 2*B*, lanes 1–4).

To show that the correction of the accumulation of the INCL-specific bands was mediated by uptake of the recombinant palmitoyl-protein thioesterase through the mannose-6-phosphate receptor pathway, we incubated INCL lymphoblasts with palmitoyl-protein thioesterase-containing COS conditioned medium in the presence of mannose 6-phosphate or mannose alone (Fig. 3). Mannose 6-phosphate specifically blocked the effect of added palmitoyl-protein thioesterase (Fig. 3, lane 3), whereas mannose had no effect (lane 4). Furthermore, addition of an excess of recombinant palmitoyl-protein thioesterase purified from Sf9 cell medium (previously shown not to be competent for uptake through the mannose-6-phosphate receptor pathway (9)) had very little effect on the

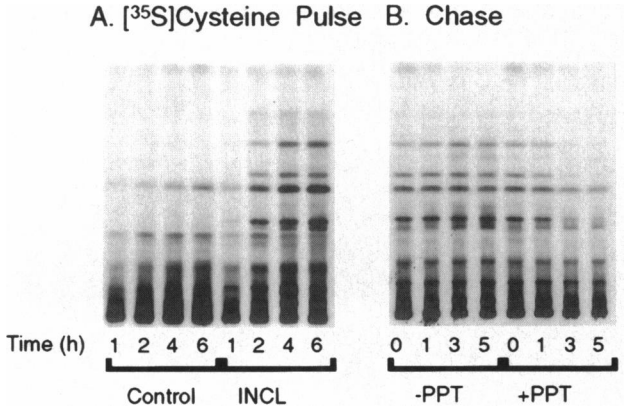

FIG. 2. Pulse–chase analysis of [^{35}S]cysteine-labeled lipids from normal and INCL lymphoblasts and correction of the metabolic defect in INCL lymphoblasts by recombinant palmitoyl-protein thioesterase. (*A*) Cells from normal control (lanes 1–4) or INCL patients (lanes 5–8) labeled with [^{35}S]cysteine for various times up to 6 h. (*B*) Cells from INCL patients labeled for 3 h and then incubated with an excess of unlabeled cysteine in the absence (lanes 1–4) or presence (lanes 5–8) of recombinant human palmitoyl-protein thioesterase (500 ng per ml) supplied from transfected COS cell-conditioned medium.

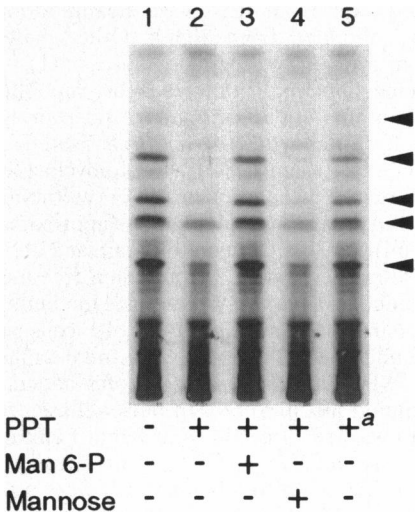

FIG. 3. Correction of the metabolic defect in INCL lymphoblasts by recombinant palmitoyl-protein thioesterase is blocked by mannose 6-phosphate. Lymphoblasts from an INCL patient were incubated for 20 h in the presence or absence of recombinant human palmitoyl-protein thioesterase (125 ng per ml) (supplied from transfected COS cell-conditioned medium), mannose 6-phosphate (5 mM) or mannose (5 mM) as indicated, then labeled with [^{35}S]cysteine, extracted with chloroform/methanol, and analyzed by thin-layer chromatography and fluorography. In lane 1, INCL cells were incubated with COS-cell conditioned medium from vector-transfected cells (producing no recombinant palmitoyl-protein thioesterase). a, Incubation with human palmitoyl-protein thioesterase (200 ng/ml) supplied from baculovirus-infected Sf9 cell medium, which is fully active but taken up poorly through the mannose-6-phosphate-dependent pathway (9).

accumulation of the INCL-specific compounds (Fig. 3, lane 5). These data show that the palmitoyl-protein thioesterase must first be internalized through the mannose-6-phosphate receptor pathway to degrade the [^{35}S]cysteine-labeled thioesters and rule out a significant action on these compounds at the cell surface.

In a separate experiment, we quantitated the approximate concentration of palmitoyl-protein thioesterase required in the medium to prevent the formation of the INCL-specific bands. When INCL cells were cultured in the presence of COS-cell conditioned medium containing various amounts of palmitoyl-protein thioesterase, we found that recombinant palmitoyl-protein thioesterase at less than 125 ng/ml was effective in preventing the accumulation of the INCL-specific bands (Fig. 4). Since only 50% of the enzyme molecules are modified by mannose 6-phosphate (9), the effective concentration is actually half that value.

The INCL-specific bands could result either directly or indirectly from the absence of palmitoyl-protein thioesterase. To determine whether the bands represent proximal substrates for palmitoyl-protein thioesterase, we incubated the lipid extracts with purified recombinant bovine palmitoyl-protein thioesterase *in vitro* (Fig. 5*A*). We observed a time-dependent decrease in the INCL-specific bands (lanes 2–6), whereas no decrease was seen in the presence of palmitoyl-protein thioesterase that had been inactivated by heat treatment (lane 7). Therefore, the INCL-specific bands represent *in vitro* substrates for palmitoyl-protein thioesterase. To confirm that the INCL-specific bands are thioester compounds, we incubated the lipid extracts in the presence of neutral hydroxylamine, a treatment that hydrolyzes thioesters but not oxyesters (15) (Fig. 5*B*). The INCL-specific bands were sensitive to neutral hydroxylamine but not to 1 M Tris·HCl (pH 8.0), confirming that the compounds represent [^{35}S]cysteine-labeled thioesters.

We hypothesized that the INCL-specific [^{35}S]cysteine-labeled thioesters are derived from acylated proteins that are

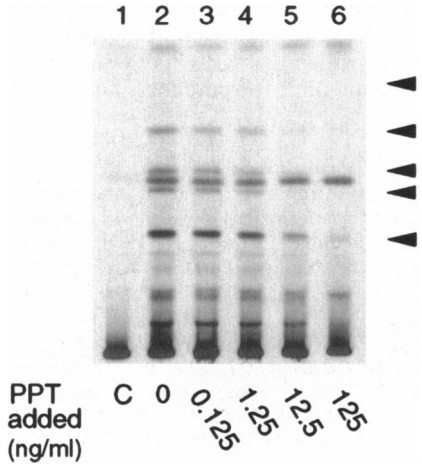

FIG. 4. Palmitoyl-protein thioesterase inhibits accumulation of [^{35}S]cysteine-labeled lipids in INCL lymphoblasts in a concentration-dependent manner. INCL lymphoblasts were incubated with increasing amounts of conditioned medium from palmitoyl-protein thioesterase-transfected COS cells (from 0 to 125 ng of palmitoyl-protein thioesterase per ml, lanes 2–6, as indicated) for 40 h and labeled for 4 h in the presence of [^{35}S]cysteine. Lipids were then analyzed. Lane 1 contains lipid extract from normal control lymphoblasts.

normally degraded in the lysosome. If this hypothesis were correct, one would predict that the [^{35}S]cysteine would first have to be incorporated into protein for accumulation of acyl[^{35}S]cysteine to occur. Therefore, we incubated the INCL lymphoblasts with cycloheximide starting 30 min prior to the labeling period. This treatment was sufficient to block the incorporation of [^{35}S]cysteine into newly synthesized protein (Fig. 6*B*, lanes 4 and 5) but did not significantly affect cellular

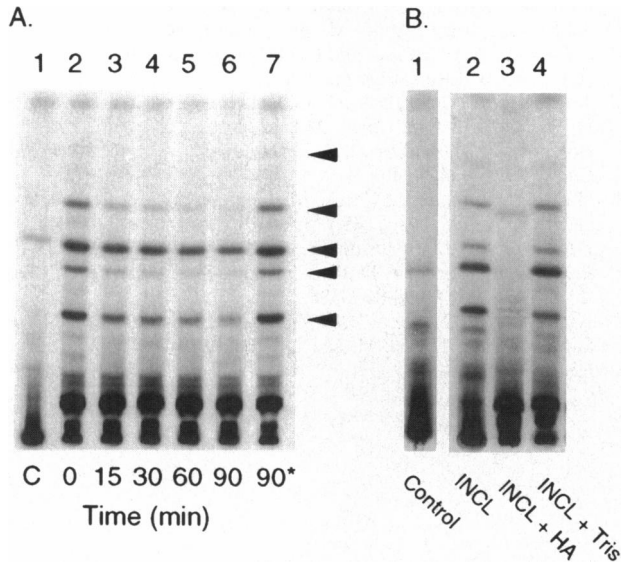

FIG. 5. Hydrolysis of [^{35}S]cysteine-labeled lipids *in vitro* by palmitoyl-protein thioesterase (*A*) and neutral hydroxylamine (*B*) and analysis by high-performance thin-layer chromatography and fluorography. Lipid extracts corresponding to 5×10^5 INCL lymphoblasts per lane (5000 cpm) were incubated with 0.25 μg of recombinant bovine palmitoyl-protein thioesterase at 37°C for the times indicated in *A*, lanes 2–6). (*A*) Lanes: 1, lipid extract of normal control (C) lymphoblasts; 7, lipids incubated with heat-denatured palmitoyl-protein thioesterase. (*B*) Neutral hydroxylamine treatment of [^{35}S]cysteine-labeled lipids from INCL lymphoblasts. Lanes: 1, normal control lipid extract, untreated; 2, INCL lipid extract, untreated; 3, INCL lipid extract, incubated for 1 h at 23°C with 1 M hydroxylamine (pH 8.0); 4, INCL lipid extract incubated with 1 M Tris·HCl (pH 8.0).

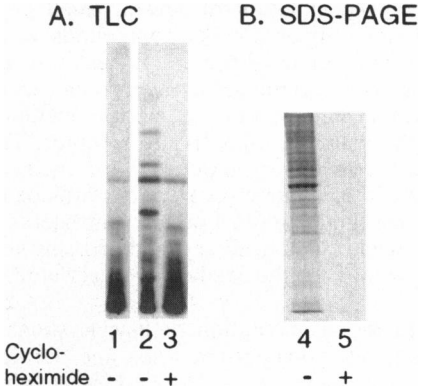

FIG. 6. Cycloheximide blocks formation of [^{35}S]cysteine-labeled thioesters. (*A*) High-performance thin-layer chromatography and fluorography of [^{35}S]cysteine-labeled lipids from INCL lymphoblasts incubated in the absence (lane 2) or presence (lane 3) of cycloheximide (50 μg/ml) during the preincubation (30 min) and labeling periods (4 h). Lane 1 contains untreated normal lymphoblasts shown for comparison. (*B*) SDS/PAGE fluorogram of [^{35}S]cysteine-labeled proteins (5 μg) extracted from untreated (lane 4) or cycloheximide-treated (lane 5) INCL lymphoblasts.

uptake of [^{35}S]cysteine, which was greater than 80% of control values in the absence of cycloheximide (data not shown). Under these conditions, the INCL-specific bands did not accumulate, and the pattern of [^{35}S]cysteine-labeled lipids in the INCL cells was essentially identical to that of the controls (Fig. 6*A*, lanes 1–3). These results strongly suggest that the INCL-specific [^{35}S]cysteine lipids are generated through an S-acyl protein intermediate.

What are likely chemical structures of these INCL-specific [^{35}S]cysteine-labeled thioester compounds? Given the specificity of palmitoyl-protein thioesterase for long-chain fatty acids of 14–20 carbons (12), we predict that the accumulating thioesters represent long-chain fatty acyl esters linked to the thiol groups of cysteine, cysteine-containing peptides, or their metabolites. To begin to address this question, we treated a "model" palmitoylated protein ([^{3}H]palmitate-labeled H-Ras) with a mixture of proteases and analyzed the reaction products by thin-layer chromatography after chloroform/methanol extraction. We obtained a discrete set of labeled bands, two of which comigrated with bands in INCL cells, and several other bands that did not correspond to any INCL-specific bands (data not shown). These results demonstrate that acylated peptides migrate with R_f values similar to the novel [^{35}S]cysteine-labeled compounds that accumulate in INCL cells and suggest the possibility that some of the INCL-specific bands represent acylated peptides derived from abundant acylated proteins.

We are presently unable to unequivocally identify *S*-palmitoylcysteine as one of the [^{35}S]cysteine-labeled compounds by comparison with a chemically synthesized standard. (Attempts at the synthesis of *S*-palmitoylcysteine by routine methods have thus far been surprisingly unsuccessful, due to rapid rearrangement of the *S*-palmitoylcysteine to *N*-palmitoylcysteine during work-up of the reaction products. Therefore, novel methods must be developed for the synthesis of *S*-acylcysteines.) None of the INCL compounds comigrates with *N*-palmitoylcysteine or *S*-palmitoyl-CoA by thin-layer chromatography, and the *N*-acetylated derivatives of the INCL compounds do not comigrate with *N*-acetyl-*S*-palmitoylcysteine (data not shown). Identification of the chemical structures of these novel compounds will require accumulation of sufficiently large quantities of pure material for direct analysis.

DISCUSSION

The current data demonstrate that the deficiency of lysosomal palmitoyl-protein thioesterase activity in INCL lymphoblasts

results in the accumulation of several small [^{35}S]cysteine-labeled thioester compounds. The compounds were shown to be substrates for palmitoyl-protein thioesterase *in vitro*. In cultured cells, their accumulation was corrected by addition of palmitoyl-protein thioesterase to culture medium through uptake via the mannose-6-phosphate receptor. The appearance of these compounds requires prior incorporation of cysteine into protein. These observations provide strong evidence that lysosomal palmitoyl-protein thioesterase functions to hydrolyze acyl chains from acylated proteins and define a major new pathway for the catabolism of acylated proteins in the lysosome.

How might the accumulation of *S*-acylcysteine thioesters lead to the storage body accumulation and the neurodegeneration that is characteristic of INCL? One possibility is that the thioesters are a major component of the storage material, representing hydrophobic peptides that, by virtue of the fatty acid component, are resistant to proteolysis and accumulate, leading to detrimental consequences for the cell. The accumulation of "indigestible" peptide material is a central feature of other neurodegenerative diseases, such as Alzheimer disease (16) and the prion diseases (17, 18). Another possibility is that the *S*-acylcysteine thioesters do not constitute the major storage material but rather cause a secondary lysosomal dysfunction that eventually leads to the formation of storage bodies. It is interesting that chronic intrathecal administration (to rats) of leupeptin, a thiol protease inhibitor that inhibits many lysosomal proteases, leads to the appearance of granular osmiophilic storage bodies nearly indistinguishable from those of INCL patients (19). Perhaps *S*-acylcysteine thioesters inhibit lysosomal thiol proteases, by acylation of the protease active site thiols. This is an easily testable hypothesis.

How does the palmitoyl-protein thioesterase gain access to the acylated cysteine residues in lipid-modified proteins, when these residues normally face the cytoplasmic face of the plasma membrane? One potential explanation is that acyl groups bound to S-acylated proteins are normally removed during the process of autophagy, in which entire membrane segments, including trapped cytoplasmic material, are degraded. Absence of palmitoyl-protein thioesterase activity would thus be predicted to cause dysfunction specifically in phagolysosomes. It is interesting that retinal degeneration is a prominent early feature of INCL (20) and that phagocytic destruction of rod outer segments (which are rich in rhodopsin, a palmitoylated protein) is a particularly active process in the retina.

It is possible that correction of the accumulation of *S*-acylcysteine thioesters might prevent subsequent formation of storage bodies and neuronal degeneration in INCL. Since *S*-acyl thioesters of cysteine are high-energy compounds, it might even be feasible to identify nontoxic nucleophilic compounds that would promote the hydrolysis of the *S*-acylcysteine thioesters and prevent later manifestations of INCL. The current work provides the basis for a simple assay for the screening of compounds for potential therapeutic use in this disorder.

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