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Tripeptidyl Peptidase I, the Late Infantile Neuronal Ceroid Lipofuscinosis Gene Product, Initiates the Lysosomal Degradation of Subunit c of ATP Synthase[1]

Junji Ezaki, Mitsue Takeda-Ezaki, and Eiki Kominami[2]

Department of Biochemistry, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421

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The specific accumulation of a hydrophobic protein, subunit c of ATP synthase, in lysosomes from the cells of patients with the late infantile form of NCL (LINCL) is caused by a defect in the CLN2 gene product, tripeptidyl peptidase I (TPP-I). The data here show that TPP-I is involved in the initial degradation of subunit c in lysosomes and suggest that its absence leads directly to the lysosomal accumulation of subunit c. The inclusion of a specific inhibitor of TPP-I, Ala-Ala-Phe-chloromethylketone (AAF-CMK), in the culture medium of normal fibroblasts induced the lysosomal accumulation of subunit c. In an *in vitro* incubation experiment the addition of AAF-CMK to mitochondrial-lysosomal fractions from normal cells inhibited the proteolysis of subunit c, but not the β-subunit of ATP synthase. The use of two antibodies that recognize the aminoterminal and the middle portion of subunit c revealed that the subunit underwent aminoterminal proteolysis, when TPP-I, purified from rat spleen, was added to the mitochondrial fractions. The addition of both purified TPP-I and the soluble lysosomal fractions, which contain various proteinases, to the mitochondrial fractions resulted in rapid degradation of the entire molecule of subunit c, whereas the degradation of subunit c was markedly delayed through the specific inhibition of TPP-I in lysosomal extracts by AAF-CMK. The stable subunit c in the mitochondrial-lysosomal fractions from cells of a patient with LINCL was degraded on incubation with purified TPP-I. The presence of TPP-I led to the sequential cleavage of tripeptides from the N-terminus of the peptide corresponding to the amino terminal sequence of subunit c.

Key words: *CLN2*, lysosomes, neuronal ceroid lipofuscinosis, subunit c, tripeptidyl peptidase I.

The neuronal ceroid lipofuscinoses (NCLs) are a closely related group of recessively inherited neurodegenerative diseases (*1–3*). NCLs are currently classified into six forms, based on age at onset, clinical presentations, and genetic linkage. Among these are infantile NCL (INCL, *CLN1*) (*4, 5*), classical late infantile NCL (LINCL, *CLN2*) (*6*), juvenile NCL (JNCL, *CLN3*) (*7, 8*), adult NCL (CLN4) (*9*), and two variant forms of LINCL (*CLN5* and *CLN6*) (*10, 11*). The diseases are pathologically characterized by the massive lysosomal storage of an autofluorescent lipopigment in neurons and a wide variety of extraneuronal cells which show characteristic ultrastructural appearances (*12–14*). Biochemical analyses of the storage bodies have shown that the major stored component is protein (*15*). In many types of NCL except for the infantile form, subunit c of the mitochondrial ATP synthase complex (F_1F_0ATPase) is also stored in the cells (*16–19*). Subunit c of mitochondrial ATP synthase is very hydrophobic and is classified as a proteolipid based on its lipid-like solubility in chroloform/methanol mixtures; it is composed of 75 amino acid residues with a molecular mass of 7,608 Da and is evolutionarily highly conserved (*20*).

Our earlier studies, which involved immunochemical and biochemical analyses of cultured fibroblasts with LINCL, showed that a high concentration of subunit c is specifically stored in the lysosomes and that this accumulation was the result of a specific delay in degradation rather than an increase in the rate of biosynthesis (*19, 21*). The detection of the lysosomal transfer of labeled subunit c from the mitochondria after a long chase period suggests that subunit c is transferred to lysosomes via an autophagic process (*21*). *In vitro* experiments, using substrates and soluble lysosomal fractions which had been isolated separately from control and patient cells, confirmed that the lysosomal fractions derived from patient cells were unable to degrade subunit c, derived either from controls or patients, but were able to degrade other cytoplasmic proteins (*22, 23*). These results suggest that lysosomal proteolytic dysfunction is

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[2] To whom correspondence should be addressed. Tel: +81-3-5802-1030, Fax: +81-3-5802-5889, E-mail: kominami@med.juntendo.ac.jp

Abbreviations: AAF-CMK, Ala-Ala-Phe-chloromethylketone; Cln2p, protein of CLN2 gene; DMEM, Dulbecco's modified Eagle medium; JNCL, juvenile form of neuronal ceroid lipofuscinosis; LINCL, classical late infantile form of neuronal ceroid lipofuscinosis; MCA, 7-(4-methyl)-coumarylamide; NCL, neuronal ceroid lipofuscinosis; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TPP-I, tripeptidyl peptidase I; tyrostatin, *N*-isovaleryl-tyrosyl-leucyl-tyrosinal.

likely to be the factor responsible for the specific delay in the degradation of subunit c. However, no significant differences were observed in the activities of well known lysosomal proteases, including cathepsins D, B, L, H, and C (*24*).

The *CLN2* gene has recently been shown to be mutated in LINCL patients (*6*, *25*). The predicted amino acid sequence of the CLN2 protein (Cln2p) shows significant homologies to pepstatin-insensitive prokaryotic carboxyl proteases (*26*, *27*, *6*). Specific antibodies were raised against Cln2p and their use indicated that Cln2p is missing in fibroblasts with LINCL, and that lysosomal CLN2p is essential for the degradation of the subunit c of ATP synthase, but not for the degradation of the β subunit of ATP synthase (*28*). It has recently been reported (*29*) that tripeptidyl peptidase I is identical to Cln2p from a sequence comparison of TPP-I and CLN2, and that TPP-I activities are deficient in LINCL fibroblasts.

The present study was implemented in order to examine whether TPP-I/Cln2p activity is involved in the specific degradation of subunit c of ATP synthase. The inhibition of TPP-I activities in cultured cells and *in vitro* incubation experiments was consistent with a specific reduction in the degradation of subunit c, leading to its lysosomal accumulation.

MATERIALS AND METHODS

Materials—Percoll was obtained from Amersham Pharmacia Biotechnology (Uppsala, Sweden). Nylon membranes (GVHP) were from Nihon Millipore (Tokyo). Antibodies against TPP-I/Cln2p402-518 were obtained as previously described (*28*). Antibodies against synthetic peptides which correspond to the amino-terminal sequence of subunit c of mitochondrial ATP synthase (residue 1–11) were obtained as previously described (*19*). Antibodies against synthetic peptides corresponding to the middle portion of subunit c of mitochondrial ATP synthase (residue 36–48) were a gift of Dr. Rowan (The Hospital for Sick Children, London, United Kingdom). Antibodies against the β subunit of ATP synthase were produced in rabbits, as described previously (*22*). Antibodies against subunit IV of cytochrome oxidase were gifts from Dr. Takamiya (Juntendo University). Antibodies against human cathepsin D were purchased from Athens Research and Technology (USA). Ala-Ala-Phe-7-(4-methyl)coumarylamide (Ala-Ala-Phe-MCA) and Ala-Ala-Phe-chloromethylketone (AAF-CMK) were purchased from Sigma (Pool, UK).

Fibroblast Culture—Skin fibroblasts WG308, derived from LINCL patients, were obtained from the McGill University Repository for Mutant Human Cell Strains. In the case of cell type WG308, a mutation within the codon (TGT) encoding for cysteine^{365} in CLN2 has been demonstrated (*6*). Fibroblasts from patients with LINCL and controls were cultured at 37°C under 5% CO_2 in air in Dulbecco's modified medium, supplemented with 10% (v/v) heat-inactivated fetal calf serum and antibiotic-antimycotic solution (GIBCO).

Preparation of Mitochondrial-Lysosomal Fractions (ML-Fractions) and Isolation of Mitochondrial and Lysosomal Fractions—ML-fractions from fibroblasts were obtained as described previously (*21*). Confluent fibroblasts were washed twice in ice-cold 0.25 M sucrose (pH 7.4) and homogenized in the same solution with 20 strokes of a glass homogenizer (Dounce, size A). The homogenate was centrifuged at 1,000 ×*g* for 2 min to remove nuclei and unbroken cells. The unbroken cells were resuspended in 0.25 M sucrose and the homogenization cycle was repeated four times. The combined post-nuclear supernatant was centrifuged at 11,000 ×*g* for 20 min, and the precipitate obtained was used as ML-fractions. Mitochondrial and lysosomal fractions were isolated separately by Percoll density gradient centrifugation as described previously (*21*). Lysosomal fractions were suspended in 3 volumes of H_2O, sonicated for 2 min, and centrifuged at 105,000 ×*g* for 60 min, and the supernatants were used as soluble lysosomal fractions. Activities of cathepsin B and β-hexosaminidase were nearly completely recovered in the soluble fractions.

In Vitro Incubation Experiments—ML-fractions were incubated with different concentrations of AAF-CMK or the purified TPP-I at 37°C for 24 h at pH 5. Mitochondrial fractions (2.5 μg) were incubated with different concentrations of the purified TPP-I and 1.5 μg of soluble lysosomal fractions at 37°C for 24 h at pH 5. After incubation, sodium dodecyl sulfate (SDS) treatment buffer was added, the samples were boiled for 3 min at 100°C, then analyzed by immunoblotting. Densitometry measurements of the signal on the immunoblots were performed using a MasterScan gel analysis system and software (Scanalytic Inc, Billerica, USA).

Analytical Methods—For the detection of subunit c, proteins were separated by Tricine-SDS–polyacrylamide gel electrophoresis (Tricine-SDS-PAGE) (*30*) in 16.5% (w/v) acrylamide. Other proteins were analyzed by SDS-PAGE according to the method of Laemmli (*31*). Immunoblot analyses were performed by the ECL immunoblotting protocol (Amersham International, Buckinghamshire, UK) or the method of Towbin *et al.* (*32*), except that 2,4-dichloro-1-naphthol was used as the substrate for the horseradish peroxidase conjugate of anti–rabbit IgG (*33*). Samples were separated by SDS-PAGE and transferred to nitrocellulose (*32*). The nitrocellulose sheet was suspended in Tris-buffered saline (10 mM Tris, pH 8.0, 150 mM NaCl) containing 1% bovine serum albumin for 1 h at room temperature, then incubated at room temperature for 2 h with various affinity-purified IgGs (5 μg/ml in Tris-buffered saline). The nitrocellulose sheet was washed three times with Tris-buffered saline containing 0.05% Tween 20. Protein concentration was determined by BCA protein assay following the manufacturer's protocol (Pierce, Rockford, IL, USA). Fluorometric assay for TPP-I using Ala-Ala-Phe-MCA as the substrate was carried out as described previously (*34*).

Purification of TPP-I—TPP-I was purified from rat spleen according to the method of Vines and Warburton (*35*). The purity of the enzyme is shown in Fig. 3. All procedures in the purification of enzymes were carried out at 4°C.

Digestion of Peptides—Synthetic peptides which correspond to the amino-terminal sequence (residue 1–11) and the middle portion (residue 38–47) of subunit c of mitochondrial ATP synthase (100 μg) were digested with 1 or 2 μg of TPP-I in 0.1 M sodium acetate pH 4.0 for 0.5–12 h. The samples were dissolved in water/0.1% TFA and subjected to reverse phase HPLC on using a 0–28% acetonitrile/0.1% TFA gradient over a 40 min period.

RESULTS

Lysosomal Accumulation of Subunit c in Cultured Fibroblasts by Treatment with AAF-CMK, a Specific Inhibitor of TPP-I—To determine whether TPP-I activities are involved in the degradation of subunit c of mitochondrial ATP synthase, the effect of AAF-CMK on the lysosomal accumulation of subunit c was investigated in normal fibroblasts. AAF-CMK was added to a culture medium of normal fibroblasts at different concentrations. After 20 days of culture in the presence or absence of inhibitor, the cells were fractionated in a Percoll density gradient to give the mitochondrial and lysosomal fractions as previously described (*22*). Figure 1 summarizes the reactivities of the antibodies against various mitochondrial proteins (subunit c, β subunit of ATP synthase, and subunit IV of cytochrome oxidase). The presence of AAF-CMK caused the lysosomal storage of subunit c in a dose-dependent manner. In the presence of AAF-CMK, a significant amount of subunit c was detected in the lysosomal fractions, whereas in cells to which only dimethyl sulfoxide (DMSO), the solvent for AAF-CMK had been added, subunit c was found exclusively in the mitochondrial fractions. Both the β subunit of ATP synthase and cytochrome oxidase subunit IV were detected only in mitochondrial fractions of control and AAF-CMK–treated cells, and were barely detectable in lysosomes. In a previous study, pulse-chase experiments (*21*) revealed that subunit c appeared in the lysosomes of patients with LINCL after 7 and 14 days of chase. In the experiments described herein, no significant lysosomal deposition of subunit c was detected after 4 days of culturing in the presence of AAF-CMK, and after 14 days of culture, no detectable amounts of subunit c in the lysosomal fractions could be detected (not shown).

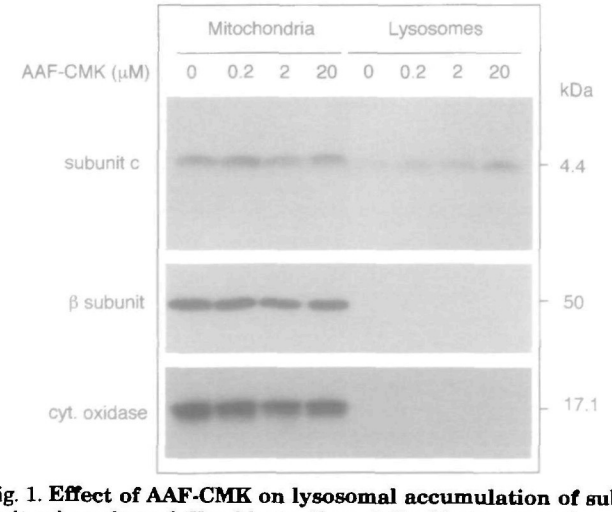

Fig. 1. **Effect of AAF-CMK on lysosomal accumulation of subunit c in cultured fibroblasts.** Normal fibroblasts were cultured for 20 days in the absence or presence of different concentrations of AAF-CMK. Cells were harvested and fractionated in Percoll density gradients into mitochondrial and lysosomal fractions as described previously (*21*). Fractions were pooled separately and washed by repeated centrifugation. Ten micrograms of proteins were separated on 16.5% Tricine-SDS-PAGE for subunit c of ATP synthase and subunit IV of cytochrome oxidase or 12.5% SDS-PAGE for β-subunit of ATP synthase. The gels were analyzed by immunoblotting with antibodies against the aminoterminal portion of subunit c of ATP synthase c, the β-subunit of ATP synthase, and subunit IV of cytochrome oxidase.

Specific Inhibition of Degradation of Subunit c by AAF-CMK—We next investigated the effect of AAF-CMK on the proteolysis of endogenous subunit c by means of an *in vitro* cell-free incubation system (*23*, *28*). ML-fractions prepared from normal fibroblasts were incubated for 24 h at 37°C and pH 5 and the degradation of subunit c was analysed by immunoblotting analysis. The optimum pH for degradation of subunit c was 5 (*22*). The *in vitro* incubation experiments and immunoblotting analyses were repeated several times, and representative data are shown in Fig. 2. Subunit c of the ML-fraction from control fibroblasts was completely degraded at pH 5 after incubation in the absence of inhibitor, but the degradation of subunit c was effectively inhibited by AAF-CMK (Fig. 2). The quantity of remaining sub-

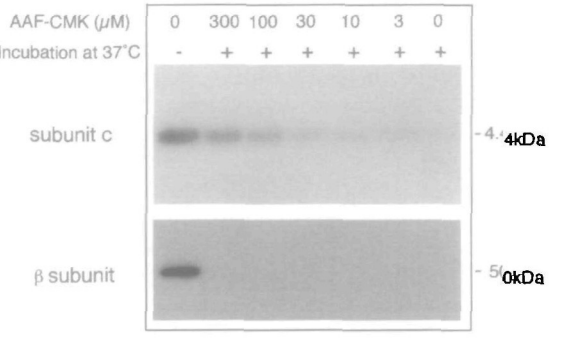

Fig. 2. **Effect of AAF-CMK on the *in vitro* degradation of mitochondrial proteins.** Mitochondrial-lysosomal fractions from normal fibroblasts (10 μg each) were incubated at the indicated concentrations of AAF-CHM at pH 5 for 24 h. The samples were separated on 16.5% Tricine-SDS-PAGE for subunit c of ATP synthase and 12.5% SDS-PAGE for β-subunit of ATP synthase. The gels were analyzed by immunoblotting with artibodies against the aminoterminal portion of subunit c of ATP synthase c, the β-subunit of ATP synthase, and subunit IV of cytochrome oxidase.

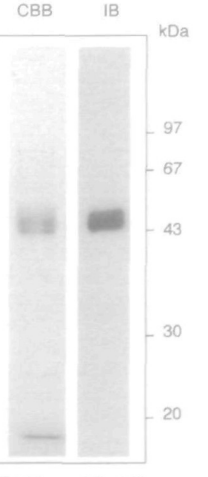

Fig. 3. **The purity of TPP-I purified from rat spleen and its reactivity against anti-TPP-I/Cln2p.** TPP-I was purified from rat spleen by the reported method (*35*). TPP-I (10 μg) was separated by 10% SDS-PAGE and stained with Coomassie Brilliant Blue (CBB). TPP-I (2 μg) was separated by 10% SDS-PAGE and analyzed by immunoblotting with the anti–TPP-I/Cln2p antibody (IB).

unit c increased, depending on the concentration of AAF-CMK. AAF-CMK had no effect on the degradation of β subunit of ATP synthase even at high concentrations. In Fig. 2, the rate of degradation of subunit c was followed by immunoblotting with antibodies against synthetic peptides which correspond to the amino-terminal sequence of subunit c of mitochondrial ATP synthase (residue 1–11).

TPP-I Initiates the Degradation of the Entire Molecule of Subunit c—To determine whether the proteolysis of the aminoterminal segment by TPP-I is the rate-limiting step in the degradation of the entire molecule of subunit c, the mitochondrial fraction from normal fibroblasts was incubated with different concentrations of the purified TPP-I and the soluble lysosomal fractions.

For this experiment, TPP-I was purified to near homogeneity from rat spleen, as described previously (*35*). The purified enzyme, which has an apparent molecular mass of 46 kDa, reacted with anti–TPP-I/Cln2p (Fig. 3). Antibodies had been raised against the GST fusion protein, which encodes the carboxyl terminal domain of TPP-I/Cln2p (GST/Cln2p$_{402-518}$), as described previously (*28*). AAF-CMK strongly inhibited the purified enzyme, whereas pepstatin or tyrostatin, an inhibitor of prokaryotic pepstatin-insensitive carboxyl peptidases, had little effect (*36*). The mitochondrial fractions were used as substrate in place of purified subunit c, since it is difficult to purify a very hydrophobic protein, such as this one, in a native state from the mitochondrial inner membrane. The addition of purified enzyme to the mitochondrial fractions resulted in the degradation of subunit c in a dose-dependent manner, as determined with antibodies against synthetic peptides that correspond to the aminoterminal sequence of subunit c (Fig. 4A). However, the only a slight loss of subunit c was observed with antibodies against synthetic peptides that correspond to the middle portion of subunit c. Moreover, immunoblotting analysis showed that two bands reacted with the anti-middle portion of subunit c on incubation with high concentration of TPP-I, suggesting the production of subunit c is lacking the aminoterminal portion under these conditions.

Next, the mitochondrial fractions from normal fibroblasts were incubated with different concentrations of the purified TPP-I in the presence of lysosomal extracts (Fig. 4B). The addition of the soluble lysosomal fractions, which contain various proteinases, resulted in rapid and extensive degradation of subunit c. The disappearance of subunit c could be observed with either antibodies against the aminoterminal sequence of subunit c or antibodies against the middle portion of subunit c, suggesting that the entire molecule of subunit c had been degraded. However, on incubation of the mitochondrial fraction with the soluble lysosomal fractions and TPP-I treated with AAF-CMK, the degradation of subunit c was markedly inhibited. The loss of subunit c was faint or did not occur at all, as evidenced by the use of antibodies against the aminoterminal and middle portion of subunit c, respectively (Fig. 4B). We found that AAF-CMK does not inhibit the activities of lysosomal cysteine proteinases and asparatic proteinases, such as cathepsins B, L, H, and D (not shown). The results herein suggest that further degradation of subunit c following an initial cleavage by TPP-I occurs rapidly with the participation of other lysosomal proteinases, and the cleavage by TPP-I is rate-limiting

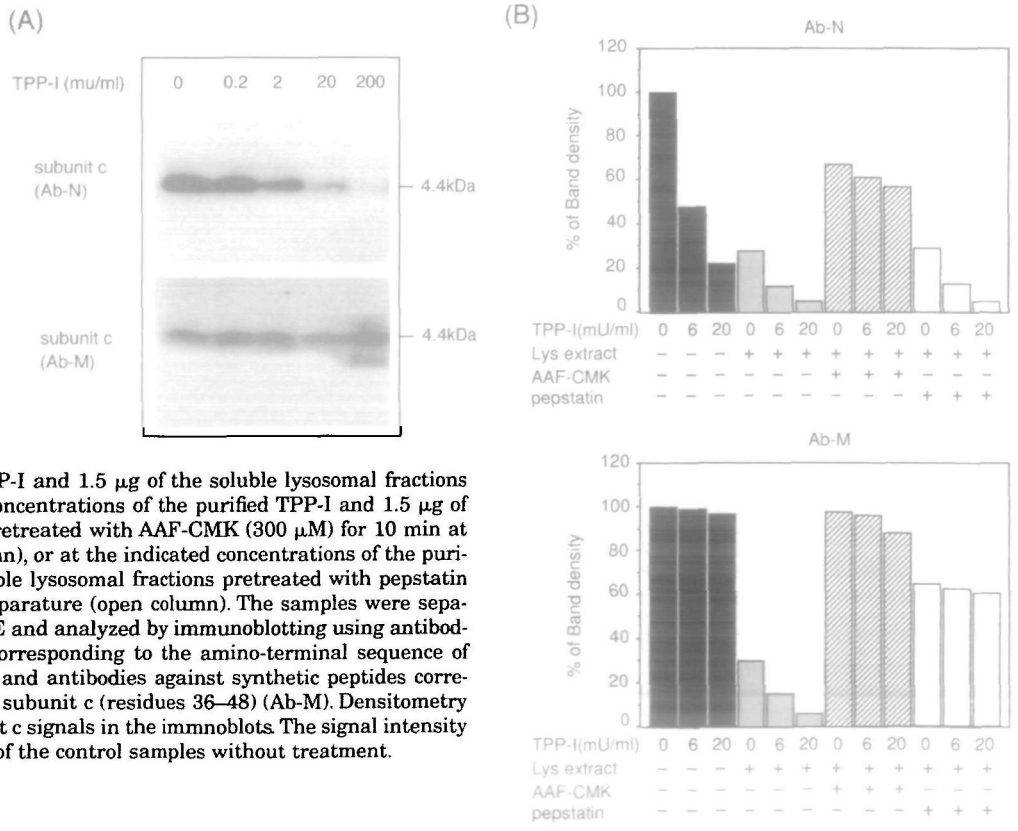

Fig. 4. **Degradation of the entire molecule of subunit c initiated by proteolysis of the aminoterminal segment by TPP-I.** (A) Mitochondrial and lysosomal fractions were separately isolated as described in "MATERIALS AND METHODS." Mitochondrial fractions from control fibroblasts (10 μg each) were incubated with the indicated concentrations of purified TPP-I at pH 5 for 24 h. (B) Mitochondrial fractions from control fibroblasts (2.5 μg each) were incubated at the indicated concentrations of the purified TPP-I alone (black column) at pH 5 for 24 h, at the indicated concentrations of the purified TPP-I and 1.5 μg of the soluble lysosomal fractions (gray column), at the indicated concentrations of the purified TPP-I and 1.5 μg of the soluble lysosomal fractions pretreated with AAF-CMK (300 μM) for 10 min at room temparature (hatched column), or at the indicated concentrations of the purified TPP-I and 1.5 μg of the soluble lysosomal fractions pretreated with pepstatin (0.1 μM) for 10 min at room temparature (open column). The samples were separated on 16.5% Tricine SDS-PAGE and analyzed by immunoblotting using antibodies against a synthetic peptide corresponding to the amino-terminal sequence of subunit c (residues 1–11) (Ab-N) and antibodies against synthetic peptides corresponding to the middle portion of subunit c (residues 36–48) (Ab-M). Densitometry was used to quantitate the subunit c signals in the immnoblots. The signal intensity was expressed as the percentage of the control samples without treatment.

in terms of the lysosomal degradation of subunit c. The inhibition of asparatic proteinases, such as cathepsins D and E, in the lysosomal extracts by pepstatin had no effect on the initial aminoterminal proteolysis of subunit c, but led to a significant decrease in the further degradation (Fig. 4B).

Restoration of the Degradation of Subunit c in Patient Cells with LINCL by the Addition of Purified TPP-I—We previously showed that subunit c in the ML-fraction of fibroblasts from a patient with LINCL was stable, even after incubation in the cell-free *in vitro* system, while the addition of the ML-fraction from normal cells caused subunit c to be completely digested in patient cells (*22*). If TPP-I represents the proteolytic source contained in the ML-fraction from normal cells, the addition of purified TPP-I to the ML-fraction of patient fibroblasts should permit the recovery of the degradative capacity of subunit c. ML fractions prepared from fibroblasts of a patient with LINCL were incubated at pH 5 for 24 h at 37°C in the presence or absence of various concentrations of purified TPP-I (Fig. 5). As described previously, subunit c in the ML-fractions from patient cells was not degraded even after 24 h of incubation at pH 5. The addition of purified TPP-I to the ML fraction of patient cells resulted in the degradation of subunit c in a dose-dependent manner. About half of the subunit c was degraded by 10 mU/ml of TPP-I after a 24-h incubation at pH 5, and after the incubation, the loss of subunit c was also observed by using antibodies specific to the middle portion of subunit c (not shown). These results suggest that the poor subunit c-degradating activity of fibroblasts with LINCL could be restored by the additon of purified TPP-I, and that TPP-I is the enzyme which is involved in the initial cleavage of subunit c of ATP synthase.

Degradation of the Peptide Containing the N-Terminus of Subunit c by TPP-I—TPP-I was reported to cleave tripeptides, such as angiotensin II and glucagon, from the N-terminus of oligopeptides (*35*, *37*). It is possible, therefore, that TPP-I cleaves tripeptides from the N-terminus of subunit c. To address this issue, the effect of the purified TPP-I on synthetic polypeptides corresponding to the amino terminal sequence (residues 1–11, DIDTAAKFIGAA) and the middle portion (residues 38–46, RNPSLKQQL) of subunit c were tested. It is thought that these regions of subunit c are exposed to the matrix or inter-membrane space (*38*). Synthetic polypeptides of N-terminus of subunit c (100 μg) were incubated with 2 μg of purified TPP-I at 37°C for the indicated time at pH 4. The reaction products were separated by reverse-phase HPLC and determined by amino acid analysis (Fig. 6). After 30 min of incubation, the first tripeptide (DID) and the peptide with the three N-terminal amino acids removed (TAAKFIGAAC) were detected, and after 2–12 h of incubation the peptide with the first six N-terminal amino acids removed (KFIGAAC) could be identified. The specific inhibitor of TPP-I, AAF-CMK, completely prevented the appearance of cleavage products. It is likely that TPP-I sequentially cleaves the aminoterminal portion

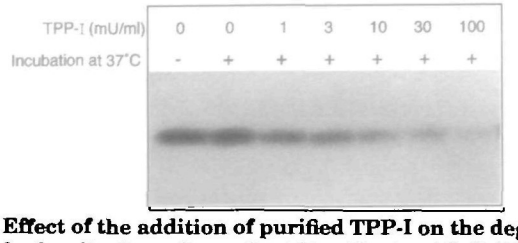

Fig. 5. **Effect of the addition of purified TPP-I on the degradation of subunit c from the patient fibroblasts with LINCL.** Mitochondrial-lysosomal fractions from fibroblasts of a patient with LINCL (WG308) (10 μg each) were incubated at the indicated concentrations of the purified TPP-I at pH 5 for 24 h. The samples were separated on 16.5% Tricine SDS-PAGE for subunit c of ATP synthase, and analyzed by immunoblotting with antibody against the aminoterminal portion of subunit c of ATP synthase c.

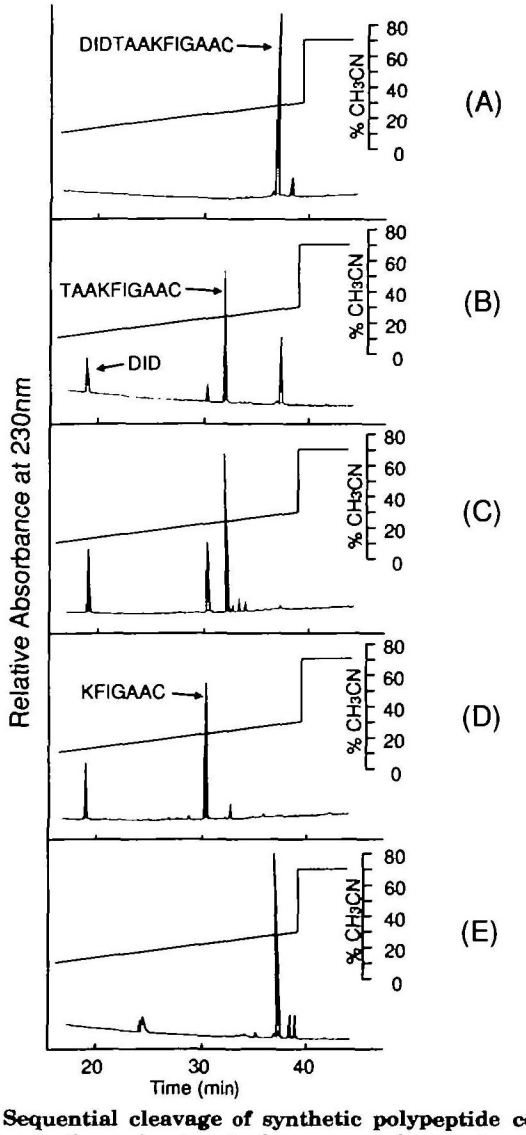

Fig. 6. **Sequential cleavage of synthetic polypeptide corresponding to the amino terminal sequence of subunit c (DIDTAAKFIGAAC) by the purified TPP-I.** One-hundred micrograms of synthetic peptide corresponding to the amino terminal sequence of subunit c (DIDTAAKFIGAAC) was incubated with 2 μg of TPP-I in sodium acetate buffer, pH 4.0 at 37°C for 30 min (B), 2 h (C), 12 h (D), or 2 h in the presence of 200 μM AAF-CMK (E). Reactions were quenched by adding TFA to a concentration of 0.1%, and reaction mixtures were analyzed by HPLC using a Cosmosil C18 column. The digests were eluted with 0–28% acetonitrile gradient containing 0.1% TFA. The elution position of undegraded synthetic polypeptides is shown in (A). Peaks were collected and identified by protein sequence analysis. The sequences of these polypeptides are indicated in the figure.

of subunit c, releasing tripeptides. A synthetic polypeptide corresponding to the middle portion of subunit c (100 μg) was resistant to TPP-I: only a very small amount of the first tripeptide (RNP) was obtained by incubation with 2 μg of purified TPP-I at 37°C for 24 h at pH 4 (not shown).

DISCUSSION

Several lines of evidence suggest that TPP-I is identical to Cln2p and that it is deficient in LINCL. (i) A comparison of the amino acid sequences of TPP-I with sequences derived from an EST database suggest that TPP-I is identical to the non-mutated form of TPP-I/Cln2p in LINCL (*29, 39*). (ii) Fibroblasts of patients with LINCL are deficient in TPP-I activity (*29, 36*). (iii) LINCL fibroblasts are defective in their ability to degrade small peptides (*29*), and this deficiency can be induced in normal cells by the use of a specific inhibitor. We were able to show that TPP-I from human fibroblasts (*36*) and purified from rat spleen (Fig. 3) reacted with anti-TPP-I/Cln2p antiserum, and to confirm a marked reduction of both TPP-I/Cln2p protein and TPP-I activity with the accumulation of subunit c of ATP synthase in fibroblasts of patients with LINCL (*28, 36*). The molecular weight of TPP-I/Cln2p in fibroblasts was 46 kDa (*28*), which is consisitent with the molecular weight of rat TPP-I (*35*). Pulse-chase experiments in a previous study (*28*) revealed that Cln2p/TPP-I is synthesized as a 67 kDa precursor and subsequently processed to 46 kDa mature proteins. In LINCL fibroblasts, neither the labeled precursor nor the mature form could be detected.

TPP-I is a lysosomal peptidase that removes tripeptides from the aminoterminus of peptides (*35, 40*). TPP-I is unusual in that it is not inhibited by any of the classical inhibitors of serine, cysteine, asparatic, or metalloproteases (*35–37*). TPP-I is potently inhibited by chloromethylketone analogues of tripeptidyl substrates (*35–37*). In our studies with the enzyme purified from rat spleen, the enzyme was not inhibited by tyrostatin, a competitive inhibitor of bacterial pepstatin-insensitive carboxyl peptidase (*36*) or pepstatin. Bacterial pepstatin-insensitive carboxyl peptidases can be classified into two groups on the basis of tyrostatin sensitivity. Carboxyl proteases from *Pseudomonas* (*26*) and *Xanthomonas* (*27*) are inhibited by tyrostatin (*41*), but carboxyl protease from *Bacillus coagulans* J-4 (*42*) is not (personal communication, Oda). Recently we have shown (*36*) that TPP-I has endopeptidase activity with substrate specificity similar to that of a carboxyl protease from Bacillus coagulans J-4. However, the issue of whether TPP-I is a carboxyl protease in mammals remains obscure. For this, identification of the active site of TPP-I will be required.

A previous study clearly showed that TPP-I/Cln2p is involved in the degradation of subunit c of ATP synthase, not in the β subunit of ATP synthase (*28*). This study provides the following evidence that TPP-I/Cln2p is directly involved in both the *in vitro* and *in vivo* degradation of subunit c. (i) The inclusion of a specific inhibitor of TPP-I, AAF-CMK, in the culture medium of normal fibroblasts could induce lysosomal accumulation of subunit c, but not of other mitochondrial proteins, the β-subunit of ATP synthase, or subunit IV of cytochrome oxidase (Fig. 1). (ii) In *in vitro* incubation experiments using mitochondrial-lysosomal fractions from normal fibroblasts, AAF-CMK exerted a marked inhibitory effect on the proteolysis of subunit c, but not the β subunit of ATP synthase (Fig. 2). (iii) A failure in the proteolytic degradation of subunit c in mitochondrial lysosomal fractions from patient cells with LINCL could be recovered by the addition of the purified TPP-I (Fig. 5). (iv) TPP-I sequentially cleaved tripeptides from the N-terminus of the peptide corresponding to the amino terminal sequence of subunit c (Fig. 6). These findings support the view that TPP-I activity is required for the degradation of subunit c of ATP synthase.

For analyses of the *in vitro* degradation of subunit c, two antibodies which recognize the aminoterminal and the middle portion of subunit c were used, indicating that TPP-I caused limited proteolysis of subunit c at the aminoterminal region (Fig. 4A). In *in vitro* experiments using the mitochondrial fractions as substrate and soluble lysosomal fractions from normal fibroblasts, there was great difference in the rates of degradation of the entire molecule of subunit c between the addition of AAF-CMK and non-addition (Fig. 4B). A combination of soluble lysosomal fractions and active TPP-I caused extensive degradation of the entire molecule of subunit c, whereas soluble lysosomal fractions without active TPP-I failed to degrade the entire molecule of subunit c effectively (Fig. 4B). The results suggest that the further degradation of subunit c following the initial cleavage by TPP-I occurs rapidly, possibly *via* the participation of other lysosomal proteinases, and that cleavage by TPP-I is rate-limiting in lysosomal degradation of subunit c. Therefore, a deficiency in TPP-I activity causes the lysosomal deposition of subunit c. In our recent studies (*43*), the CNS neurons in cathepsin D-knockout mice showed a new form of lysosomal accumulation disease with a phenotype resembling neuronal ceroid lipofuscinosis. Subunit c was found to accumulate in the lysosomes of neurons, but the activity of TPP-I was rather increased in the brain, suggesting that cathepsin D might be responsible for further degradation of subunit c following the initial cleavage by TPP-I. In fact, the inhibition of asparatic proteinase(s) in the lysosomal extracts by pepstatin caused a dramatic reduction of further degradation of subunit c without effect on the initial cleavage by TPP-I (Fig. 4B).

However, the lysosomal accumulation of subunit c of mitochondrial ATP synthase has been observed to be a defect, not only in the case of proteases, TPP-I or cathepsin D, but also in the defect of CLN3 and CLN5 (*7, 10*). CLN3 and possibly CLN5 represent genes which code for lysosomal membrane molecules (*8, 10*). Further studies on the interaction between TPP-I and these membrane components, relative to subunit c degradation, will be required for a further understanding of the mechanism of the lysosomal deposition of subunit c

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