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Contribution of Peroxisome-specific Isoform of Lon Protease in Sorting PTS1 Proteins to Peroxisomes

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Using an organelle proteomics approach, we previously studied the rat peroxisome in order to characterize the proteins participating in its biogenesis. A peroxisome-specific isoform of Lon (pLon) protein was accordingly identified. However, the precise role of pLon in peroxisomes remains to be elucidated. Here, we demonstrate that pLon plays a role in processing and activating a specific regulatory protein belonging to the peroxisome targeting signal (PTS) 1-containing proteins. Proteomic analysis of proteins co-immunoprecipitated with Lon suggested that Lon interacts with PMP70 and several enzymes involved in β-oxidation, including acyl-CoA oxidase (AOX). The processing of AOX for its activation in peroxisomes was strongly inhibited in cells expressing a dominant negative form of pLon. Furthermore, a catalase possessing a modified PTS1 sequence was misdistributed in this cell line. pLon exhibits little, if any, *in vitro* AOX processing activity, and does not process PTS2-containing 3-ketoacyl-coenzyme A thiolase (PTL). Therefore, pLon may specifically control, sort and process PTS1 proteins. Based on the relationship between pLon and the β-oxidation enzymes that regulate peroxisomal morphology, the observation of enlarged peroxisomes in cells expressing recombinant pLon suggests that pLon is a critical factor determining peroxisome morphology.

Key words: peroxisome, β-oxidation, PTS1, protease, proteomics.

Abbreviations: CBB, Coomassie brilliant blue; HEK, human embryonic kidney; RT–PCR, reverse transcriptase–PCR; siRNA, small interference RNA.

The peroxisome is an organelle involved in a variety of metabolic processes including peroxide-based respiration, the β-oxidation of long-chain fatty acids and the synthesis of bile acids and plasmalogens (*1*). Peroxisomal proteins constituting the enzymes catalysing these reactions are translated in the cytoplasm; these proteins possess either peroxisome-targeting signal (PTS) 1 or 2 (*2*). PTS1 has been characterized as a protein that attaches to the C-terminal conserved amino acids sequence, S/A-K-L (*3*). The mechanism of the translocation of PTS1-containing proteins has been intimately studied using yeast genetics and cultured mammalian cells. The cytosolic soluble receptor peroxin (Pex)5p recognizes PTS1-containing proteins as cargo, which is carried to the peroxisomal membrane (*4*). Pex5p is thought to be recycled by shuttling between the peroxisomal surface and the cytoplasm complexed with several peroxin and peroxisomal matrix proteins (*5*). Compared to the number of PTS1-containing proteins, only a few peroxisomal proteins are synthesized as precursors containing the N-terminal PTS2 motif R/K-L/V/I-5aa-H/Q-L/A/F. These proteins undergo proteolytic digestion prior to translocation to the peroxisome (*6*). Recently, Tysnd1 has been identified as a processing enzyme for PTS2-containing proteins. Moreover, another peroxisomal enzyme containing a PTS1 sequence, and associated with β-oxidation, has been demonstrated to be processed and activated by this protease (*7*).

Previously, using an organelle proteomics approach, we identified peroxisomal Lon (pLon) protein in rat liver peroxisomes (*8*). Lon is characterized as an ATP-dependent protease that is conserved in a wide variety of species (*9*). In bacteria, Lon is known as La protease and plays a role in excluding abnormal proteins or chaperons (*10*). Mitochondrial Lon (mLon) in mammalian cells has also been characterized as a protease (*11*). La and mLon possess the ability to bind to chromosomal and mitochondrial DNA, respectively. La exhibits its ATPase activity by binding to DNA (*12*); mLon contributes to mitochondrial DNA maintenance (*13*). pLon is specifically localized in the peroxisomes, organelles that contain no DNA molecules; both its function in mammalian cells and its enzymatic properties remain to be determined. In plants, three forms of Lon, namely, Lon1–3, have been identified. Lon1, which possesses a C-terminal PTS1 sequence, has been demonstrated to be identical to the mammalian pLon (*14*).

Although recent papers provided evidences that pLon is related to peroxisomal morphology in yeast (*15*), or autophagy of proliferated peroxisome in rat liver cells (*16*), the precise role of each Lon molecule has not been analysed.

In the present study, we used biochemical and cell biological techniques to functionally characterize pLon.

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We report that several enzymes related to β-oxidation were identified as the proteins binding to pLon, and that PTS1-containing proteins are regulated by pLon expression. Furthermore, pLon might contribute to peroxisomal division and morphology.

MATERIALS AND METHODS

Reagents, Cell Culture and Transfection—Materials unless otherwise specified, all reagents were purchased from Sigma (St Louis, MO, USA) or WAKO Pure Chemicals (Osaka, Japan). COS-7, HeLa, HEK293 and 293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 100 μg/ml streptomycin and 100 U/ml penicillin. Transfection of plasmids into COS-7 or 293T cells was carried out by electroporation using a Gene-Pulser (Bio-Rad, Hercules, CA, USA).

cDNA Cloning and Vector Construction—For cloning human cDNAs of pLon, mLon, acyl-CoA oxidase (AOX) and 3-ketoacyl-coenzyme A thiolase (PTL), PCR was carried out using the following primer pairs. pLon: 5′-ATTGACGC AAATGGGCGGTAGGCGTGT-3′ and 5′-AAACTCGAGAT TTGGACCTACAGTTTGC-3′; mLon: 5′-GTTTGAATTCA TGACGATCCCCGATGTGTTTCCG-3′ and 5′-GAAAGGA TCCCCGTTCCACGGCCAGCGCCTC-3′; AOX: 5′-GTTTG GATCCATGAACCCGGACCTGCGC-3′ and 5′-GAAACTC GAGTCAGAGCTTGGACTGCAG-3′; and PTL: 5′-GACCT GCCATATGCAGAGGCTGCAGGTAGTGC-3′ and 5′-CTG GATCCTCACTCAGTTCCCAGGGTATTC-3′. The amplified products were first subcloned into a TOPO™ TA cloning vector (pCR®2.1-TOPO; Invitrogen, Carlsbad, CA, USA) and then sequenced. The pLon and AOX cDNAs were inserted into a pQCIXP vector (Clontech, Franklin Lakes, NJ, USA) and a pCMV-3Tag-2 vector (Stratagene, La Jolla, CA, USA) and expressed as FLAG-tagged and 3×Myc-tagged proteins, respectively, at the N-terminus. The PTL cDNA was inserted into a pcDNA3 vector (Invitrogen) and expressed as 1× Myc-tagged proteins at the C-terminus. The point and internal deletion constructs (pLonS743A and pLonΔ75–490) were introduced using the QuickChange kit (Stratagene) following the manufacturer's protocol. All nucleotide sequences were determined and verified using an ABI PRISM Dye Terminator Cycle Sequencing Kit (Perkin-Elmer Life Sciences, Montreal, ON, Canada) and an ABI PRISM 3100-Avant genetic analyser.

Antibodies—A rabbit polyclonal anti-pLon antibody was prepared as described previously (*8*). The other antibodies used were obtained commercially from various companies as follows: anti-FLAG M2, anti-PMP70 and anti-β-actin (Sigma); anti-cytochrome *c* (SC-7159; Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-AOX (ABNOVA, Taipei City, Taiwan); anti-catalase (Abcom, Cambridge, MA, USA) and anti-myc (9E10; Roche, Indianapolis, IN, USA).

Semi-Quantitative RT–PCR—In order to assess the relative expression levels of pLon and PMP70 transcripts, reverse transcriptase–polymerase chain reaction (RT–PCR) was performed for each panel of eight different human culture cell and tissue cDNAs (human tissue and cell line MTC panel; Clontech) by using the following primer pairs. pLon: 5′-GAAATTACACATATCT CAGG-3′ and 5′-TCATCTAATAGGAACACTGG-3′; and PMP70: 5′-AACGAGTGCAATTGGAGCTCAGGG-3′ and 5′-TTCTCCAGCACCAGGTATCAAGGG-3′. The normalized cDNA was amplified under the following conditions: denaturation at 94°C for 1 min; 30 cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 1 min; and extension at 72°C for 5 min. The PCR products were electrophoresed on 1.5% agarose gels containing ethidium bromide.

Cloning of the HEK293 Stable Cell Line—The mammalian expression vector pQCXIP (Clontech) was used for expressing FLAG-pLon derivatives and myc-AOX in the HEK293 cells. An empty vector was used as a control. The HEK293 cells were stably infected with an amphotropic receptor. The AmphoPack-293™ cell line (Clontech), *i.e.* packaging cells, was transfected with the appropriate retroviral constructs by electroporation. Culture supernatants were collected at 48 h post-transfection and centrifuged. The HEK293 cells were infected with the viral supernatants in the presence of 8 μg/ml polybrene (Sigma) for 12 h, after which the medium was changed. Following the infection, the transfected cells were selected using 2 μg/ml puromycin.

Small Interference RNA (siRNA)—Synthetic siRNA duplexes were used for the knockdown of pLon expression in HeLa cells (Dharmacon, Boulder, CO, USA). The targeted sequence of human pLon was 5′-GCAGAGAACA CATCTTAGA-3′ (from nucleotide 1,799 to 1,917). siRNA specific for green fluorescent protein (GFP) was used as a control. The HeLa cells were transfected with siRNA by using oligofectamine (Invitrogen).

Immunoprecipitation and Immunoblot Analysis—The following procedures were carried out at 0–4°C. The transfected cells were lysed in a lysis buffer containing 20 mM Tris–HCl (pH 7.5), 1 mM EDTA, 10 mM dithiothreitol (DTT), 1% Triton X-100, 150 mM NaCl, 10 mM NaF, 1 mM Na_3VO_4 and complete protease inhibitor cocktail (Roche). The lysate was used as a whole-cell lysate (WCL). For immunoprecipitation experiments, the WCL was centrifuged and the supernatant was incubated for 2 h with the primary antibody or an anti-FLAG affinity gel (Sigma). Protein G-Sepharose beads (Amersham Biosciences, Piscataway, NJ, USA) were added and the resulting mixture was centrifuged at 4°C for an additional hour. The beads were then washed three times with lysis buffer. The samples were boiled in sodium dodecyl sulphate (SDS) sample buffer, separated by SDS–polyacrylamide gel electrophoresis (PAGE) and transferred to an Immobilon P membrane (Millipore, Bedford, MA, USA). Immunoblot analysis was carried out with primary antibodies, as described in the figure legends. Immunoreactive bands were visualized using horseradish peroxidase-conjugated (HRP) anti-rabbit or anti-mouse IgG and an enhanced chemiluminescent reagent (Amersham Biosciences).

Purification of FLAG-pLon and Its Binding Proteins—A peroxisome-enriched fraction was prepared from the HEK293 cells (2×10^8 cells) that stably expressed FLAG-pLon; the cells were lysed with lysis buffer containing 0.5% sodium deoxycholate (DOC). The lysate was centrifuged and the supernatant was incubated with a FLAG-affinity gel (50 μl bed volume) for 2 h. The gel was

applied to an empty mini column (Bio-Rad) and washed several times with lysis buffer. FLAG-pLon was eluted with 45 μl of 100 mM glycine–HCl at pH 3.0. The eluate was neutralized with 5 μl of 1 M Tris–HCl (pH 9.0) and resolved by SDS–PAGE. All bands visualized using Coomassie brilliant blue (CBB) were subjected to tandem mass spectrometry (LC/MS/MS) analysis after in-gel digestion with trypsin, as described previously (*8*).

Fluorescence and Confocal Microscopic Analysis—Transfected and untransfected cells were fixed with 5% formaldehyde in phosphate-buffered saline (PBS) for 10 min, washed with PBS, permeabilized with 0.1% Triton X-100 in PBS for 10 min and then washed with PBS again. Following blocking with 3% bovine serum albumin (BSA) in PBS for 30 min, the primary antibodies (described in the figure legends) were applied for 1 h. After washing with PBS, the cells were incubated with the appropriate secondary antibodies conjugated with Alexa fluorescent dyes (Molecular Probes, Eugene, OR, USA) for 45 min. If required, the cells were treated with MitoTracker Red (Molecular Probes); the cell nuclei were simultaneously stained with 2 μM Hoechst 33342 (Molecular Probes). Finally, the cells were rinsed three times with PBS and mounted on microscope slides with ProLong Antifade reagent (Molecular Probes). Images were captured using a Zeiss Axiovert 200 fluorescence microscope or an LSM 510 Meta laser scanning confocal microscope with a 40×, 63× or 100× PlanApo objective lens (1.0 numerical aperture) (Zeiss Axioskop; Carl Zeiss Inc., Feldbach, Switzerland) using two separate lasers and emission filters: the 488-nm argon laser line for excitation of Alexa Fluor 488, and the 543 nm laser line for the excitation of Alexa Fluor 594. Figures were prepared using Adobe Photoshop (Adobe Systems, Mountain View, CA, USA).

Preparation of Light Mitochondria (ML) Fraction and Peroxisomes—Cells were harvested in an isotonic mitochondrial buffer [200 mM mannitol, 50 mM sucrose, 1 mM EDTA, 5 mM HEPES (pH 7.5) and 0.1% ethanol] supplemented with a complete protease inhibitor cocktail, and then homogenized in a Dounce homogenizer on ice. The suspension was centrifuged at 1,500*g* for 10 min using a swing-out centrifuge at 4°C. The supernatant was centrifuged at 2,000*g* under the same conditions. Finally, the supernatant was centrifuged at 12,600*g* at 4°C for 22 min to pellet the ML fraction. The peroxisomes were then isolated by sucrose density gradient centrifugation (193,000*g* for 17 h). The peroxisome-containing fraction was determined by the distribution of catalase activity, as described previously (*8*).

β-Oxidation Assay—β-oxidation activity was measured using 100 μl of 100 μM [1-^{14}C]palmitoyl-CoA (74 kBq/μmol) (Amersham Biosciences), 2 mM $MgCl_2$, 0.5 mM coenzyme A, 2 mM NAD^+, 2 mM KCN, 2 mM dithiothreitol, 0.1% (w/v) BSA, 0.25 M sucrose, 50 mM Tris–HCl (pH 7.4), 2 mM ATP and 100 μM FAD. In this assay, 30 μg samples of the peroxisome preparations were employed. After incubation for 60 min at 37°C, the reactions were terminated by adding 700 μl of ice-cold water, 150 μl of 10% (w/v) BSA and 200 μl of 3 M perchloric acid to the reaction mixture. The mixture was centrifuged and the supernatant was extracted three times with 3 ml of hexane. The acid-soluble radioactivity in the medium was measured using a scintillation counter.

In Vitro Protease Activity of pLon—From the lysates of HEK293 stable cell lines, the FLAG-pLon derivatives were immunoprecipitated with an anti-FLAG affinity gel. Using the anti-myc antibody, myc-AOX and myc-PTL were immunoprecipitated from the lysate of COS-7 cells transfected with each respective expression vector; both myc-AOX and myc-PTL were immobilized with the protein G-Sepharose. Both sets of beads were mixed and extensively washed with a lysis buffer, and the buffer was then replaced with PBS. After removing PBS, 20 μl of protease assay buffer [50 mM Tris–HCl (pH 8.0), 2 mM $MgCl_2$, 150 mM NaCl, 2 mM ATP and 1 mM DTT] was added, and the mixture was incubated at 37°C for 1 h. Finally, all assay solutions and beads were boiled and applied to SDS–PAGE gels.

RESULTS

Primary Structure, Expression, and Sub-cellular Localization of Recombinant pLon—As mentioned earlier, pLon was identified through our whole peroxisome proteomics studies; pLon contains a typical PTS1 sequence at the extreme C-terminus. In addition, pLon possesses an AAA (ATPases associated with diverse cellular activities) domain and a protease catalytic domain, both of which are conserved among the Lon family proteins of various species (Fig. 1A) (*9*). The gene expression of pLon was essentially ubiquitous in human tissues and cultured cells, as confirmed by semi-quantitative RT–PCR (Fig. 1B). pLon and PMP70, a peroxisomal membrane-specific protein, exhibited a co-ordinated expression profile—both being highly expressed in the liver, kidney and pancreas. As previously demonstrated, endogenous pLon is specifically localized in the peroxisome (*8*). In order to analyse the precise role of the protein, recombinant pLon was expressed in COS-7 cells and fluorescence and confocal microscopic analyses were subsequently performed (Fig. 1C). The N-terminal FLAG-tagged wild-type protein, but not the mutant lacking the PTS1 region, was localized in the peroxisome; therefore, our constructed expression vectors were considered to be suitable for further analysis of pLon in the peroxisome.

Identification of Proteins Interacting with pLon—In order to gain insights into the function of pLon in mammalian cells, we analysed the proteins that co-precipitated with pLon by performing immuno-affinity purification. Initially, we generated HEK293 cell lines that stably expressed wild-type pLon and its internal deletion mutant, pLonΔ75–490, by using the retrovirus expression system. The mutant cell line was accidentally generated by the aberrant recombination of the infected wild-type pLon gene; this cell line was used for further analysis, as described below. Both the proteins were expressed and were verified to be localized in the peroxisome (Fig. 2A and B).

Next, cells expressing FLAG-tagged pLon were lysed, the peroxisome fraction was prepared and immunoprecipitation was carried out using the anti-FLAG affinity gel. Proteins that were eluted with glycine buffer were

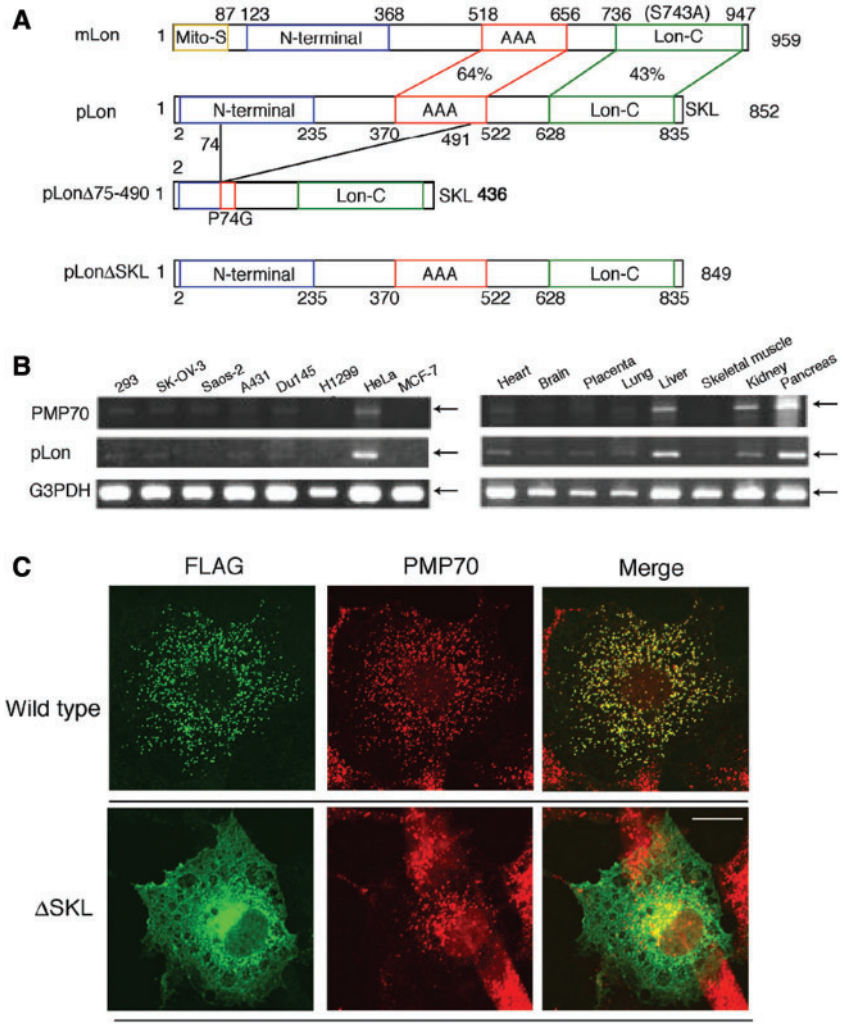

Fig. 1. **Expression profiling of peroxisomal Lon protease (pLon) and its sub-cellular localization.** (A) Schematic representation of the primary structure of mLon, pLon, and pLon derivatives observed in this study. The C-terminal SKL site represents PTS1. Number of amino acid residues at the boundaries and typical functional domains are indicated. Amino acid sequence identities of AAA and C-terminal regions of each Lon are indicated. S743A is a mutant with a replaced critical amino acid in the protease catalytic domain. N-terminal, similar to the N-terminal domain of Lon protease; AAA, AAA superfamily of ATPases; Lon-C, C-terminal proteolytic domain of Lon protease. (B) The mRNA expression level of pLon and a peroxisomal membrane-specific protein PMP70 in various human cell lines (left panel) and tissues (right panel), as determined by RT–PCR. Glycerol-3-phosphate dehydrogenase (G3PDH) was used as a control. (C) COS-7 cells transfected with plasmids encoding full-length FLAG-tagged pLon or FLAG-tagged pLonΔSKL lacking the C-terminal PTS were processed for immunofluorescence microscopy by using an anti-FLAG antibody (Ab) (green) at 48 h after transfection. Peroxisomes were stained with anti-PMP70 Ab (red); merged images are shown in the right panels. Scale bars, 20 μm.

analysed using SDS–PAGE. In addition to pLon, other visual protein bands were analysed by LC/MS/MS in order to identify their amino acid sequences.

The identified proteins included several enzymes associated with the β-oxidation of fatty acids in peroxisomes, including AOX, PTL and hydroxyacyl-coenzyme A dehydrogenase (Fig. 2C). We therefore focused on the relationship between these enzymes and pLon.

Association of pLon and AOX—In order to examine the binding specificity of pLon to AOX, several plasmids that are transiently expressed in cells were constructed. As a control, we also used a plasmid construct that expressed an mLon mutant lacking the mitochondrial-targeting signal (MTS) domain (Fig. 1A). The full-length AOX protein (72 kDa) is known to undergo enzymatic digestion to form ~51-kDa and 21-kDa subunits after sorting to the peroxisome (Fig. 3A).

Initially, the endogenous pLon and AOX were associated in the peroxisomes of the HEK293 cells; this was confirmed by immunoprecipitation with the anti-pLon antibody (Fig. 3C). In order to detect the essential AOX-binding domain of pLon, recombinant Lon derivatives were expressed in COS-7 cells, and immunoprecipitation with the anti-FLAG antibody was performed. The wild-type and the ΔSKL mutant of pLon, but neither the internal deletion mutant of pLon nor the MTS-lacking

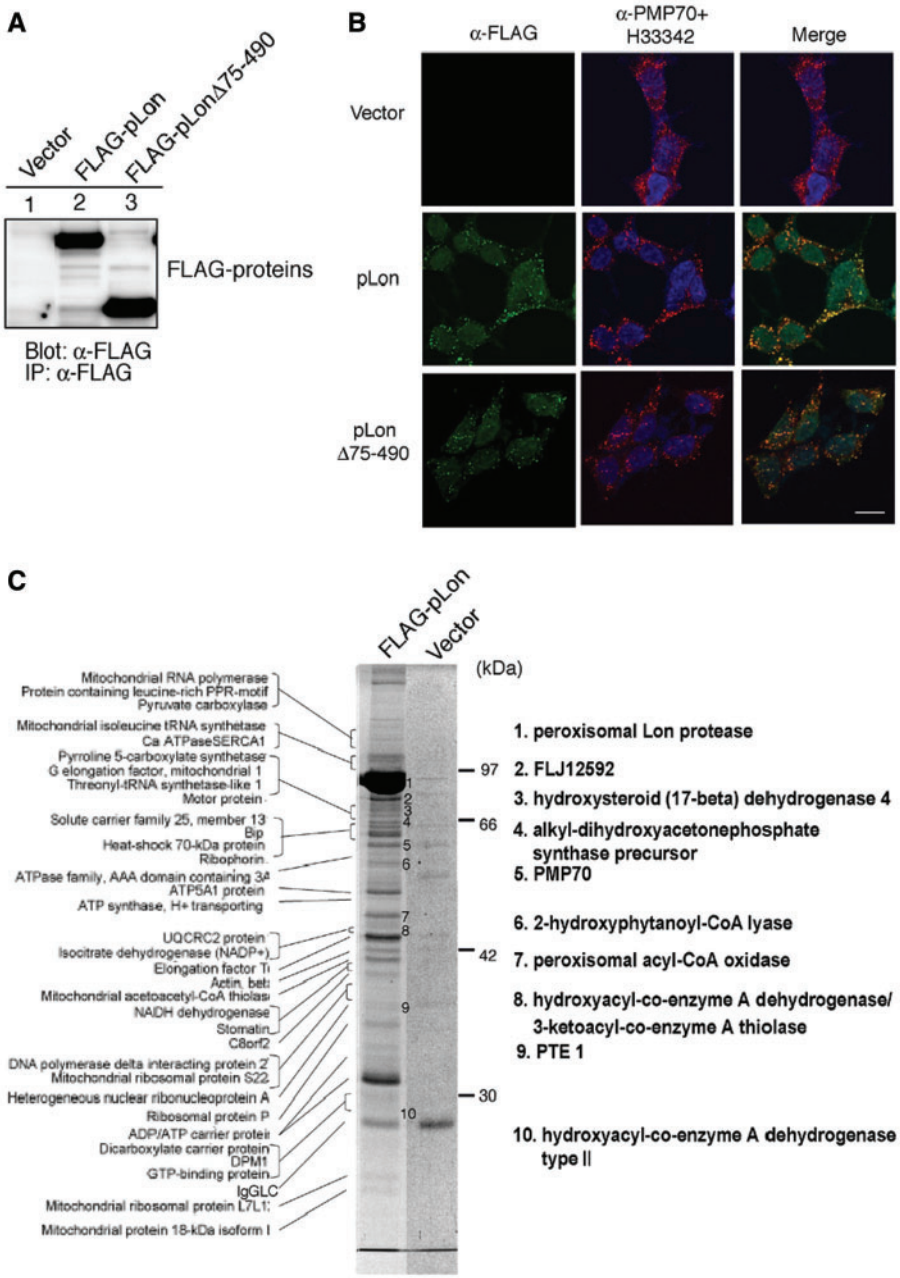

Fig. 2. **Identification of protein binding to pLon.** (A) Establishment of stable expression of FLAG-tagged pLon (lane 2) and FLAG-tagged pLonΔ75–490 (lane 3) by using a retrovirus expression vector in HEK293 cells. Control cells were mock infected with a retrovirus possessing an empty vector (lane 1). Immunoprecipitation (IP) and immunoblot experiments were carried out for each cell lysate by using an anti-FLAG antibody (Ab). (B) Cells were processed for co-immunofluorescence staining by using anti-FLAG (green) and PMP70 (red) Abs. Hoechst 33342 was used for staining the nucleus. Merged images are shown in the right panels. Scale bars, 10 μm. (C) Purification of the pLon complex. FLAG-tagged pLon was purified from the peroxisomal fraction of the HEK293 cells stably transfected with the expression plasmid. Affinity-purified protein eluted with 0.1 M glycine–HCl buffer (pH 2.5) was analysed by SDS–PAGE. Purified pLon and co-precipitated bands indicated by the arrows were in-gel digested, and peptides were analysed by tandem mass spectrometry. The names of peroxisomal proteins identified from the numbered gel pieces and other proteins are indicated on the right and left sides of the panel, respectively. As a control, mock-transfected HEK293 cells transfected with the empty vector were used (right lane).

mLon, were associated with the full-length myc-AOX (Fig. 3C). Therefore, the unique region of pLon at the N-terminal region might be the binding domain for AOX.

Effect of pLon on the Processing of Peroxisomal Proteins—In addition to AOX, another peroxisomal enzyme is known to function following enzymatic processing (*5*). PTL, containing a typical PTS2 motif at its N-terminus, was incorporated into the pLon complex (Fig. 4A). Recently, Tysnd1 has been identified as an essential factor for accurately sorting PTS1- and

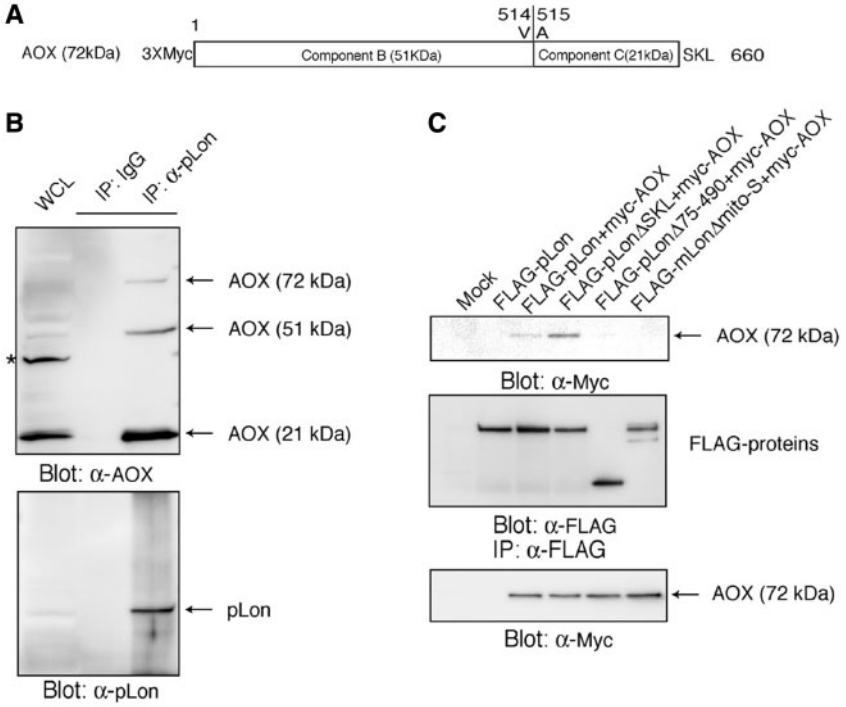

Fig. 3. **Specific interaction between pLon and peroxisomal AOX.** (A) Schematic representation of the primary structure of AOX. The 72-kDa precursor form and the converted forms in the peroxisomes, the N-terminal 51-kDa component B and the C-terminal 21-kDa component C are indicated. The position of the proteolytic digestion site of components B and C is 514 V and 515 A, respectively. (B) Association between endogenous pLon and AOX *in vivo*. Immunoprecipitation (IP) experiments were carried out using the ML fraction of the HEK293 cells with control IgG and anti-pLon antibodies (Abs). Immunoblot (IB) analysis was carried out using anti-AOX (upper panel) and anti-pLon Abs (lower panel). The asterisk indicates a non-specific band. (C) The binding of pLon, but not of mLon, to myc-AOX. Co-immunoprecipitation studies were carried out using the anti-FLAG Ab for all cell lysates obtained from the COS-7 cells that over-expressed myc-AOX and each derivative molecule of FLAG-tagged Lon. Immunoblot analysis carried out using anti-myc (upper panel) or anti-FLAG Abs (middle panel). Expression of myc-AOX in each whole-cell lysate was confirmed (bottom panel).

PTS2-containing proteins to peroxisomes (*7*). Next, we analysed the effect of pLon on the processing of these proteins. Either myc-pLon or myc-PTL was expressed in the HEK293 cells stably expressing the wild-type pLon or the Δ75–490 mutant of pLon. AOX was appropriately processed in the control and pLon-expressing cells. However, the 51-kDa component B band was not detected in the pLonΔ75–490-expressing cells (Fig. 4B, left panel). On the other hand, the 41-kDa mature form of PTL was equally generated in all the cell lines (Fig. 4B, right panel). Furthermore, the suppression of pLon by specific RNAi inhibited the generation of components B and C of the endogenous AOX (Fig. 4C). In particular, the amount of component C was very low in the cells with suppressed pLon expression. It may be assumed that the suppression of pLon expression led to AOX instability. Therefore, pLon may play a role in the peroxisomal translocation of AOX and in its subsequent processing, which leads to appropriate AOX activation. These results suggest that pLon might play a role in accurately sorting AOX, but not PTL, to the peroxisome.

We next measured the β-oxidation activity of [1-^{14}C]palmitoyl-CoA in the ML fraction derived from each cell line. The β-oxidation activity in the ML fractions of the pLonΔ75–490 cells was lower than that in the wild-type cells. Although the processing efficiency of AOX in the FLAG-tagged pLon-expressing cells was relatively higher than that in the control cell line, the β-oxidation activity unexpectedly decreased in the pLon-over-expressing cells (Fig. 4D). An explanation for this result is given below.

In Vitro Protease Activity of pLon—Lon is well known as an ATP-dependent protease conserved from prokaryotes to higher vertebrates. mLon, which possesses a structure similar to that of pLon, has been studied in detail with respect to its enzymatic characterization (*11*, *13*). Our preliminary results revealed that the activity of purified FLAG-tagged pLon obtained from cultured cells was very low when a synthetic casein peptide was used as a substrate (data not shown). The characterization of recombinant pLon purified from *Escherichia coli* will be discussed elsewhere.

In the cells expressing pLon derivatives, the processing pattern of AOX was dramatically altered (Fig. 4B). Therefore, we demonstrated *in vitro* that FLAG-tagged pLon processes the myc-AOX or myc-PTL purified from the cells over-expressing these proteins. Although the AOX processing activity of pLon was low, the detection of component B varied with the amount of pLon, but not with that of pLonS743A, which is a point mutant at the centre of the protease catalytic domain (Fig. 5A and B). On the other hand, the band of mature PTL (41 kDa) was

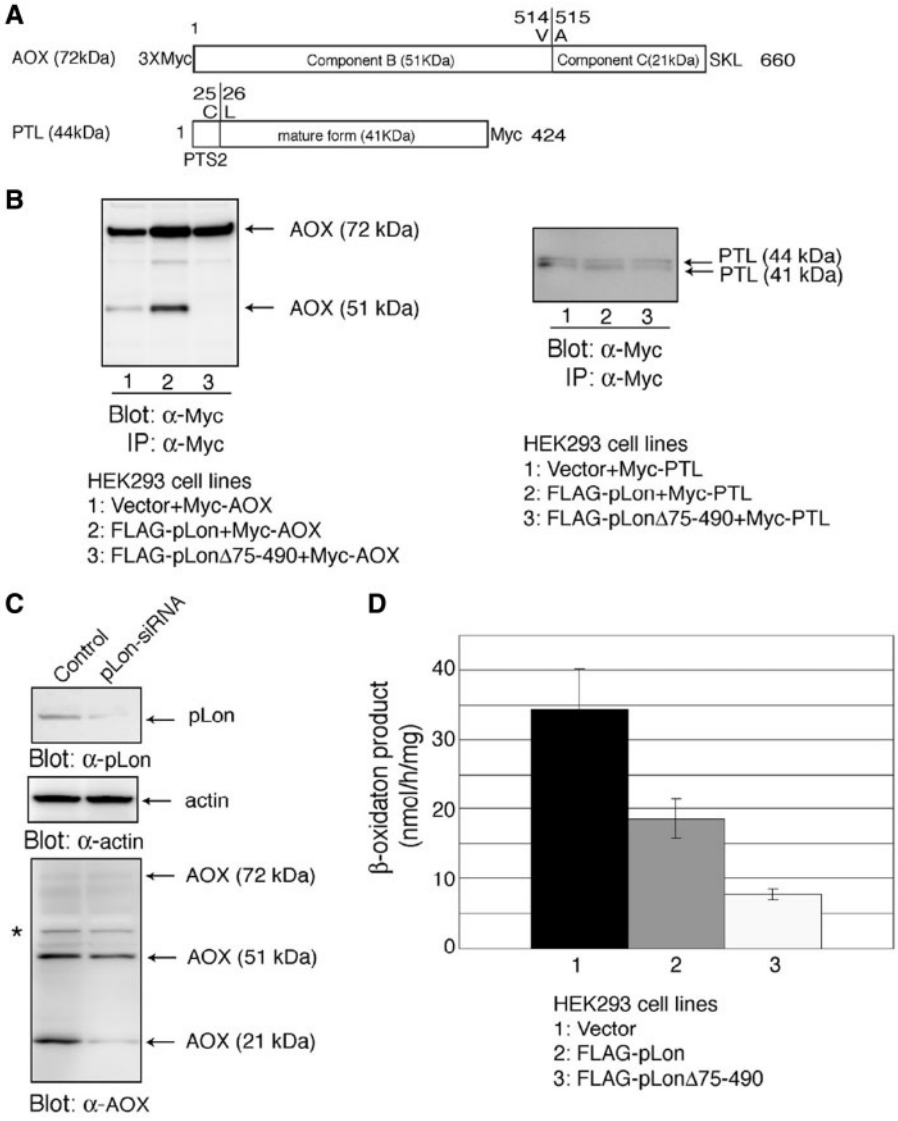

Fig. 4. **Effect of pLon expression on the processing of peroxisomal proteins.** (A) Schematic representation of the primary structures of AOX and PTL. The 44-kDa precursor form and the converted forms in the peroxisomes, C-terminal 41-kDa mature form and C-terminal PTS2 are indicated. The position of the proteolytic site of PTS2 is 25 C and 26 L. (B) Each established HEK293 cell line that stably expressed FLAG-tagged pLon (lane 2), FLAG-tagged pLonΔ75–490 (lane 3) and mock control (lane 1) shown in Fig. 2 was transfected with the expression vector encoding myc-AOX (left panel) and myc-PTL (right panel). Immunoprecipitation (IP) and immunoblot (IB) experiments were carried out for each cell lysate by using anti-myc antibody (Ab). Each intact or processed band is indicated by an arrow. (C) Partial inhibition of AOX processing in the cells in which pLon expression was suppressed by siRNA. Whole-cell lysates prepared from HeLa cells transfected with control or pLon siRNA were subjected to SDS–PAGE and IB analysis with anti-pLon (upper) and anti-AOX (lower) Abs. The asterisk indicates a non-specific band. (D) Effect of the pLon mutant on the β-oxidation activity. The β-oxidation activity of the ML fractions prepared by differential centrifugation was measured using [1-^{14}C]palmitoyl-CoA, as described in the 'MATERIALS AND METHODS' section. For this purpose, 30 μg of ML fractions was used. Error bars show the standard deviation.

not detected even after adding pLon (Fig. 5C). Thus, pLon might be an AOX-specific processing protease.

Effect of pLon on the Distribution of Catalase—One of the important roles of the peroxisome is hydrogen peroxide detoxification by catalase that contains a modified PTS1 (*18*). We next focused on the effect of pLon on the catalase in cultured cells. The sub-cellular localizations of endogenous AOX (72 kDa) and catalase in each cell line were observed (Fig. 6A). The FLAG-tagged pLonΔ75–490-over-expressing cells exhibited a remarkable decrease in AOX and mislocalization of catalase to the cytoplasm. In addition to the biochemical results, the fractionation studies revealed that the catalase in the pLonΔ75–490-expressing cells did not localize in the peroxisome; this result was confirmed by microscopic analysis (Fig. 6B). Moreover, catalase activities in the sub-cellular fractions obtained from each cell line corresponded to these results (Fig. 6C). Therefore, pLon may contribute to the regulation of translocation of catalase in peroxisomes.

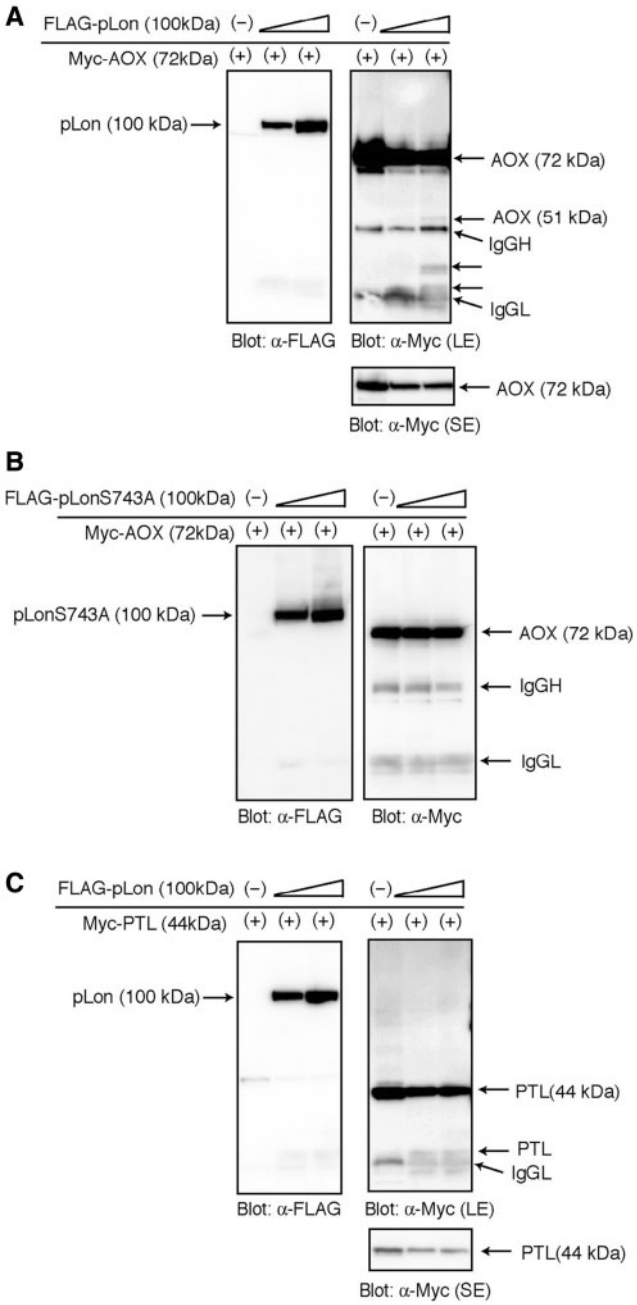

Fig. 5. ***In vitro* processing activity of FLAG-pLon against myc-AOX but not against myc-tagged PTL.** (A–C) Purified wild-type FLAG-pLon (A, C) and S743A mutant (B) were obtained by immunoprecipitation from the lysates of HEK293 cells with stable expression. Immunoprecipitated myc-tagged AOX (A, B) and myc-tagged PTL (C) expressed in the COS-7 cells were immobilized on protein G-Sepharose beads conjugated with anti-myc antibody. Both types of bead were mixed, incubated in the assay buffer and subjected to SDS–PAGE. Immunoblot (IB) analyses were performed using the indicated antibodies. SE and LE refer to short and long exposure times for IB, respectively. The cleavage bands and IgGs are indicated by arrows.

Effect of pLon on Peroxisome Morphology—Studies on the mechanism of peroxisome division that have been conducted using yeast demonstrated that some of the mutants of β-oxidation enzymes exhibit abnormal peroxisome morphology (*15*, *19*). Our study indicated that pLon affects the distribution of peroxisomal β-oxidation enzymes. The established cell line that over-expressed FLAG-tagged pLon contained large peroxisomes. However, the number of peroxisomes in this cell line was less than that in wild-type cells (Fig. 7). Although the precise reason for this phenomenon is not clear, it could be attributable to a disturbance in the normal regulation of peroxisome division as a consequence of pLon overloading at the peroxisomal membrane. Despite an increase in the peroxisomal distribution of the enzymes, the activities of β-oxidation and catalase in the peroxisomes of this cell line were lower than those in the control cells. Therefore, these enzymes might exist in an inactive form in the enlarged peroxisome.

DISCUSSION

In mammals, one of the physiological functions of mLon is to selectively degrade abnormal polypeptides such as oxidized aconitase for quality control (*17*). Furthermore, mLon binds to mitochondrial DNA and is associated with its maintenance (*13*). On the other hand, little is known regarding the function of pLon in mammalian cells. Recently, pLon in the yeast *Hansenula polymorpha* was demonstrated to play a role in the degradation of unfolded and non-assembled peroxisomal matrix proteins (*15*). In the present study, we have demonstrated that human pLon contributes to the accurate sorting and processing of the PTS1-containing protein AOX in the peroxisome. One of the important functions of the peroxisome is the β-oxidation of fatty acids. Many peroxisomal enzymes associated with this activity have been identified (*18*). In general, peroxisomal proteins have a typical targeting signal sequence known as PTS1 or PTS2 (*5*). The mechanism by which the PTS1-containing proteins are translocated to the peroxisomes is well established; the cytosolic receptor Pex5p recognizes the PTS1 sequence and carries the protein to the peroxisomal membrane as a cargo (*19*). The translocation of cytosolic AOX to the peroxisomes probably depends on this pathway. However, the enzymatic activation of AOX was clearly inhibited in the cells expressing the pLon mutant, which was incapable of binding to AOX (Fig. 4B). AOX bound to pLon, but not to mLon; thus, the unique N-terminal domain of pLon, which differs from that of mLon, may be the essential domain for this binding (Fig. 3C). Cytosolic AOX did not accumulate, but was rapidly degraded, in the cells expressing pLonΔ75–490; this mutant might have a dominant negative effect on the endogenous pLon protein (data not shown). Thus, cytosolic AOX may be stabilized after binding to pLon; the complex thus formed is accordingly recognized as a cargo by Pex5p, which facilitates translocation to the peroxisomes.

The PTS2-containing protein PTL was also identified as a protein binding to pLon (Fig. 2C). However, the expression of pLon did not influence the maturation of active PTL (Fig. 3C). Recently, Tysnd1 has been identified as a cysteine protease for PTS1- and PTS2-containing proteins (*7*). AOX and PTL are extensively

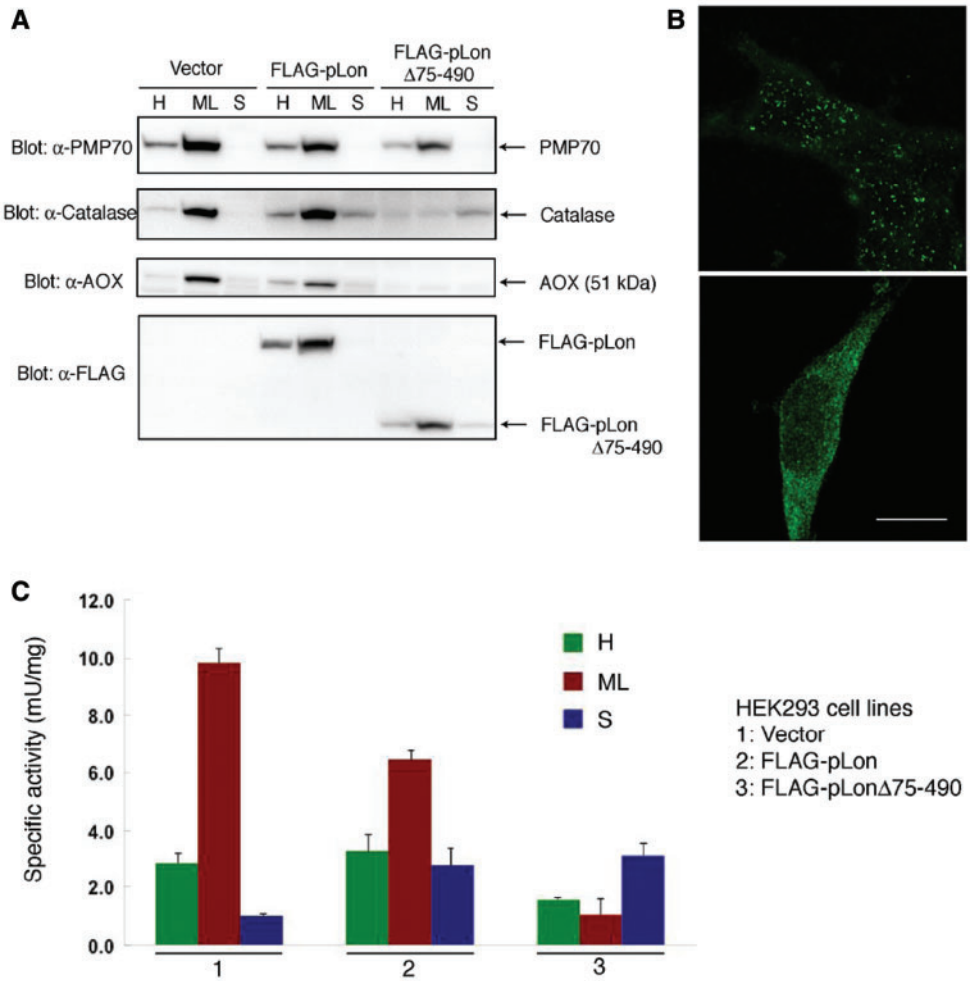

Fig. 6. **Effect of the pLon mutant on the distribution of peroxisomal proteins.** (A) Sub-cellular localization of the peroxisomal proteins in the control cells (vector) or the cells expressing FLAG-tagged pLon or FLAG-tagged pLonΔ75–490. The cells were subjected to sub-cellular fractionation, and the indicated proteins (20 μg each) were detected by immunoblotting. H, homogenate fraction; ML, mitochondrial and peroxisomal fraction; S, microsomal and cytosolic fraction. (B) Expression of catalase in the cells over-expressing FLAG-tagged pLon (upper panel) or FLAG-tagged pLonΔ75–490 (lower panel). The cells were immunostained with anti-catalase antibody. Scale bar, 10 μm. (C) Sub-cellular distribution of catalase activity. Catalase activities in the sub-cellular fractionations were measured. The values are expressed as mean ± SE.

processed in the cells expressing Tysnd1. Although Tysnd1 has been demonstrated to digest and process PTL and AOX *in vitro*, there exist some controversial results on the digesting site of these substrates. Tysnd1 may therefore be a general processing protease for peroxisomal proteins. Although there was little *in vitro* protease activity of pLon with AOX as the substrate, the activity observed was specific for AOX processing (Fig. 5). Because pLon is highly similar to other well-characterized Lon proteases such as mLon, we expected that pLon may exhibit ATP-dependent protease activity *in vitro*. We confirmed that the purified recombinant pLon produced by bacteria possesses ATP-dependent protease activity using synthetic peptide and FITC-casein. However, the cleavage of recombinant AOX and PTL was almost undetectable. Similarly, we were barely able to detect processed AOX (51 kDa) and specific cleaved bands in an *in vitro* protease assay of FLAG-pLon immuno-purified from lysates of the culture cells (Fig. 5A). Moreover, we were unable to detect the processed AOX under the same assay conditions using a point mutant of pLon (S743A) containing a substituted critical amino acid in the protease domain (Fig. 5B). It is conceivable that bacterial-produced recombinant pLon may be unable to form a homohexamer that is required for its activation and substrate recognition. It may therefore be necessary to identify more suitable assay conditions for pLon. The characterization of the recombinant pLon enzyme purified from bacteria will be discussed elsewhere.

In addition to the β-oxidation enzymes, pLon plays a role in appropriately targeting peroxisomal catalase that possesses a modified PTS1(*20*). We were unable to characterize catalase as a protein binding to pLon by mass spectrometric analysis. However, our preliminary data of immunoblotting analysis suggests that catalase might bind to pLon. Therefore, the distribution of catalase in the peroxisomes was found to be severely

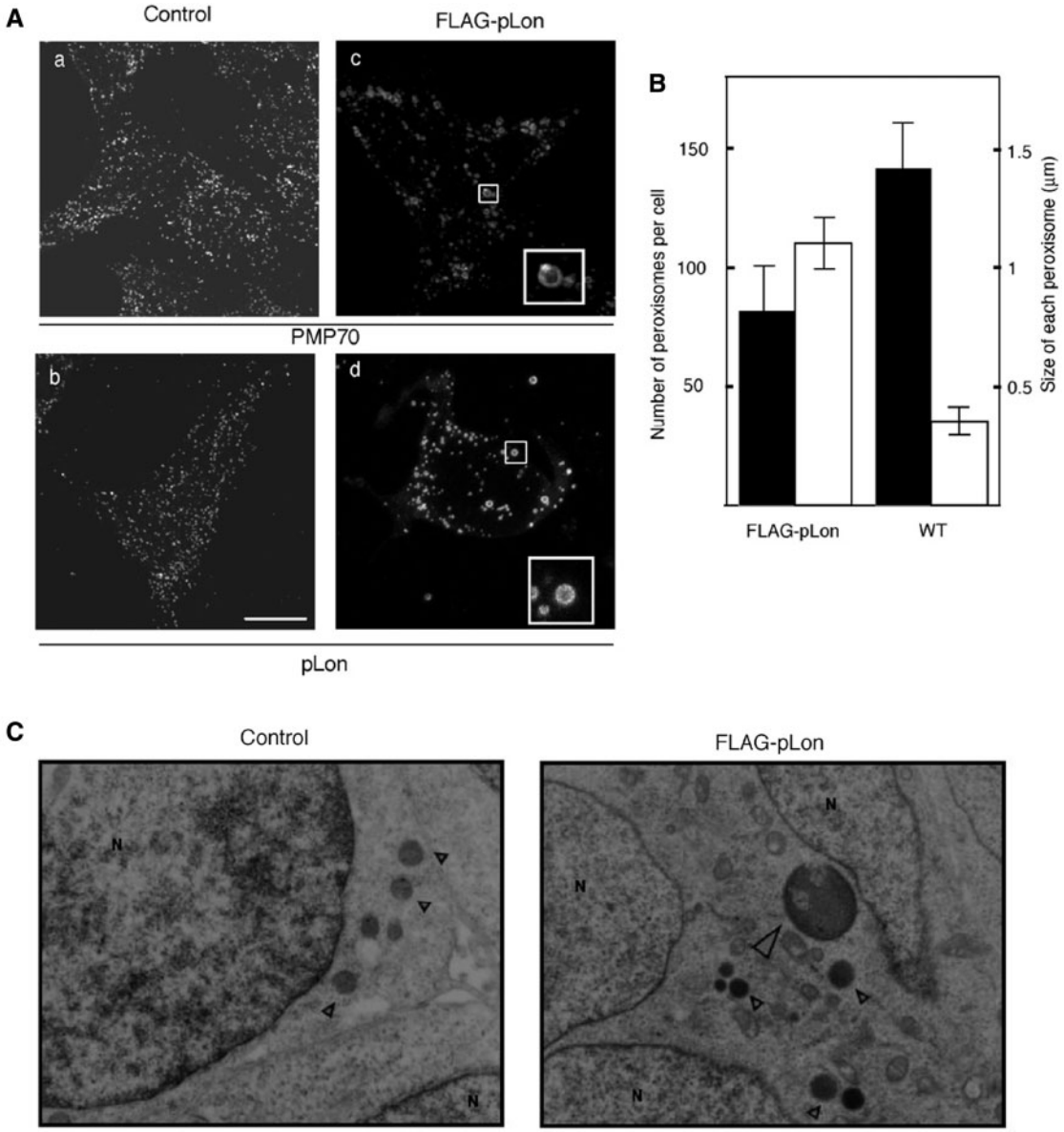

Fig. 7. **Effect of pLon on the morphological alteration of peroxisomes.** (A) Changes in the size and the number of peroxisomes. PMP70 and Lon were visualized using anti-PMP70 (a, c) and anti-Lon (b, d) antibodies (Abs), respectively. The expression of FLAG-tagged Lon (c, d) resulted in the enlargement of peroxisomes; this enlargement was not observed in the control cells (a, b). The magnified images of the area enclosed in the central boxes in the right panels are shown in the inset. Scale bar, 10 μm. (B) Graph showing the average number (black bar) and diameter (white bar) of peroxisomes in the indicated cell lines. Data were taken from 20 randomly selected cells in one experiment, and three independent sets of experiments were carried out. The values are expressed as the mean ± SE. (C) Electron micrographs of the peroxisomes in the control (WT) and FLAG-tagged pLon-expressing HEK293 cells. The DAB-stained cells were subjected to electron microscopy. An enlarged peroxisome is observed in the cells expressing FLAG-tagged pLon. Arrowheads indicate peroxisomes. N, nucleus; magnification, 16,000×.

disrupted, and its activity decreased in the cells expressing the dominant negative form of pLon (Fig. 6). Catalase, one of the most important peroxisomal enzymes, detoxifies hydrogen peroxide and protects cells from its toxic effects (*18*). Therefore, a normal pLon expression is probably necessary for the viability or homoeostasis of living cells.

The peroxisome is a dynamic organelle that undergoes changes in size, shape and number. Morphologically aberrant peroxisomes are observed in peroxisome biogenesis disorders and single-protein deficiencies in inherited metabolic diseases in humans. The PEX family of proteins and the β-oxidation enzymes have been identified as characteristic features of peroxisomal morphology (*17*, *21*). Some of the yeast mutants of these proteins exhibited abnormally large peroxisomes that were induced by aberrant peroxisome division (*15*, *19*). Moreover, some extra-cellular stimuli such as the addition of serum, UV irradiation and the production of hydrogen peroxide have been demonstrated to provoke

the formation of tubular peroxisomes in mammalian cells (*18*). PEX11 and dynamin-like proteins participate in these events (*22*). In our experiments, stably expressed FLAG-tagged pLon was correctly localized in the peroxisomes. However, the peroxisomes observed in these cells were larger and fewer in number (Fig. 7). Although pLonΔ75–490 may function as a dominant negative mutant of endogenous pLon, over-expressing the wild-type pLon did not necessarily have a positive effect on the distribution of active peroxisomal enzymes such as AOX and catalase. This is because the activities of these enzymes in the pLon-over-expressing cells were lower than those in the control cells (Figs 4 and 6). This phenomenon is considered to be induced since non-functional peroxisomes, probably generated as a consequence of containing too much recombinant pLon, lack the normal capacity to divide.

The division of peroxisomes co-ordinated with cell growth or the cell cycle may be indispensable for their normal functioning, for example, the activity of β-oxidation enzymes. A number of recent studies on the mechanism of peroxisome division have revealed that some β-oxidation enzymes play a role in regulating this process (*18*, *23*). We observed that pLon expression—either directly or indirectly—caused an enlargement of peroxisomes; however, the reason for this enlargement is not clear. We expected that the activity of β-oxidation in the cells expressing FLAG-pLon would increase. However, our contradictory results suggest that the relationship between the morphology of the peroxisome and the mechanism of protein sorting may be more complicated than previously thought. Large peroxisomes enriched with pLon might contain large amounts of β-oxidation enzymes. However, these proteins could be inactive due to aggregation or denaturation. Alternatively, peroxisomes enlarged due to the over-expression of pLon might not be able to incorporate other proteins into their structure.

Recently, it was reported that the expression of pLon increased in rat liver treated with the reagent for peroxisome proliferation, di-(2-rthyl-hexyl)phthalate (DEHP) (*16*), which might enhance the degradation of β-oxidation enzymes. The treatment with DEHP also induced the expression of other β-oxidation enzymes and enlargement in the size and number of peroxisomes that were targets of autophagy. pLon might contribute to the degradation of β-oxidation enzymes, and restore the size and number of peroxisomes to normal. Our observation of the expression of FLAG-pLon may induce a situation that resembles the case of treatment with DEHP. Therefore, the processed fragment of AOX increased in the peroxisome, and the size of the peroxisome was enlarged without DEHP treatment. It may be difficult to demonstrate the effects of long-time expression of pLon on β-oxidation enzymes using culture cells. A further study using other biological tools is required to elucidate this issue.

In conclusion, the pLon discovered using an organelle proteomics approach may have additive and positive effects on facilitating the sorting of PTS1-containing proteins to the peroxisomes of mammalian cells.

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