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Novel peroxisomal protease Tysnd1 processes PTS1- and PTS2-containing enzymes involved in β-oxidation of fatty acids

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Peroxisomes play an important role in β-oxidation of fatty acids. All peroxisomal matrix proteins are synthesized in the cytosol and post-translationally sorted to the organelle. Two distinct peroxisomal signal targeting sequences (PTSs), the C-terminal PTS1 and the N-terminal PTS2, have been defined. Import of precursor PTS2 proteins into the peroxisomes is accompanied by a proteolytic removal of the N-terminal targeting sequence. Although the PTS1 signal is preserved upon translocation, many PTS1 proteins undergo a highly selective and limited cleavage. Here, we demonstrate that Tysnd1, a previously uncharacterized protein, is responsible both for the removal of the leader peptide from PTS2 proteins and for the specific processing of PTS1 proteins. All of the identified Tysnd1 substrates catalyze peroxisomal β-oxidation. Tysnd1 itself undergoes processing through the removal of the presumably inhibitory N-terminal fragment. Tysnd1 expression is induced by the proliferator-activated receptor α agonist bezafibrate, along with the increase in its substrates. A model is proposed where the Tysnd1-mediated processing of the peroxisomal enzymes promotes their assembly into a supramolecular complex to enhance the rate of β-oxidation.

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Introduction

In human beings and rodents, peroxisomes are involved in a variety of anabolic and catabolic pathways (e.g. cholesterol biosynthesis, fatty acid oxidation, purine metabolism, hydrogen peroxide detoxification, bile acid synthesis, plasmalogen synthesis, amino acid metabolism (Titorenko and Rachubinski, 2004). As peroxisomes lack DNA and protein synthesis capabilities, all peroxisomal proteins are synthesized in the cytosolic compartment and post-translationally sorted to the peroxisome (Subramani *et al*, 2000). Two distinct peroxisomal signal targeting sequences (PTSs) and their variants, the C-terminal PTS1 (Gould *et al*, 1989; Miyazawa *et al*, 1989) and the N-terminal PTS2 (Swinkels *et al*, 1991), have been defined. Almost all peroxisomal enzymes have the PTS1 signal [SA]-K-L, which is recognized by the cytosolic soluble receptor Pex5p that carries the cargo subsequently to the peroxisomal membrane. Only a few peroxisomal proteins are targeted via the N-terminally located PTS2 motif [RK]-[LVI]-[X5]-[HQ]-[LAF]. A small number of peroxisomal matrix proteins that lack both PTS1 and PTS2 signals are targeted to the organelle by poorly defined internal PTSs (Hettema *et al*, 1999). The import of precursor PTS2 proteins into the peroxisomes is accompanied by the proteolytic removal of the N-terminal targeting sequence. For example, peroxisomal 3-ketoacyl-CoA thiolase A (thiolase), an enzyme catalyzing the last step of the fatty acid β-oxidation pathway, is synthesized as a larger precursor. Upon proteolytic processing of a 26-amino acid (aa) N-terminal sequence, the precursor is converted to the mature protein (Hijikata *et al*, 1987). So far, the protease responsible for the processing has not been identified (Liz and Sousa, 2005). Although the PTS1 signal is preserved upon translocation, many PTS1 proteins, including peroxisomal β-oxidation enzymes acyl-coenzyme A oxidase 1 (Acox1), hydroxysteroid (17-beta) dehydrogenase 4 (Hsd17b4), also known as D-bifunctional enzyme and sterol carrier protein 2 (Scp2) undergo in the peroxisomal matrix a highly selective and limited cleavage. Reminiscent of the PTS2 proteins, the processing enzyme(s) is still unknown (Otera *et al*, 2001; Liz and Sousa, 2005).

Up to now, only two proteases, the insulin-degrading enzyme (IDE) (Authier *et al*, 1994) and a peroxisome-specific form of Lon protease (Kikuchi *et al*, 2004), have been experimentally detected in peroxisomes. IDE does not work on large proteins and instead cleaves peptides less than 50 aa in length (Duckworth *et al*, 1998; Kurochkin, 2001), whereas

an ATP-dependent Lon protease is likely to function as a chaperone and degrade unfolded proteins (Kikuchi *et al*, 2004). Recently (Kurochkin *et al*, 2005), in a computational search for novel peroxisome PTS1-targeted protein candidates, we identified a protein product of the mouse 1300019N10 clone weakly similar to a protease-related protein derived from *Arabidopsis thaliana*. It is now called Tysnd1 or trypsin domain containing 1, and its orthologs were found in rat, cattle, dog and human. In this paper, we provide evidence that a protein product of Tysnd1 gene is localized to the peroxisomes. Our study demonstrated that Tysnd1 is responsible for both removal of PTS2-containing leader peptide from prethiolase and for specific processing of all PTS1 proteins involved in the peroxisomal β-oxidation pathway of fatty acids. This finding, together with the induction of Tysnd1 by peroxisome proliferator-activated receptor α (PPARα) agonist, suggests that Tysnd1-mediated site-specific proteolysis is a part of regulatory mechanisms that control the peroxisomal β-oxidation of fatty acids.

Results

Anti-Tysnd1 antibody

The rabbit polyclonal antibody was raised against a region close to the C terminus of the mature Tysnd1 protein (aa 501–515; Figure 1A). The specificity of the antibody was tested for its ability to recognize recombinant mouse Tysnd1 purified from *Escherichia coli* extracts and also the N-FLAG-Tysnd1-C construct in transiently transfected COS-7 cells. Western blot analysis of the recombinant Tysnd1 revealed a signal corresponding to the expected size of 59 kDa (Figure 1B, lane 1). No band could be detected in the lysates of mock-transfected COS-7 cells (Figure 1B, lane 2). Cells transfected with the Tysnd1 expression plasmid displayed two prominent bands (Figure 1B, lane 3). The 59-kDa band fragment corresponds to the full-length Tysnd1 and the 49-kDA fragment represents a processed form of Tysnd1 (see below). To examine the specificity of the anti-Tysnd1 antibody, we performed competition assays with the synthetic peptide that was used for the immunization. The signals were completely abolished after the preabsorption of the antibody, demonstrating its specificity (Figure 1B, lanes 4 and 6).

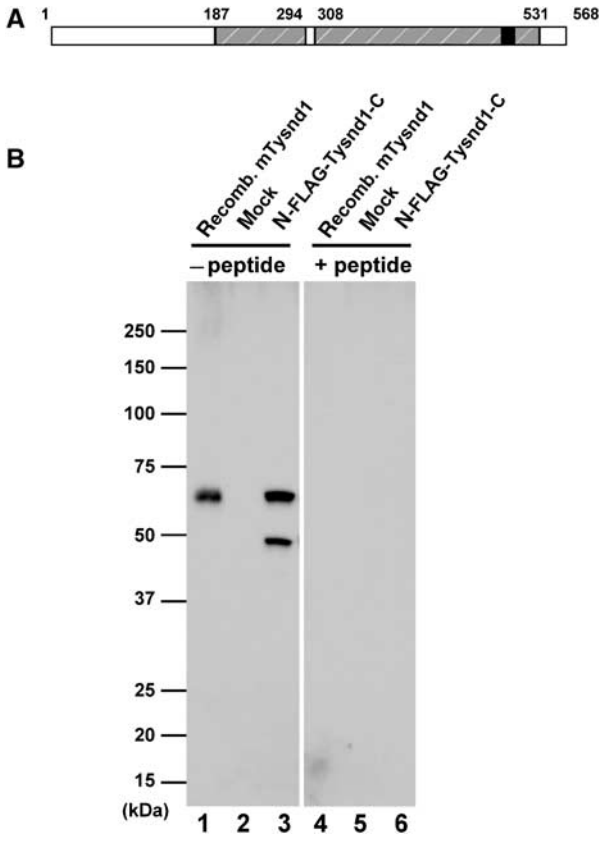

Figure 1 Specificity of antibodies raised against Tysnd1. (**A**) Schematic view of mouse Tysnd1. Two trypsin-like serine and cysteine peptidase domains (InterPro Entry domain IPR009003) at positions 187–294 and 308–531 are indicated by hatched bars. The antigenic sequence at position 501-515 is shown by a filled bar. (**B**) Immunoblotting of purified recombinant Tysnd1 (lanes 1 and 4), lysates from COS-7 cells transfected with empty vector pcDNA3.1 (lanes 2 and 5) and transfected with a vector for N-terminally FLAG-tagged Tysnd1 (lanes 3 and 6) were resolved by gradient 4–20% SDS–PAGE and assayed by Western blotting with anti-Tysnd1 antibody. The antibody used in lanes 4–6 was preabsorbed with the synthetic peptide CSNTRDNNTGATYPHL.

Localization and size of endogenous Tysnd1

Rat liver homogenate was fractionated by differential centrifugation followed by the separation of a light mitochondrial fraction on a self-generated Optiprep gradient (Figure 2A). The localization of peroxisomes in the gradient fractions was established by detection of catalase activity (Figure 2A). Peroxisomes were located near the bottom of the gradient (fractions 18–20) and were well separated from the other organelles (i.e. mitochondria and lysosomes) which were found in the top fractions (fractions 1–4), and constituted the major protein content of this sample (Figure 2B). Western blot analysis of the fractions with the anti-Tysnd1 antibody revealed immunoreactive material only in fractions 18–20 (Figure 2C), the region of the gradient containing catalase. Two prominent bands detected by Western blot correspond to the protein forms with molecular weights of 49 and 27 kDa (Figure 2C). In COS-7-transfected cells, the major Tysnd1 species detected corresponded to the intact (59 kDa) and partially processed (49 kDa) forms, whereas the 27-kDa form was undetectable (Figure 1B). Therefore, we conclude that Tysnd1 is synthesized as a 59-kDa protein and is converted to the 49-kDa and 27-kDa forms after being imported into the peroxisomes. The fact that in COS-7 cells most of the protein remained intact (59 kDa) is likely a consequence of the overexpression generated by the pcDNA3.1 vector, which may saturate the processing pathway of Tysnd1. The 27-kDa form of endogenous Tysnd1 contains presumably an entire C-terminal protease-like domain (308–531) (Figure 1A).

Localization of Tysnd1 by confocal microscopy

Intracellular localization of Tysnd1 was also investigated by confocal microscopy. A GFP-Tysnd1 fusion construct was cotransfected with a plasmid encoding the DsRed2-Peroxi. The full-length cDNA sequence of Tysnd1 was appended to the C-terminus of GFP to preserve PTS1 sequence at the C terminus of the fusion protein. The GFP-Tysnd1 fusion construct was cotransfected with a plasmid encoding DsRed2-Peroxi, a red fluorescent protein fused to the PTS1 and localizing in peroxisomes. GFP fluorescence was observed in numerous punctuated structures but not in other parts of CHO-K1 cells (Figure 3, GFP). All of the GFP-Tysnd1 fusion

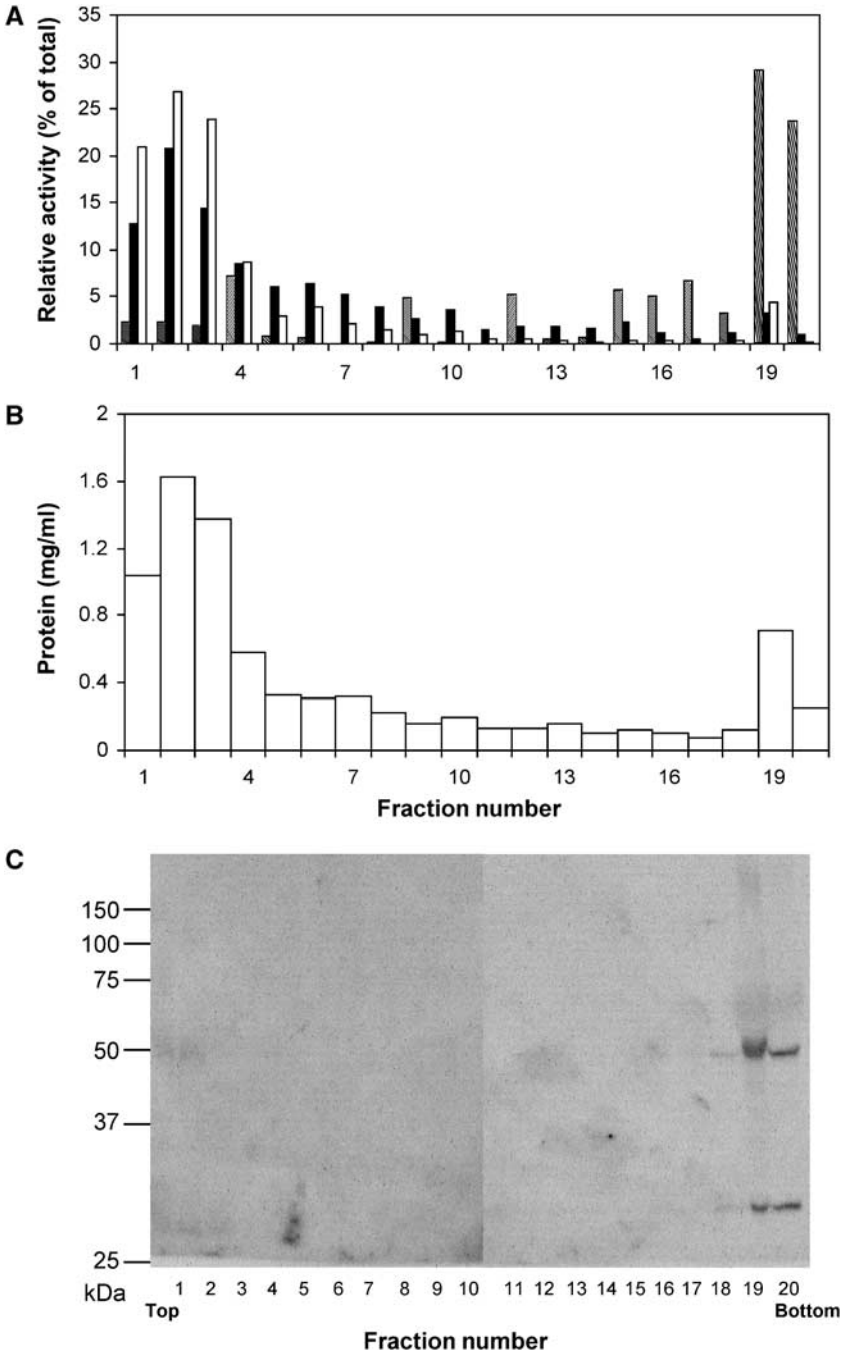

Figure 2 Subcellular fractionation reveals Tysnd1 localization to peroxisomes. Rat liver light mitochondrial fraction was fractionated by a self-formed Optiprep gradient. The fractions were collected starting from the top of the tubes. (**A**) Catalase (stripped bars), succinate dehydrogenase (filled bars) and β-galactosidase (open bars) were measured as marker enzymes for peroxisomes, mitochondria and lysosomes, respectively. Results are given as percentage of the total gradient activity. (**B**) Protein content. (**C**) Proteins from equal volumes of each fraction were separated by 12.5% SDS–PAGE and immunoblotted with an antibody against mouse Tysnd1.

protein-positive granules in the same sections were positive for pDsRed2-Peroxi protein (red color) (Figure 3, DsRed). This colocalization provides strong evidence that GFP-Tysnd1 is targeted to the peroxisomes.

Cell-based degradation of PTS2-containing peroxisomal prethiolase

To determine the role of Tysnd1 in the removal of the PTS2-containing signal sequence, we analyzed the processing of peroxisomal precursor 3-oxoacyl-coenzyme A thiolase (prethiolase). Prethiolase is synthesized as a 424-aa-long precursor, with a removable N-terminal sequence consisting of 26 aa (Hijikata *et al*, 1987). When the C-terminally HA-tagged prethiolase was overexpressed in COS-7 cells alone, we detected the unprocessed precursor form of 44 kDa. Cotransfection with increasing amounts of pcDNA3.1/TOPO-FLAG-Tysnd1 led to a gradual disappearance of the 44-kDa precursor and a concurrent increase in the mature 41-kDa protein (Figure 4A). Similar results were obtained with CHO-K1 and 293FT cells (data not shown).

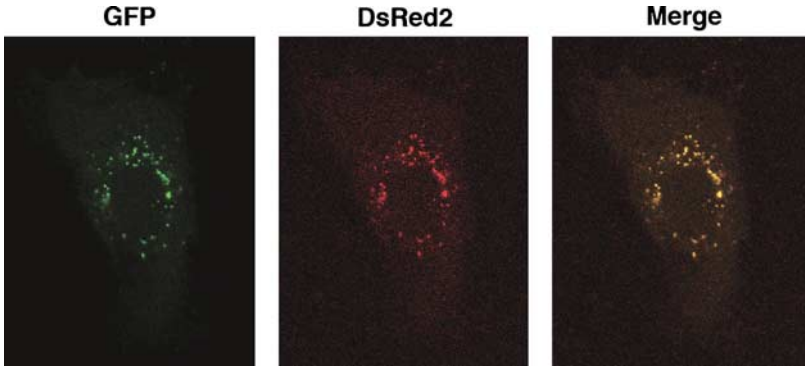

Figure 3 CHO-K1 cells were cotransfected with plasmids expressing fused GFP-Tysnd1 and DsRed2-Peroxi. Living cells were analyzed by fluorescence microscopy, and representative images for the GFP, DsRed channels and merged signals are shown. Original magnification, × 630.

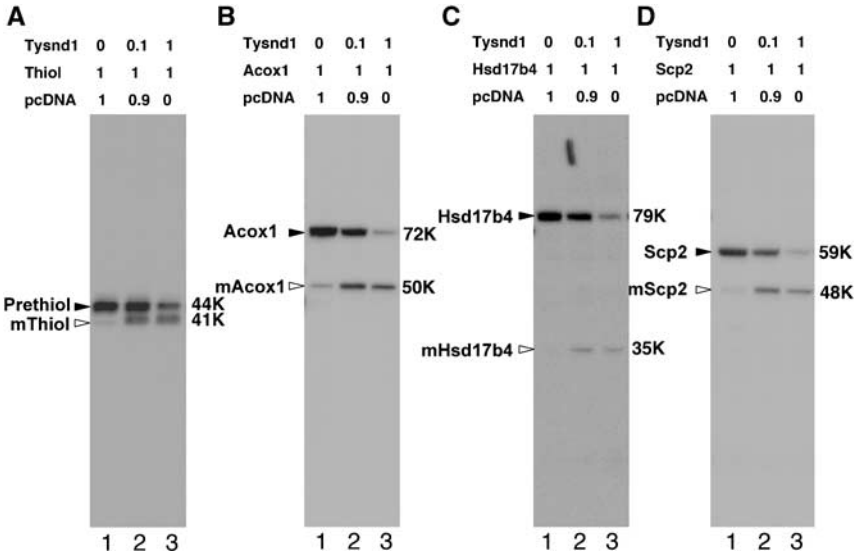

Figure 4 Effect of Tysnd1 expression on processing of peroxisomal enzymes to their mature forms. COS7 cells were transiently transfected with the indicated combinations of thiolase-HA (**A**), HA-Acox1 (**B**), HA-Hsd17b4 (**C**), and HA-Scp2 (**D**) and Tysnd1 expression plasmids. Total amounts of plasmids were kept constant at 2 μg by supplementation with the empty cDNA3.1. The arrowheads indicate positions of thiolase, Acox1, Scp2 and Hsd17b4 precursors and their mature forms (*mThiolase, mAcox1, mHsd17b4* and *mScp2*).

Processing of PTS1-containing peroxisomal enzymes

Next, we investigated whether Tysnd1 can process PTS1-containing Acox1, Scp2 and Hsd17b4 proteins. Acox1 is synthesized in cells as a precursor protein of 72 kDa (component A) and is converted in the peroxisomes into an N-terminal 50-kDa (component B) and a C-terminal 22-kDa (component C) form (Osumi *et al*, 1980). It exists in peroxisomes as a hetero-oligomer comprising A2, ABC and B2C2 (Osumi *et al*, 1980). Hsd17b4 (79 kDa) is cleaved after reaching the peroxisomes at single point to yield separate N-terminal and C-terminal components with the size of 35 and 44 kDa, respectively (Leenders *et al*, 1994). Scp2, a protein with molecular mass of 59 kDa, is proteolytically converted in the peroxisomes to an N-terminal 46-kDa and a 13-kD C-terminal fragment (Mori *et al*, 1991; Ossendorp *et al*, 1996). To assess the effect of Tysnd1 on the proteolytic conversion of Acox1, Hsd17b4 and Scp2, the expression constructs for the proteins were transfected into COS-7 cells. Acox1, Scp2 and Hsd17b4 were tagged with an HA epitope at their N-termini to preserve the PTS1 signal. On the Western blot, Acox1 appeared mostly as a 72-kDa protein band corresponding to the unprocessed enzyme form (Figure 4B, lane 1). A very faint band of approximately 50 kDa corresponds to the processed form (Figure 4B, lane 1). Cotransfection with increasing amounts of the Tysnd1 vector caused gradual reduction in the levels of the unprocessed 72-kDa form, with a concomitant increase in the 50-kDa protein (Figure 4B, lanes 2 and 3). The 22-kDa C-terminal fragment was not detected because it lacks the HA-tag. Similarly, HA-Hsd17b4 was processed when coexpressed with Tysnd1 to produce the N-terminal 35-kDa fragment (Figure 4C, lanes 1–3). Analysis of extracts from cells transfected with the HA-Scp2 expression plasmid revealed the presence of the predominant unprocessed 59-kDa form and the N-terminal fragment of the enzyme with an apparent size of 48 kDa (Figure 4D, lane 1). Coexpression of Tysnd1 led to an accumulation of the processed 48-kDa protein (Figure 4D, lanes 2 and 3). These data demonstrate that ectopically expressed Tysnd1 is involved in cellular processing of PTS1 enzymes, generating proteolytical fragments identical in size with those produced by endogenous protease.

Tysnd1 is the major enzyme involved in cellular processing of peroxisomal enzymes

To determine the physiological contribution of Tysnd1 in the processing of peroxisomal enzymes, we utilized small interfering RNA (siRNA). We chose to perform the knockdown experiment in 293FT cells. Prior testing of various mouse and human cell lines revealed that only 293FT cells showed detectable amounts of fragments produced from the full-length HA-Acox1, HA-Scp2 and HA-Hsd17b4 by endogenous protease. The introduction of siRNA against Tysnd1 (siRNA Tysnd1_1 and Tysnd1_2) into 293FT cells led to the knockdown of Tysnd1 mRNA, when compared to control siRNA, as assessed by quantitative RT–PCR analysis (Figure 5A). Transfection of Tysnd1-specific siRNAs essentially blocked the generation of the N-terminal HA-tag-containing fragments of Acox1, Scp2 and Hsd17b4 as compared with the non-silencing control siRNA (Figure 5B). Interestingly, siRNA-mediated silencing of Tysnd1, although prevented the production of an N-terminal 35-kDa fragment from Hsd17b4, led to the appearance of the 70-kDa form of the protein. This result implies induction of the proteolytic activity, removing the fragment of approximately 10-kDa from the C terminus of the protein. Considering that not all cells expressing target mRNA were transfected with siRNA and that endogenous Tysnd1 might have a long half-life time, the observed effect demonstrates that Tysnd1 plays a major, if not sole, role in processing of Acox1, Scp2 and Hsd17b4.

Direct processing of peroxisomal enzymes by Tysnd1

Results of a cell-based assay established that Tysnd1 is involved in the processing of PTS1 and PTS2 enzymes.

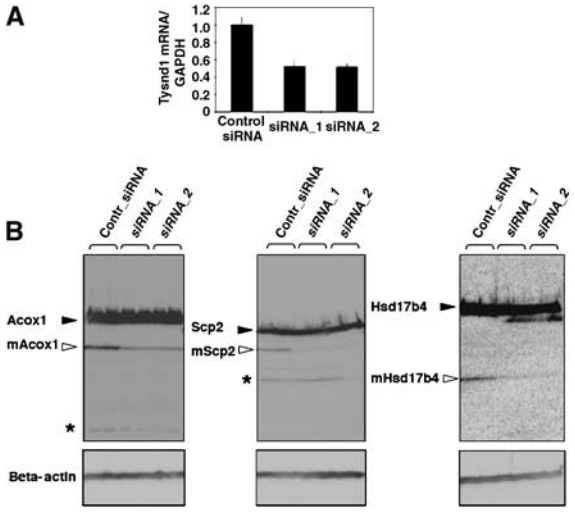

Figure 5 Inhibition of Acox1, Scp2 and Hsd17b4 processing by siRNA specific for Tysnd1. (**A**) 293FT cells were transfected with control non-silencing (*contr_siRNA*) or human Tysnd1-specific siRNA (*siRNA_1* and *siRNA_2*). Tysnd1 mRNA levels were analyzed by real-time RT–PCR after 54 h of incubation. Transfection efficiency was 70–80%. (**B**) 293FT cells were transfected with Acox1-HA, Scp2-HA or Hsd17b4-HA expression plasmids together with siRNAs specific for human Tysnd1 (*siRNA_1* and *siRNA_2*) or non-silencing siRNA (*contr_siRNA*). Duplicate transfections were performed for each siRNA sample. Cell lysates were analyzed by Western blot with anti-HA antibody. The arrowheads indicate positions of Acox1, Scp2 and Hsd17b4 precursors and their mature forms (*mAcox1, mScp2* and *mHsd17b4*). Western blot analysis for beta-actin was used as a control for siRNA specificity and sample loading. Asterisks indicate nonspecific bands.

However, there is a possibility that Tysnd1 may process its targets indirectly by activating another genuine peroxisome processing protease. To assess whether Tysnd1 directly processes peroxisomal enzymes, recombinant Tysnd1-FLAG protein was expressed in COS-7 cells and purified by affinity chromatography. Upon incubation of Tysnd1-FLAG with recombinant Acox1, Scp2 and prethiolase, cleavage products with the size identical to that generated in intact peroxisomes were formed (Figure 6A). Acox1 was cleaved to produce the C-terminal fragment of 22 kDa (Figure 6A, Acox1) and Scp2 was processed to generate the C-terminal fragment of 13 kDa (Figure 6A, Scp2). Incubation of Tysnd1 with prethiolase resulted in the removal of a 3-kDa PTS2-containing N-terminal propeptide to produce 41-kDa mature thiolase (Figure 6A, thiolase). N-terminal sequence analysis established that Tysnd1 cleaves prethiolase between residues Cys^{26} and Ser^{27} (Figure 6B), producing the mature form of thiolase found *in vivo* (Hijikata *et al*, 1987). These experiments demonstrate that Tysnd1 is a genuine peroxisomal processing protease.

Tysnd1 is a cysteine endopeptidase

In order to determine the protease class of Tysnd1, the enzyme processing activity was assessed in the presence of various protease inhibitors. Two substrates selected for this experiment were prethiolase and Acox1. In addition to being representatives of different peroxisome targeting classes (PTS1 and PTS2), prethiolase and Acox1 differ in the amino-acid sequence surrounding the processing sites (Hijikata *et al*, 1987; Miyazawa *et al*, 1987). The Tysnd1 processing activity was completely abolished with the cysteine protease inhibitor *N*-ethylmaleimide (NEM) (Figure 7). It was resistant, however, to two other cysteine proteinase inhibitors, leupeptin and *trans*-epoxysuccinyl-l-leucylamido-(4-guanidino) butane (E64). Leupeptin is known to inhibit

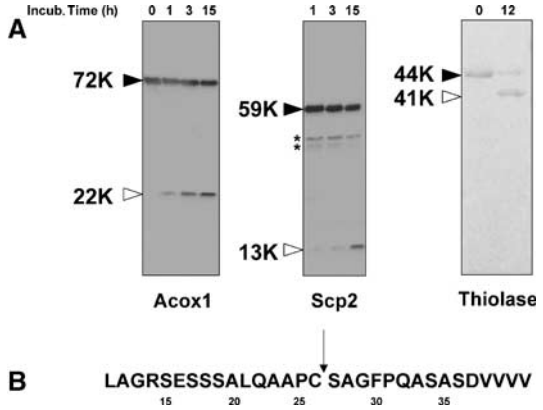

Figure 6 *In vitro* cleavage of Acox1, Scp2 and prethiolase by recombinant Tysnd1. Recombinant Acox1, Scp2 and thiolase were purified from *E. coli* extracts as described in Materials and methods. Mouse Tysnd1 was purified from COS-7 cell extracts transfected with Tysnd1-FLAG expression plasmid. (**A**) The processing of Acox1 and Scp2 was analyzed by Western blot with the anti-c-Myc antibody after the transfer of the proteins from 4–20% SDS–PAGE gel. Prethiolase cleavage was detected by 0.05% Amido Black staining of the Hybond-P membrane after the transfer of the proteins from 12.5% SDS–PAGE gel. (**B**) N-terminal sequence of processed rat thiolase precursor was determined by Edman degradation analysis. The cleavage site is designated by an arrow. The amino-acid residues are numbered according to the GenBank sequence P07871.

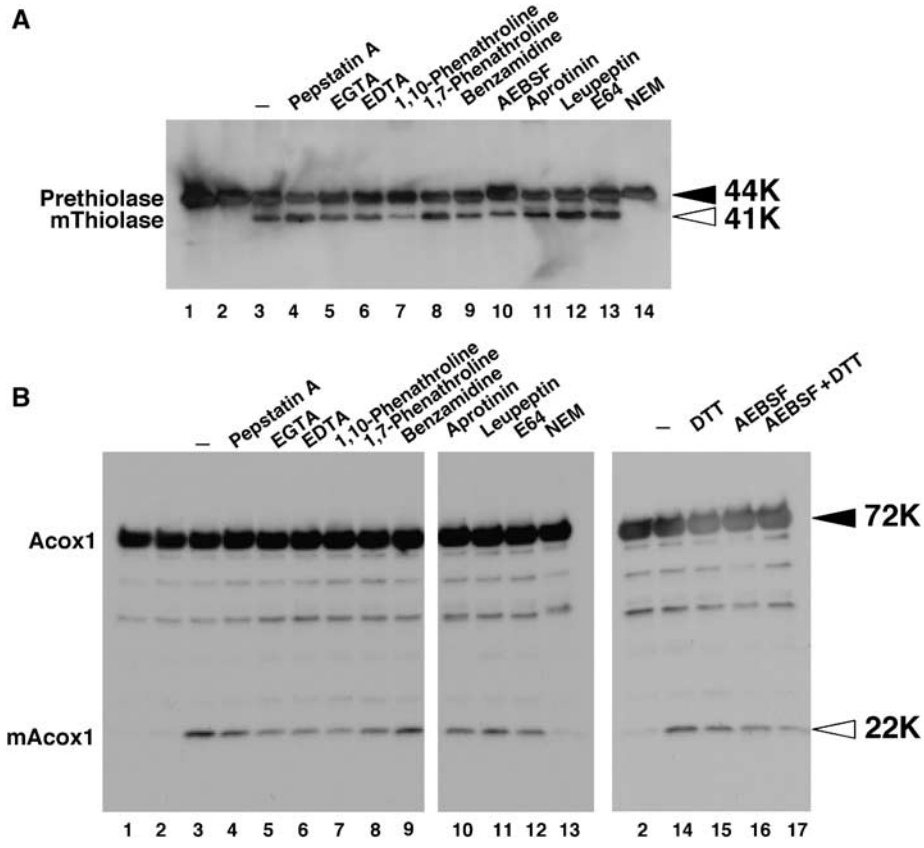

Figure 7 Effect of various protease inhibitors on processing of thiolase (A) and Acox1 (B) by Tysnd1. (**A**) Recombinant His_6-thiolase was incubated either alone for 0 h (lane 1) or 12 h (lane 2) or with Tysnd1-FLAG purified from COS-7 cell lysates for 12 h (lanes 3–14). The processing was assessed by Western blot with the anti-thiolase antibody. (**B**) Recombinant Acox1-*myc*-His_6 was incubated either alone for 0 h (lane 1) or 12 h (lane 2) or with Tysnd1-FLAG purified from COS-7 cell lysates for 12 h (lanes 3–17). The cleavage was detected using anti-c-Myc antibody.

some lysosomal serine and cysteine proteases, whereas E64 is a specific inhibitor of the papain protease family (Salvesen and Nagase, 2001). Partial inhibition of Tysnd1 by metal chelators (Figure 7), notably by 1,10-phenanthroline but not by related, non-chelating compound 1,7-phenanthroline, indicates that Zn^{2+} ions might be involved in the stabilization of Tysnd1 conformation or formation of a complex with its substrates.

Post-translational processing of Tysnd1

Like other peroxisomal proteins, Tysnd1 is synthesized in the cytosol. To ensure that the potential Tysnd1 substrates are not processed until the import to the peroxisome is completed, the proteolytic activity of Tysnd1 should be restrained until the protein reaches the peroxisomes. Most proteases are synthesized as inactive precursors and converted to the active forms upon reaching their destinations. To investigate the putative Tysnd1 processing, we transfected COS-7 cells with a eukaryotic expression vector for this enzyme containing a FLAG epitope either at the N terminus or C terminus of the protein. The FLAG epitope at the C terminus was introduced between aa 562 and 563 to preserve the native C terminus of Tysnd1, which contains the PTS1 signal. Transfection with the plasmid encoding the FLAG epitope at the N terminus of Tysnd1 resulted in the appearance of a 59-kDa protein (Figure 8A, lane 3), consistent with the calculated weight of the translated CDS of the cDNA sequence. However, an additional band of approximately 10 kDa was also detected (Figure 8A, lane 3). This small protein species corresponds to the FLAG epitope linked to approximately 100 amino-acid residues at the N terminus of Tysnd1 (Figure 8B). On the other hand, when cells were transfected with the plasmid encoding the FLAG epitope at the C terminus of Tysnd1, the antibodies detected an additional band of 49 kDa (Figure 8A, lane 2). The difference in size indicates that the 10-kDa fragment is split off from the N terminus of Tysnd1, thus corroborating the result obtained with the N-terminally tagged Tysnd1 protein. The 49-kDa form of the enzyme was purified from the transiently transfected COS-7 cells and subjected to seven cycles of Edman degradation. The determined N-terminal sequence established that Tysnd1 is cleaved between Cys^{110} and Ala^{111} (Figure 8C).

Induction of Tysnd1 by hypolipidemic drug bezafibrate

The PPARα activator bezafibrate is known to increase liver β-oxidation of fatty acids, in part owing to the induction of responsible peroxisomal enzymes prethiolase, Acox1 and Hsd17b4 (Beier *et al*, 1988). We therefore tested whether Tysnd1 is also induced by bezafibrate. In agreement with previous reports (Reddy, 2004), we observed significant induction by bezafibrate of the mouse liver Acox1 (5.86 ± 1.09), Hsd17b4 (2.84 ± 0.23) and thiolase (9.86 ± 0.59), but not Scp2 (0.79 ± 0.03) and catalase (1.34 ± 0.04) transcripts (Figure 9A). Noteworthy, the quantitative real-time RT–PCR revealed a two-fold (1.99 ± 0.1) transcriptional activation of the Tysnd1 gene (Figure 9A). Even more prominent was the

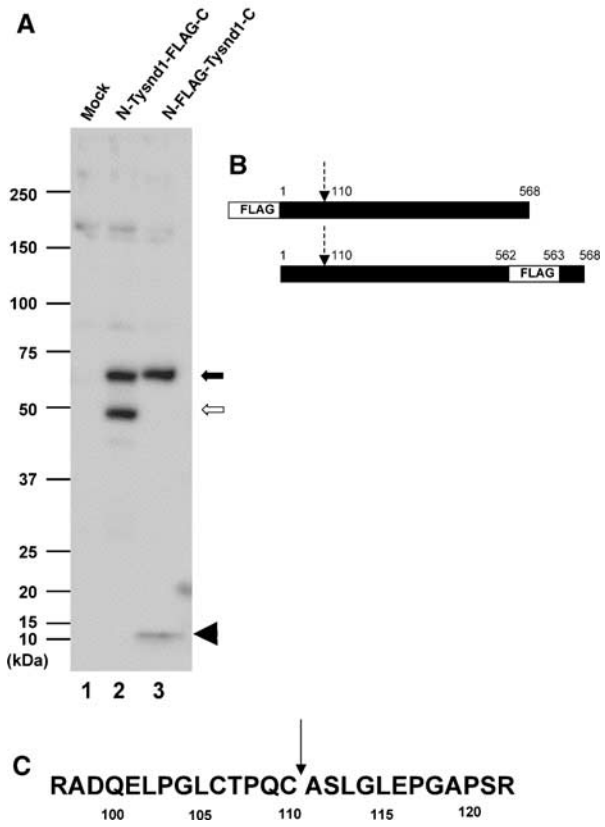

Figure 8 Intracellular processing of Tysnd1. (**A**) COS-7 cells were transiently transfected in a 6-well plate with 4 μg DNA of pcDNA3.1 (mock, lane 1), C-terminally FLAG-tagged Tysnd1 (N-Tysnd1-FLAG-C, lane 2) and N-terminally FLAG-tagged Tysnd1 (N-FLAG-Tysnd1-C, lane 3). Cell lysates were separated by 4–20% gradient SDS–PAGE and assayed by Western blotting with anti-FLAG antibodies. Solid and open arrows indicate the full-length and processed forms of Tysnd1, respectively. Arrowhead designates 10-kDa fragment derived from Tysnd1. Molecular markers in kDa are on the left. (**B**) Schematic view of the constructs used in the study. Filled bars indicate the coding sequence of Tysnd1. The numbers indicate amino-acid residues. Cleavage site of Tysnd1 is designated by the dashed arrow. (**C**) Cleavage site of Tysnd1 was determined by N-terminal sequence analysis of the 49-kDa form of the protease purified from COS-7 cells transfected with the Tysnd1-FLAG expression plasmid. The cleavage site is designated by an arrow. The amino-acid residues are numbered according to the GenBank sequence BAB23793.

induction of Tysnd1 protein, as revealed by Western blot analysis of the liver subcellular fractions. The 49-kDa Tysnd1 protein was almost undetectable in the control samples, but significantly accumulated in the bezafibrate-treated samples (Figure 9B). Tysnd1 was present in the light mitochondrial fraction that is enriched in peroxisomes. In contrast, bezafibrate treatment led only to a slight increase in the total quantity of catalase and no increase in the enzyme content of the light mitochondrial fraction (Figure 9B). These data suggest that the expression of Tysnd1 is most likely coregulated with the expression of its substrate proteins to meet the metabolic needs, particularly in conditions of enhanced β-oxidation of fatty acids.

Discussion

Confocal microscopy studies and subcellular fractionation data followed by immunoblot analysis with Tysnd1 antibodies clearly established the peroxisomal location of this novel cysteine protease. Intriguingly, Tysnd1 is involved in processing of both PTS1- and PTS2-targeted peroxisomal enzymes. Tysnd1 removes the PTS2-containing propeptide from prethiolase and processes three PTS1 enzymes, Acox1, Hsd17b4 and Scp2. This activity was demonstrated in cell-based studies, where expression of Tysnd1 led to the processing of the peroxisomal enzymes to fragments with the sizes identical to those described for their endogenous forms. siRNA-mediated knockdown of Tysnd1 in 293FT cells significantly decreased accumulation of the cleaved forms, demonstrating the physiological significance of the protease in the peroxisomal protein processing.

Importantly, experiments with recombinant proteins established that Tysnd1 acts directly on its substrates. An N-terminal sequence analysis revealed that Tysnd1 cleaves thiolase precursor protein after Cys^{26}, exactly the processing site of the endogenous liver prethiolase (Hijikata *et al*, 1987). Studies with protease inhibitors showed no significant inhibition of Tysnd1 activity by metallo-, serine and aspartic protease inhibitors. Instead, Tysnd1-mediated cleavage of prethiolase and Acox1 was completely blocked by NEM, which is a general sulfhydryl modifying agent. The failure of E-64 and leupeptin to inhibit Tysnd1 suggests that the protease is not a conventional cysteine protease, but may represent a new family of proteolytic enzymes.

Interestingly, Tysnd1 itself undergoes processing, presumably upon reaching the peroxisomes. Expression of Tysnd1 in the cell culture leads to a generation of two fragments, the N-terminal of 10-kDa and C-terminal of 49 kDa. The full-length 59-kDa form of Tysnd1 is absent in rat and mouse liver extracts. In analogy to other proteases, the 59-kDa Tysnd1 protein may present an inactive precursor form of the catalytically active 49-kDa form. The removal of the N-terminal 110-aa fragment does not affect the two protease-related domains (trypsin-like serine and cysteine peptidase domains; InterPro Entry IPR009003) at residues 187–294 and 308–531 (Figure 1A). Consequently the N-terminal segment (aa 1–110) may represent an inhibitory peptide that restrains the activity of Tysnd1 until it reaches the peroxisomes. The conversion of the inactive precursor into the active Tysnd1 might be mediated self-catalytically or by another unidentified protease. Analysis of the liver peroxisome fraction with the Tysnd1 antibodies specific to the amino-acid residues 501–515 revealed two protease forms. The first form contains both protease-related domains (49 kDa), whereas the second one (27 kDa) contains only the C-terminal protease-like domain (308–531). This suggests that Tysnd1 may undergo in peroxisomes a series of proteolytic processing events. Generation of separate protease domains from a single translation product in mammalian tissues is not without a precedent. Recently, Cal and co-workers (2003) have demonstrated that human polyserase-I and polyserase-2 (Cal *et al,* 2005) are cleaved in human tissues, with the release of three independent serine protease units.

The complex nature of Tysnd1 processing may reflect a cellular control mechanism that enables the regulation of multiple biological processes in the peroxisomes. It is possible that Tysnd1 cleavage between its two protease-like domains represents a mechanism for inactivation of the enzyme. In transient overexpression assay, Tysnd1 fragments aa 95–303 and 299–568 containing the first and second

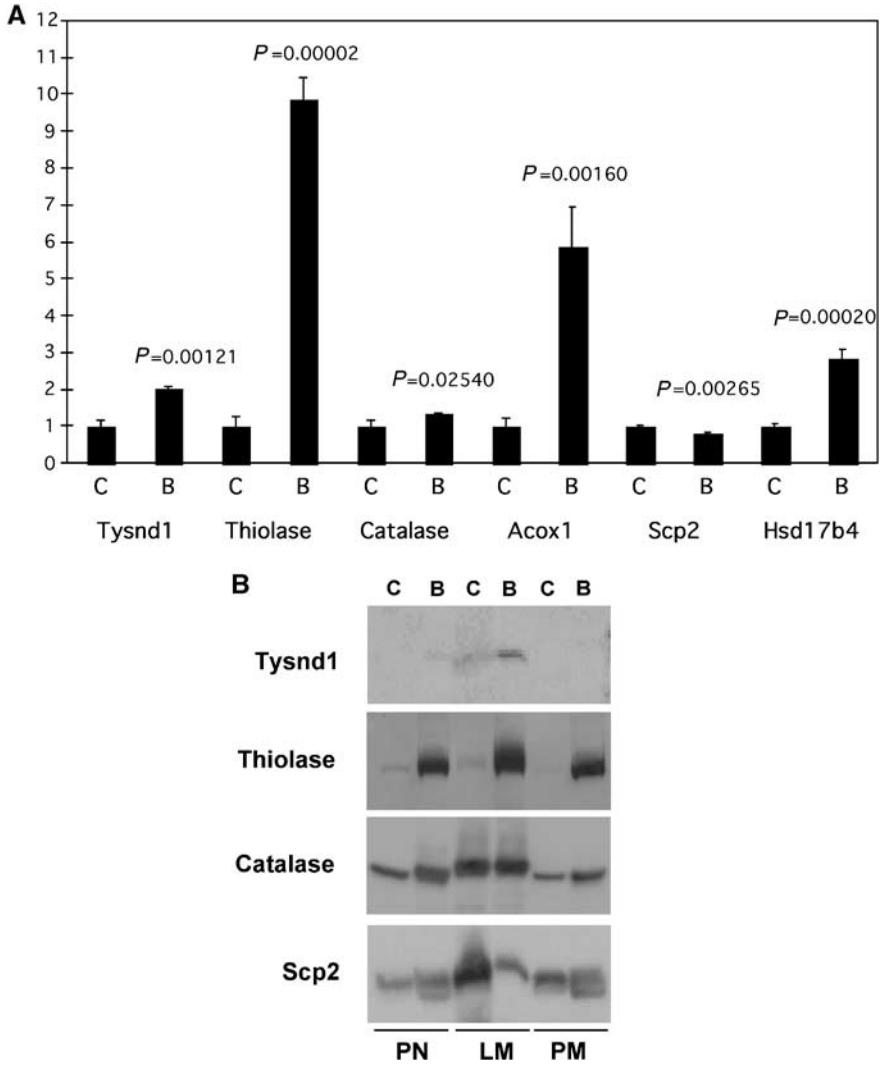

Figure 9 Induction of Tysnd1 in the liver of mice treated with bezafibrate. C57BL/6 mice were maintained for 3 weeks on a standard diet containing 0.5% (v/w) bezafibrate. (**A**) Histogram of quantitative real-time RT–PCR shows Tysnd1, thiolase, catalase, Acox1, Scp2 and Hsd17b4 mRNA expression in the liver of control (C) ($n=3$/group) and bezafibrate-fed (B) ($n=3$/group) mice. Data are normalized to the GAPDH gene and are presented as the mean $\pm$ s.d. The Excel spreadsheet package was used to perform two-tailed *t*-tests set up for samples with equal population variances. (**B**) Immunoblots of liver subcellular fractions for Tysnd1, thiolase, catalase and Scp2. Proteins from post-nuclear (PN), light mitochondrial (LM) and post-mitochondrial (PM) fractions were resolved on 4–20% SDS–polyacrylamide gels and transferred to Hybond-P polyvinylidene fluoride membranes. Note that LM fraction is enriched in peroxisomes.

protease domain, respectively, were unable to process the tested peroxisomal proteins (data not shown).

Probably, Tysnd1 recognizes common structural element in its substrates that are absent in non-substrate proteins. The sequences around the cleavage sites in prethiolase (AAPC*SAGF) (Hijikata *et al*, 1987 and this study), Hsd17b4 (AAPA*ATSG) (Leenders *et al*, 1994) and Scp2 (AAPT*SSAG) (Mori *et al*, 1991) share the Ala–Ala–Pro motif. The AAP motif is also present in Tysnd1 itself (aa 299–301 in human and aa 281–283 and 300–302 in mouse and rat enzyme). The cleavage around the common AAP motif in Tysnd1 would produce the C-terminal fragment of 27.8 kDa. This is exactly the size of Tysnd1 fragment detected in the liver peroxisomes (Figure 2C), implying its generation by self-cleavage. Bioinformatic analysis established that, in addition to enzymes participating in the β-oxidation (thiolase, Scp2 and Hsd17b4), several other peroxisomal proteins contain the AAP motif. Of these, only calcium-independent phospholipase A2 gamma (PNPLA8) can be processed at the site close to the AAP sequence (Murakami *et al*, 2005), making the enzyme a potential Tysnd1 substrate. However, in Acox1 (PQQV*AVWP), the motif is not found around the reported processing site (Miyazawa *et al*, 1987). It is therefore possible that separate Tysnd1 domains are responsible for the recognition of different substrate sequences. Alternatively, the Tysnd1 substrate cleavage mechanism may involve the recognition of secondary and tertiary structures rather than recognition of a single peptide bond. Substrate mutational studies are required to establish the Tysnd1 recognition motif that would allow the prediction of novel substrates, including inhibitor and activators for pharmacological studies.

Interestingly, the processing of recombinant Acox1 and Scp2 by Tysnd1 was very inefficient as compared with the processing of thiolase (Figures 6 and 7). An *in vivo* conversion rate of Acox1 to its mature form was also reported to be much slower than that of thiolase (Tsukamoto *et al*, 1990). Of

note is the observation that a large fraction of Acox1 and Scp2 was not processed in the peroxisomes (e.g. Otera *et al*, 2001), whereas thiolase was fully converted to its mature form inside the organelle (Ito *et al*, 2005). CHO cells, termed SK32, that express mutant PTS1 receptor Pex5p (G432R), were reported to differentially process thiolase and Acox1 (Ito *et al*, 2005). Whereas at 37°C the peroxisomal processing of thiolase was completely prevented, it was partially restored at 30°C. The conversion of Acox1 in the peroxisomes was completely prevented at both temperatures. Forced expression of wild-type Pex5p in the mutant cells restored the normal processing of both peroxisomal proteins (Ito *et al*, 2005). Hence, it is likely that Acox1 becomes more susceptible to the cleavage by Tysnd1 when it forms a complex with Pex5p and/or other components of the translocation machinery.

The physiological significance of the intra-peroxisomal processing of PTS1 and PTS2 proteins is not yet understood. In the case of mitochondria, the majority of precursor proteins contain cleavable amino-terminal extension sequences for mitochondrial targeting. It is believed that the removal of the signaling sequence by mitochondrial processing proteases is necessary for protein folding and further sorting within the organelle (Gakh *et al*, 2002).

Unlike mitochondrial proteins, peroxisomal proteins are successfully translocated without prior unfolding (Titorenko and Rachubinski, 2004). Recently, Nair *et al* (2004) demonstrated that the PTS2 receptor Pex7p follows, like PTS1 receptor Pex5 (Dammai and Subramani, 2001), an 'extended shuttle' mode of transport. Pex7p enters the peroxisomes during the course of PTS2 protein import and re-emerges into the cytosol to carry out further rounds of protein import. The retention of the signal sequence of PTS2 proteins may cause these proteins to enter and leave the peroxisomes together with Pex7p. Tysnd1-mediated removal of the leader propeptide, therefore, would allow accumulation of PTS2 proteins in the organelle. In the case of PTS1 proteins, the functional significance of peroxisomal processing is less clear. For example, the proteolytic cleavage of Scp2 produces two fragments each possessing distinct activities. The 46-kDa fragment is enzymatically active as a branched-chain fatty acid thiolase (Seedorf *et al*, 1994; Antonenkov *et al*, 1997). The 13-kDa fragment, which is also known as nonspecific lipid transfer protein (nsLTP), may function in the transfer of substrates for example, fatty acyl-CoA derivatives to Acox1 (Wouters *et al*, 1998). The intact full-length form of Scp2 (59 kDa) can still perform both functions. Similarly, cleavage of Hsd17b4 is not necessary for the activity of the protein (Van Grunsven *et al*, 1999). In the case of Acox1, the role of peroxisomal processing is even less clear, as the produced fragments remain associated (Osumi *et al*, 1980).

Importantly, all Tysnd1 substrates identified in this study are components of β-oxidation pathway of fatty acids, which consists of four steps (Supplementary Figure S1). In the first step, Acox1 catalyzes dehydrogenation of acyl-CoA esters to their corresponding *trans*-2-enoyl-CoAs. Hsd17b4 catalyzes enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase reactions in the second and third steps. 3-ketoacyl-CoA thiolase and Scp2 are involved in the final cleavage step of straight-chain and branched-chain 3-keto-acyl-CoA, respectively (Wanders, 2004). It is increasingly recognized that many key biological processes are carried out within very large multiprotein complexes. Using FRET microscopy, Wouters *et al* (1998) demonstrated molecular association of Acox1, Hsd17b4, thiolase and nsLTP in the peroxisomes. The complex is thought to allow an efficient transfer of the lipid intermediates between the enzymes because of transient increase in their local concentration.

The peroxisomal processing by Tysnd1 may provide a feed-forward type regulatory mechanism that enhances the assembly of the β-oxidation enzymes into a complex. In this model, protein fragments produced through Tysnd1-mediated site-specific proteolysis acquire the ability to interact with its partners in the complex (Figure 10). Tysnd1 functions in this scenario as an enhancer of peroxisomal fatty acid β-oxidation. Indeed, Tysnd1 expression was induced by the peroxisome proliferator bezafibrate, which also increased the expression of its substrate enzymes (Figure 9). Coexpression patterns of mouse genes sampled from 55 tissues (Zhang *et al*, 2004) that are similar to the expression pattern of Tysnd1 indirectly support the proposed regulatory role. The Tysnd1 (XM_125636.1), thiolase (XM_135249.1) and Scp2 (XM_135267.1) transcripts are coexpressed at moderate to high levels in aorta, brown fat, small intestine, liver, prostate and adrenal gland, as one would expect from a protease–substrate relationship.

The processing of peroxisomal proteins by Tysnd1 may have potential diagnostic and therapeutic implications on the pathogenesis of fatty acid β-oxidation, ketogenesis and cholesterol synthesis in metabolic diseases such as dietary obesity, fatty liver disease, or hypercholesterolemia. It was shown recently that Hsd17b4 activity is necessary for the male reproductive function by maintaining lipid homeostasis in Serotoli cells of the testis (Huyghe *et al*, 2006). Lack of mature Scp2 in mouse is implicated in the accumulation of phytanic acid and the development of adult Refsum disease-like syndromes (Mukherji *et al*, 2002). Functional defects in Tysnd1 may therefore result in male infertility and Refsum-like disease symptoms. Tysnd1 differential expression in microvascular endothelial cells (Keen *et al*, 2004) may also imply a role of Tysnd1 dysfunctions in the pathogenesis of cardiovascular diseases. The generation and characterization

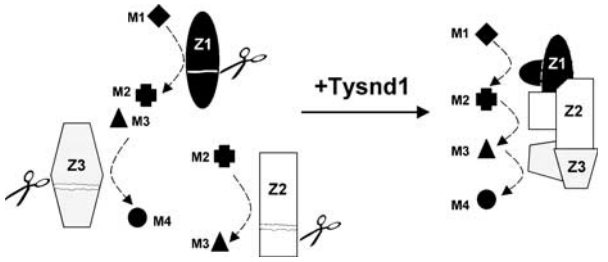

Figure 10 Immature, non-processed peroxisomal enzymes do not interact with each other. Thus, the metabolites (M1–M4) can diffuse freely for a prolonged period of time until they meet the subsequent enzyme (Z1–Z3) in the chain of the reactions. Therefore, the efficiency of the β-oxidation is low (left panel). Tysnd1-catalyzed processing (as indicated by the scissors) provokes conformational changes in its substrate proteins. These changes are thought to facilitate interaction between enzymes involved in the entire chain of the β-oxidation pathway. As a result, the product of one enzymatic reaction is transferred directly to a subsequent enzyme in the reaction chain without dissociating from the complex (right panel). This type of substrate channeling enhances the rate of peroxisomal fatty acid β-oxidation and implies a key regulatory role of Tysnd1 for the entire process.

of Tynsd1 knockout mice will help to clarify the role of Tysdn1 in both physiological and pathological processes.

Materials and methods

Details of the plasmid constructs, cloning methods, cells and transfections, expression and purification of the recombinant substrate proteins, and immunoblotting analysis are available in Supplementary data.

Localization of GFP fusion protein

CHO-K1 cells were plated onto glass coverslips and transiently transfected with expression vectors pEGFP-Tysnd1 and pDsRed2-Peroxi (variant 2 of the red fluorescent protein from *Discosoma sp*) encoding peroxisome-targeted DsRed2 reporter protein (BD Biosciences Clontech). Live cell microscopy of EGFP fusion and DsRed2-Peroxi proteins was performed 48 h after transfection on a laser scanning confocal microscope TCS SP2 (Leica) using a $\times 63$ objective. EGFP fluorescence and DsRed2 fluorescence were detected at 500–530 nm and 555–650 nm wavelength range after excitation at 488 and 543 nm, respectively.

Purification of peroxisomes

Peroxisomes were purified by a method based on the combination and modification of three previously described protocols (Van Veldhoven *et al*, 1996; McClelland *et al*, 2003; Antonenkov *et al*, 2004). See Supplementary data for more details.

Marker enzymes and protein content

To determine the localization in the Optiprep gradient fractions of peroxisomes, mitochondria and lysosomes, we measured activities of catalase (Johansson and Borg, 1988), succinate dehydrogenase (Pennington, 1961) and β-galactosidase (b-Gal assay kit, Invitrogen), respectively. The protein content was determined by the Bio-Rad DC protein assay reagent kit (Bio-Rad).

Polyclonal antibodies

Mouse Tysnd1—Rabbit polyclonal antibodies were raised against keyhole limpet hemocyanin (KLH)-conjugated polypeptide SNTRDNNTGATYPHL corresponding to the amino acids 501–515 of Tysnd1 and affinity-purified by SCRUM Inc., Tokyo. For Western blot analyses, the antibodies were used at a concentration of 2 μg/ml. For the antibody preabsorption assay, synthetic peptide CSNTRDNNTGATYPHL was dissolved in Me_2SO at a concentration of 2 mg/ml and then mixed with the primary antibody solution before Western blotting (40 μg peptide/5 μg antibody; about 700-fold molar excess of peptide). *Mouse prethiolase*—Rabbit polyclonal antibodies were raised against KLH-conjugated polypeptide KLKPAFKDGGSTTAGN corresponding to the amino acids 259–274 of mouse prethiolase and affinity-purified by SCRUM Inc., Tokyo. For Western blot analyses, the antibodies were used at concentrations of 2 μg/ml.

RNA isolation and real-time RT–PCR

The mice livers were immersed in RNA-later solution (Takara). Total RNA was isolated using the RNeasy Miniprep kit (Qiagen). A similar RNA isolation protocol was applied to cells transfected with siRNA. For further details, see Supplementary data.

Small interfering RNA

RNA-mediated interference for downregulating human Tysnd1 expression was done using siRNA duplexes purchased from Qiagen (detailed in Supplementary data).

Purification of recombinant Tysnd1 from COS-7 cells is detailed in Supplementary data.

Assay for in vitro Tysnd1 processing activity

Eight microliters of purified recombinant Tysnd1 were incubated with 6 μl of recombinant Acox1, Scp2 or Acaa1 in 50 mM Hepes, pH 8.0, 115 mM NaCl and 0.2 mM DTT at 37°C. The reaction was stopped by the addition of Laemmli sample buffer and heating for 5 min at 95°C. The reaction products were separated by 4–20% SDS–PAGE and blotted onto Hybond-P membrane. The cleavage products of recombinant Acox1 and Scp2 were detected using anti-c-Myc monoclonal antibody (Nacalai Tesque). The processing product of prethiolase was visualized by anti-thiolase antibody or alternatively by staining Hybond-P membrane with 0.05% Amido Black.

NH_2-terminal sequence analysis

Proteins were separated by 4–20% SDS–PAGE, blotted onto Hybond-P membrane and stained with 0.05% Amido Black. The bands of interest were excised and subjected to multiple cycles of Edman degradation on an Applied Biosystems model Procise 494HT sequencer.

Protease inhibition assay

For inhibition assays, purified recombinant Tysnd1 was preincubated for 30 min at 25°C with various protease inhibitors for 30 min. After addition of the substrate protein, the incubation proceeded for 12 h at 37°C. The following protease inhibitors were used (final concentration): pepstatin A (1 μM), EGTA (2 mM), EDTA (2 mM), 1,10-phenanthroline (1 mM), 1,7-phenanthroline (1 mM), benzamidine (1 mM), AEBSF (1 mM), aprotinin (4 μg/ml), leupeptin (10 μM), E64 (20 μM) and NEM (1 mM). The processing products were assayed as described above.

Bezafibrate treatment and subcellular fractionation

Six 7-week-old C57BL/6J male mice were used for the experiment. The control group ($n=3$) was maintained on a standard diet, whereas experimental group ($n=3$) was fed with a standard diet containing 0.5% (v/w) bezafibrate. For quantitation of mRNA levels by real-time RT–PCR analysis, each liver sample was processed individually as described above. For Western blot analysis, the livers from three animals belonging to the same group were mixed and subcellular fractionation was performed as described under 'Purification of peroxisomes'. Post-mitochondrial fraction represents a supernatant after centrifugation of the PN fraction at 20 000 *g* for 20 min.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Competing interests statement

The authors declare that they have no competing financial interests.

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