The Expression of Tripeptidyl Peptidase I in Various Tissues of Rats and Mice*

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Summary. To understand the precise distribution of tripeptidyl peptidase I (TPP-I), a defect of which has been shown to induce late infantile neuronal ceroid lipofuscinosis, various tissues from rats and mice were analyzed using biochemical and immunohistochemical techniques. Western blot analyses showed that a protein band immunoreactive to anti-TPP-I appeared in tissue extracts of both animals at a molecular weight of approximately 47 kD. Protein levels of TPP-I differed among tissues; they were high in the rat brain, liver, stomach, kidney, thyroid and adrenal glands and in the mouse brain, stomach, kidney, and testis. The proteolytic activity of TPP-I was detectable; it differed in the tissues examined and did not always reflect the expression levels of the protein in the tissues. In particular, the TPP-I activity was low in the brains of both animals and high in the rat testis, although its protein levels were high in the former tissue and low in the latter. Double immunostaining showed the immunoreactivity for TPP-I to be well localized in granular structures of epithelial cells in renal tubules and the cerebral choroid plexus, both of which were also stained with lamp2, a lysosomal membrane protein marker, indicating that TPP-I is a lysosomal enzyme. The immunoreactivity was intense in F4/80-immunopositive macrophages/microglial cells located in various tissues including the thymus, spleen, liver, alimentary tract, and central nervous system. Although the immuno-reactivity differed depending on the tissues and even within the same tissues between the species, it was detected in all tissues examined, especially in nerve cells, some types of endocrine cells, and oocyte cells such as gastric parietal cells and bone osteoclasts. However, the immunoreactivity was faint and week in rat thyroid gland, although its protein level was high in the tissue. These lines of evidence suggest that TPP-I, a lysosomal serine protease, is widely distributed in rat and mouse tissues, although its expression levels vary among them.

Late infantile neuronal ceroid lipofuscinosis (LINCL), a recessive fatal neurological disease, is characterized by the lysosomal accumulation of the subunit c of mitochondrial ATP synthase in neurons and other types of cells in the central nervous system (CNS) and peripheral tissues (SLEAT et al., 1997; WILLIAMS et al., 1999). The causal gene of LINCL (CLN2) encodes a 46 kD glycoprotein which is absent from the cells and CNS tissues of LINCL patients and is similar in amino acid sequence to bacterial endopeptidases (SLEAT et al., 1997; LIU et al., 1998). An EST database analysis showed that tripeptidyl peptidase I (TPP-I), a lysosomal protease that cleaves triptides from the N-terminus of polyptides, is identical to a pepstatin-insensitive carboxyl peptidase which is mutated in LINCL (VINES and WARBURTON, 1999). Moreover, fibroblasts from LINCL patients have less than 5% of the normal TPP-I activity (VINES and WARBURTON, 1999). Although TPP-I is not inhibited by classical inhibitors of serine, cysteine, aspartate or metallo-proteinases, LIN et al. (2001) have shown that the CLN2 product is synthesized as an inactive proenzyme that is autocatalytically converted to an active serine protease.

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EZAKI et al. (1999) have shown that TPP-I is synthesized as a 67 kD precursor in normal human skin fibroblasts, processed to a 46 kD protein, and localized with cathepsin D in the high density lysosomal fraction. Moreover, it has been shown that a defect of TPP-I causes the specific accumulation of subunit c of mitochondrial ATP synthase in lysosomes of cells, while the presence of the enzyme leads to the sequential cleavage of tripeptides from the N terminus sequence of subunit c (EZAKI et al., 2000). The presence of TPP-I in human subjects has been shown by immunohistochemistry and immunoblotting in various regions of the CNS and visceral organs including the heart, liver, colon, kidney, pancreas, pituitary and adrenal (KIDA et al., 2001; KURACHI et al., 2001). Our previous study also demonstrated that TPP-I is localized together with cathepsins H and C in lysosomes of macrophages and lamellar bodies of type II epithelial cells in rat pulmonary alveoli (YAYOI et al., 2001). TPP-I is thus involved in the lysosomal degradation in various tissue cells, although it plays a crucial role in the initial cleavage of subunit c of mitochondrial ATP synthase.

In the course of studies on the distribution of TPP-I in rat and mouse tissues, we noticed that its protein expression and proteolytic activity differed by tissue. We therefore examined the precise localization of TPP-I in various tissue cells of rats and mice, using immunohistochemical and biochemical techniques. The results showed that TPP-I, which first was solely localized in lysosomes of various tissue cells, had a wide distribution in rat and mouse tissues, although the proteolytic activity of the enzyme did not always coincide with the expression levels of the protein.

MATERIALS AND METHODS

Animals

Ten adult Wistar rats and C3HBL/6 mice were respectively used for the present study: 4 rats and 4 mice for immunohisto/cytchemistry, and 6 rats and 6 mice for biochemical analyses.

Antibodies

Polyclonal antibodies against the carboxyl terminal portion of TPP-I (TPP-I/Cln2p402-518) were prepared and purified by affinity chromatography, as described previously (EZAKI et al., 1999, 2000). Rabbit antisera to synthetic human glucagons, somatostatin, insulin, and pancreatic polypeptide (Santa Barbara, CA, USA), and monoclonal antibodies against mouse macrophages/microglial cells (F4/80) (Serotec, Oxford, UK) and mouse lamp2, a lysosomal membrane protein (Developmental Studies Hybridoma Bank (DSHB), University of Iowa, USA) were obtained commercially.

Immunohisto/cytchemistry

The animals were deeply anesthetized with pentobarbital (25 mg/kg i.p.) and fixed by cardiac perfusion with 4% paraformaldehyde buffered with a 0.1 M phosphate buffer, pH 7.2, containing 4% sucrose for light microscopic immunohistochemistry. Various tissues including the brain, retina, choroid plexus, liver, pancreas, alimentary tracts, kidney, urinary bladder, pituitary gland, adrenal gland, thyroid gland, testis, ovary, uterus, thymus, and spleen were quickly excised from the animals and further immersed in the same fixative for 2 h. The samples from each animal were processed for paraffin embedding, cut at 5 μm with a microtome, and placed on silane-coated glass slides, while some samples were frozen by liquid nitrogen after cryoprotection with sucrose solutions, cut at 10 μm with a cryostat, and placed on the glass slides. In addition to these tissues, mice at 10 days and 8 weeks of age were perfused with the same fixative, and their excised tibial bones and cochleas were further placed in the fixative. Before embedding in paraffin, bone and cochlear tissues were decalcified with a 10% EDTA solution for 3 days.

Deparaffinized and frozen sections from each sample were immunostained according to the method by NITATORI et al. (1995) or GOTO et al. (1999). Briefly, they were treated with 0.3% H2O2 in methanol for 30 min and incubated with 2% normal goat serum for 20 min at room temperature. They were then incubated at 4°C with anti-TPP-I (6.5 μg/ml) for one to three days. Four sets of two serial sections from pancreatic samples were prepared; each set was placed at a mirror image position on glass slides and incubated with combinations of anti-TPP-I and anti-glucagon (1:1000), anti-pancreatic polypeptide (1:1000), antismatostatin (1:1000), and anti-insulin (1:2000) at 4°C for one to three days, respectively. Further incubations were performed with biotinylated goat antirabbit IgG for 1 h and peroxidase-conjugated streptavidin (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA, USA) for 1 h at room temperature. After each step, sections were rinsed thoroughly in 0.1 M phosphate-buffered 0.5 M saline (pH 7.2) (PBS) containing 0.1% Tween 20 (TPBS) (Sigma, Tokyo). Staining for peroxidase was performed using 0.0125% 3,3'-diaminobenzidine tetrahydrochloride (DAB) and 0.002% H2O2 in 0.05 M Tris-HCl buffer (pH 7.6) for 10 min.

For double immunostaining, the sections were incubated with a mixture of anti-TPP-I and F4/80
(1:50) or lamp2 (1:50) at 4°C for 24 h and then with goat anti-rabbit IgG coupled with Arexa 594 (Molecular Probes, USA) and goat anti-rat IgG coupled with FITC (Organon Teknika Corp., PA, USA) for 1 h. The stained sections were mounted with an antifade solution and examined by a confocal laser microscope (Olympus, LSM-GB200, Tokyo).

**Immunoblot analysis**

Anesthetized rats and mice were killed by decapitation and each excised tissue was independently homogenized in 2 ml of 0.05 M Tris-buffered 0.15 M saline (TBS) containing 1% Triton X-100 and a protease inhibitor cocktail (Losch, Basel, Switzerland) using a Poltron homogenizer at 80% of the maximum speed. After being centrifuged twice at 10,500 g for 10 min at 4°C, the supernatants were measured for protein concentrations using the BCA protein assay system (Pierce, IL, USA) and applied to immunoblotting. The samples were then analyzed by 12.5% SDS-PAGE. Electrophoretic transfer of proteins from polyacrylamide gels to a PVDF membrane (Immobilon-P; Millipore Co., Tokyo) was performed according to the method by Towbin et al. (1979). The sheets were soaked in PBS containing 5% bovine serum albumin (Sigma) to block non-specific binding, and then incubated with antisera. Immunodetection was carried out with a chemiluminescent ECL kit (Amersham Bioscience Corp., NJ, USA) according to the manufacturer's recommended protocol. To obtain lysosome-rich fractions, liver samples from the mice were homogenized in 0.25 M sucrose with a glass homogenizer and centrifuged at 1,200 g for 5 min. The supernatant was loaded on a cushion of 15% Percoll in 0.25 M sucrose, centrifuged at 50,000 g for 30 min, and then fractionated into 12 fractions from bottom to top. The heavier fractions showing cathepsin B activity, which was detected using Z-Arg-Arg-MCA (Peptide Inst., Osaka) were then incubated with a lysis buffer containing 150 mM NaCl, 50 mM Tris, 1% Triton X-100 including the proteinase inhibitor cocktail (Losch), and subjected to SDS-PAGE and immunoblotting.

**Enzyme assay**

Each sample obtained from the anesthetized rats and mice was independently homogenized with a Poli-toron homogenizer in a lysate buffer consisting of 0.05 M Tris-Cl, pH 7.5, 0.15 M NaCl and 1% Triton X-100 for 30 min on ice. For measurement of TPP-I, the lysates were then diluted with a standard buffer consisting of 0.4 M sodium acetate buffer, pH 5.5, containing 4 mM EDTA. Following centrifugation at 12,500 g for 20 min, the activity of TPP-I in various tissue extracts of both animals was assayed using Ala-Ala-Phe-MCA (Peptide Inst.) as a substrate as described previously (Page et al., 1993).

**RESULTS**

**Protein expression of TPP-I and its proteolytic activity in various tissues**

To determine the expression levels of the TPP-I protein, various tissue extracts from rats and mice were subjected to immunoblotting. A protein band immunoreactive to anti-TPP-I appeared at a molecular weight of approximately 47 kDa in both rat and mouse tissues (Figs. 1A, 2A). The molecular weights of rat and mouse TPP-I agree with that of human fibroblast TPP-I (Izaki et al., 1999). The protein band was present in each sample from the rat and mouse tissues examined, although its amounts largely differed by tissue. The brain, heart, stomach and kidney in both animals expressed the TPP-I protein at high levels, whereas its protein expression was low in the thymus, testis, and skin of rats and the pituitary, testis, liver, pancreas, duodenum, jejunum and spleen of mice.

Since the expression of the TPP-I protein differed depending on the tissues, we next examined the proteolytic activity of TPP-I in each tissue from the animals. TPP-I activity in each was much higher in rats than in mice, while it largely differed depending on the tissue in both animals (Figs. 1B, 2B). High activity was detected in the kidneys of rats and mice and in the testes of rats, where the protein expression was low. In both animals, the protein amounts of TPP-I were high in the brain and heart, but the activity was low in these tissues. Thus, the proteolytic activity of TPP-I did not always reflect its expression levels in the tissues of both animals.

**Immunohistochemical localization of TPP-I in various tissues**

Since the relationship between the protein expression and proteolytic activity of TPP-I was incongruous, we then examined the immunohistochemical localization of TPP-I in various tissues of the animals. The figures basically demonstrated immunostaining in mouse tissues when staining patterns were similar between the animals.

To confirm the specificity of the anti-TPP-I used, double immunostaining using anti-TPP-I and lamp2, a lysosomal membrane marker, was applied to the mouse renal cortex and choroid plexus of the
cerebral ventricle (Fig. 3). Positive signals for TPP-I were clearly localized in fine and coarse granular structures which were precisely co-labeled with lamp2 in these two tissues, indicating that anti-TPP-I recognized the TPP-I protein in lysosomes. Moreover, since TPP-I has been shown to be well localized in lysosomes of macrophages (Yayoi et al., 2001), various tissues were dual-stained with anti-TPP-I and the F4/80 that recognizes macrophages/microglial cells. Intense staining of TPP-I was localized in F4/80-immunopositive cells in CNS and peripheral tissues, especially in the thymus (Fig. 4).

TPP-I immunoreactivity has been shown in neurons and glial cells of various regions in human CNS tissues (Kida et al., 2001; Kurachi et al., 2001). In the mouse and rat CNS tissues, cerebral cortical neurons, especially pyramidal neurons, contained TPP-I immunoreactive granular structures (Fig. 5A). Immunostains were also found intensely in Bergmann glia located near Purkinje cells in the cerebellum, while they were faint or weak in the perikarya of the Purkinje cell and weak but distinct in granule cells (Fig. 5B). Neurons in the cochlear spiral and retinal ganglia showed intense immunoreactivity (Fig. 5C, D). In retinal tissues, granular immunodeposits were also detected intensely in neurons of the inner nuclear layer and weakly in cells of the outer nuclear layer (Fig. 5C).

Immunodeposits for TPP-I were densely distributed in various peripheral tissues of rats and mice. In endocrine organs, the immunoreactivity was densely localized in most endocrine cells of the anterior pituitary (Fig. 6A), while it was detected in Leydig (interstitial) and Sertoli cells of the testis (Fig. 6D). Thyroid follicular epithelial cells possessed many immunodeposits for TPP-I in mice (Fig. 6B), whereas the immunoreactivity was not found in follicular epithelial cells but in interstitial macrophages in the rat thyroid gland (Fig. 6C). The immunoreactivity in the adrenal gland of mice was intensely localized in cells of the zona glomerulosa, zona fasciculata, and zona reticularis, but weakly in cells of the medulla (Fig. 6C), whereas immunodeposits were intensely found in the middle portion of the zona fasciculata and certain medullary cells in the rat adrenal gland (Fig. 8A, B). In pancreatic islets, the immunoreactivity was found in some types of endocrine cells in both animals (Fig. 6E). To identify the cell types in the islets, two serious sections mounted on a glass slide at a mirror position were stained for a combination of TPP-I and one of insulin, somatostatin, and pancreatic polypeptide/glucagon, the results indicating that the staining pattern of TPP-I in the islet coincided well with that of somatostatin (Fig. 9C, D) but not with those of insulin and pancreatic polypeptide/glucagon (Fig. 9A, B, E, F).
In alimentary tracts and their derivatives, the immunoreactivity was similarly detected between rats and mice (Fig. 6E-G). In the stomach, parietal and chief cells in the deep zone of the gastric gland possessed dense immunodeposits of TPP-I which were absent or faint in the neck and superficial cells (Fig. 6G). In the small and large intestine, TPP-I immunoreactivity was lacking or faintly found in epithelial cells (data not shown). The immunoreactivity was faintly or weakly found in pancreatic exocrine cells (Fig. 6E), while it was dense in the perivenuous hepatocytes of the liver but weak in the periportal hepatocytes (Fig. 6F). Kupffer cells clearly showed immunodeposits for TPP-I (data not shown). Although TPP-I and its activity were distinctly demonstrated in the mouse liver by immunocytochemistry and enzyme assay, respectively, no protein band was found by immunoblotting. Therefore, lysosomal fractions were obtained from the mouse liver and subjected to Western blotting. As shown in Figure 2C, a protein band immunoreactive for TPP-I was present in the mouse liver (Fig. 2C).

In the mouse kidney, immunodeposits for TPP-I were intensely found in epithelial cells of proximal tubules and collecting tubules and moderately in those of distal and collecting tubules (Fig. 7A–C). The immunoreactivity was faint or absent in epithelial cells of the loop of Henle and in cells of the glomerulus and Bowman’s capsule (Fig. 7A–C). In the rat kidney, however, the immunoreactivity was not found in epithelial cells of the proximal tubules, while it was detected in those in distal and collecting tubules, and in collecting tubes (Fig. 8D, data for the renal medulla were not shown). The immunoreactivity was also examined in cardiac tissues of the animals, but staining with anti-TPP-I showed typical cross striations of muscle fibers in the cells in addition to perinuclear granular structures (data not shown). This indicates that anti-TPP-I may cross-react with some of cytoskeletal elements in muscle fibers.
Fig. 3. Double immunostaining of TPP-I and lamp2 (LAMP-2) in the mouse renal cortex (A–C) and cerebral choroids plexus (D–F). A–C. Immunoreactivity for TPP-I is well localized in granular structures in epithelial cells of renal tubules (A), while lamp2 stains lysosomes in these cells (B). An overlaid figure shows that TPP-I immunoreactivity is specifically detected in lamp2-immunopositive lysosomes in the cells (C). D–F. Immunoreactivity for TPP-I is also localized in granular structures in epithelial cells of the choroid plexus (D), while lamp2 stains lysosomes in these cells (E). An overlaid figure shows that TPP-I immunoreactivity is precisely detected in lamp2-immunopositive lysosomes in the cells (F). A–F: ×510

Fig. 4. Double immunostaining of TPP-I (A) and F4/80 (B) in the mouse thymus. TPP-I immunoreactivity (red color) is localized in F4/80-immunostained macrophages (green color) (overlay in C). A–C: ×640
In the mouse tibial bone, immunodeposits for TPP-I were moderately found in osteoblasts and osteocytes, while they were intense in osteoclasts and chondrocytes of the articular cartilage (Fig. 6H, I).

DISCUSSION

The present biochemical and immunohistochemical studies demonstrated that TPP-I is widely distributed in various tissues of rats and mice, although its proteolytic activity does not always correspond with protein levels in the tissues. Moreover, TPP-I was confirmed by immunohistochemistry to localize in lysosomes of tissue cells.

To date, TPP-I has been identified in various tissues of mammals including the bovine anterior pituitary (DOEBBER et al., 1978), and brain (JUNAID et al., 2000), rat liver (WATANABE et al., 1992), spleen (VINES and WARBURTON, 1998), and kidney (DU et al., 2001), and human osteoclastoma (PAGE et al., 1993). These studies showed that TPP-I has a molecular weight of approximately 46–48 kD by SDS-PAGE which agrees well with that obtained in the present study. TPP-I is known to cleave tripeptides from the N-terminus of peptides and has been shown to cleave various bioactive peptides as natural substrates such as angiotensin II and III, substance P, β-amyloid, and neuromedin B (JUNAID et al., 2000; DU et al., 2001). However, the subunit c of mitochondrial ATP
Fig. 6. Immunohistochemical demonstration of TPP-I in various peripheral tissues of mice. A. Anterior pituitary. Positive signals are intensely detected in most endocrine cells. B. Thyroid. Follicular epithelial cells contain intense immunodeposits. C. Adrenal. Dense granular immunodeposits are detected in the zonae glomerulosa, fasciculata and reticularis, while they are sparse in medullary cells. D. Testis. Immunostaining is distinct in Leydig (interstitial) cells and Sertoli cells of the seminiferous tubule. E. Pancreas. The immunoreactivity is distinctly localized in some of the islet endocrine cells, but the exocrine cells are mostly negative. Interstitial macrophages display positive signals. F–I. Legend continued on the opposite page.
Fig. 7. Immunostaining of TPP-I in the mouse kidney. A. Positive signals are distinct in both the renal cortex and medulla. Epithelial cells in thick and thin portions of the loop of Henle are largely negative in staining. B. Renal cortex. Granular immunodeposits are intensely localized in epithelial cells from the initial part of the proximal tubules, but relatively faint in the distal tubules. Positive signals are absent in cells of Bowman’s capsule and a glomerulus (also see in A). C. Renal medulla. Intense staining is demonstrated in epithelial cells of renal tubes, while dot-like immunodeposits are in some cases detectable in those of the loop of Henle. A: ×80; B, C: ×300

Fig. 6 F. Liver. Immunolocalization shows a perivenous pattern in the hepatic acinus. G. Stomach. The immunoreactivity is largely deposited in the deep zone of the gastric gland, while it is faint or lacking in the neck and superficial zones. Intense immunodeposits are detected in chief cells located in the basal portion and parietal cells located from the basal to the middle portions. H and I. Tibial bone at 10 days of age. Positive signals are detectable in osteoblasts and osteocytes, while intensely stained osteoclasts are distinctly visible (H). Chondrocytes in the articular catilage possess intense positive signals, particularly in the middle portion. A, B, D, H, I: ×210; C, G: ×100; E: ×300; F: ×80
Fig. 8. Immunohistochemical staining of TPP-I in rat tissues. A and B. Adrenal. Intense positive staining is seen in the zona fasciculata but weakly so in the zona glomerulosa and reticularis, while it appears intense in the medullary (A). Dense immunodeposits are found in medullary cells (B). C. Thyroid. Immunostaining is faint or negative in follicular epithelial cells, while positive staining is detectable in interstitial macrophages. D. Kidney. Immunostaining is found only in distally in collecting tubules in the renal cortex but not in the proximal tubules, Bowman’s capsule and glomeruli. A: × 60; B-D: × 260

synthase is the accumulated substance in LINCL, suggesting that this subunit is a major natural substrate of TPP-I (Palmer et al., 1992; Ezaki et al., 1999, 2000).

By Northern blotting using TPP-I cDNA cloned from the rat liver, Du et al. (2001) demonstrated that the order for mRNA expression of the enzyme is kidney > liver > heart > brain > lung > spleen. In the rat tissues examined in the present study, the highest protein level of TPP-I appeared in the brain and heart, followed by the kidney, liver, and stomach. These tissues in mice also showed a high expression of the protein except for the liver in which the protein was detected after lysosome-rich fractions were applied to immunoblotting. Thus, the expression of the TPP-I protein was ubiquitous in all tissues of both animals, but its levels differed by tissue.

Using Ala-Ala-Phe-MCA, which has been shown to be one of sensitive substrates for TPP-I (Vines and Warburton, 1998; Junaid et al., 2000; Du et al., 2001), we also measured its proteolytic activity in various rat and mouse tissues. Similar to the expression levels of the protein, the activity varied among tissues examined. However, the TPP-I activity measured did not always reflect the expression levels of the protein in various tissues. In particular, although the expression level of the protein was high in the brains of both animals, TPP-I activity was low. It has been shown that the protein expression of TPP-I in the human cerebral cortex increases with development and reaches an adult level after the age of 2, while the protein is well localized in neurons and glial cells (Kida et al., 2001; Kurachi et al., 2001). The localization of TPP-I in brain tissues of rats and mice was similar to that in human brain, as evidenced by the present immunohistochemical study. The high protein expression in brain tissues is also supported by the fact that TPP-I mRNA is highly expressed in the rat brain (Du et al., 2001). It remains unknown why the proteolytic activity of TPP-I is low in brain tissues, compared with its high protein expression. This contradictory result, however, may predict the presence of an endogenous inhibitor for TPP-I in brain tissues. In contrast to brain tissues, the protein level in the rat testis was found to be low, but its activity was extremely high in the tissue. The present immunohistochemical data show that immunodeposits for TPP-I were intensely localized to Leydig and Sertoli cells in testicular tissues of the rats and mice, this coinciding with the protein expression of TPP-I in the mouse testis. To date, there has been no report concerning the presence of a peptidase that cleaves Ala-Ala-Phe-MCA as a substrate at an acidic pH (pH 4) as TPP-I does. However, the present data
from rat testis strongly argue for the presence of such a peptidase.

As far as we know, the present study is the first report to show the localization of TPP-I in lysosomes of in vivo tissue cells by immunohistochemistry, except for a study which notes that TPP-I is colocalized with cathepsin D in cultured human fibroblasts (Kida et al., 2001). Double immunostaining colocalized TPP-I in granular structures of epithelial cells in cerebral choroid plexus and renal tubules which were exactly labeled with lamp2, a lysosomal membrane marker. These results are consistent with previous biochemical data (Doebber et al., 1978; Watanabe et al., 1992; Page et al., 1993; Vines and Warburton, 1998, 1999; Junaid et al., 2000; Du et al., 2001).

It has been shown that positive staining for TPP-I is distinct in all cells consisting of human CNS tissues, including not only neurons but also vascular endothelial cells, glial cells, ependymal cells, epithelial cells of choroid plexuses, and meningeal cells (Kida et al., 2001; Kurachi et al., 2001). The localization patterns of TPP-I in CNS tissues of rats and mice were essentially similar to these studies using human materials (Kida et al., 2001; Kurachi et al., 2001). One clinical symptom in LINCL which CLN2p/TPP-I lacks is the loss of vision due to retinal atrophy (Jully and Palmer, 1995). TPP-I immunoreactivity was densely localized in ganglion neurons, moderately so in neurons of the inner nuclear layer, and weakly present in cells of the outer nuclear layer. A defect of CLN2p/TPP-I delays the degradation of subunit c, a major natural substrate of the proteinase, in lysosomes and induces the accumulation of lysosomes in neurons, resulting in cerebral and cerebellar cortical atrophy and retinal atrophy (Palmer et al., 1992). Kida et al. (2001) have suggested that the development of CLN2p/TPP-I expression in human brains is associated with the onset of LINCL at 2-4 years of age, indicating that it takes years till neuronal cell death occurs. This tendency of TPP-I expression has also been shown by Kurachi et al. (2001) in human brains and Suopanski et al. (2000) in rat brains. This may explain why the loss of the CLN2p/TPP-I generation induces neurogenic disorder but not dysfunction in peripheral tissues, since functional cells in peripheral tissues are always renewed within certain time periods, in contrast with neurons in CNS tissues which are known to be postmitotic cells. Our present data showing that TPP-I immunoreactivity was intensely detected in functionally differentiated cells of various tissues but not in primitive (stem) cells located in the neck portion of gastric glands and seminiferous tubules of the testis strongly argue for this assumption.

Fig. 9. Immunohistochemical staining of TPP-I (A, C, E) and insulin (B), somatostatin (D) and pancreatic polypeptide (F) in the rat pancreatic islet. Three sets of two serial sections which were mounted on glass slides at a mirror position were applied to immunostaining. Positive staining for TPP-I is localized in cells immunostained for somatostatin (C, D) but not for insulin (A, B) or pancreatic polypeptide (E, F). A-F: ×180
Localization patterns of lysosomal cysteine proteinases have been shown to differ depending on tissue cells, suggesting that these lysosomal proteinases have a substrate specificity which is involved in the functional expression of tissue cells (UCHIYAMA et al., 1994). In the liver, cathepsins B and H are well localized to perivenous hepatocytes, while cathepsin L is mainly detected in Kupffer cells (WATANABE et al., 1989; WAGURI et al., 1990, 2001). The former pattern is associated with the localization of cation independent-mannose 6-phosphate receptor (MPR) and the latter with that of cation dependent-MPR (WAGURI et al., 2001). The immunoreactivity for TPP-I has been detected in both Kupffer cells and perivenous hepatocytes in which the lysosomal volume density is much higher than in periportal hepatocytes (UCHIYAMA and ASARI, 1984). In the stomach, TPP-I immunoreactivity was localized mainly in parietal cells of gastric glands which possess numerous mitochondria to supply ATP to the proton pump for acid production. Considering that TPP-I is required to degrade the subunit c of mitochondrial ATP synthase, it is reasonable to think that TPP-I is highly expressed in parietal cells for the degradation of unneeded mitochondria by the autophagy/autolysosome system. In the tibial bone, osteoclasts — which also possess numerous mitochondria — were immunopositive for TPP-I. It has been shown that an inhibitor of TPP-I, Ala-Ala-Phe-CH₂Cl, inhibits bone resorption, suggesting that TPP-I is involved in the degradation of collagen type I (PAGE et al., 1993; VINES and WARSURTON, 1998). To date, a major candidate for bone resorption has been demonstrated to be cathepsin K, since a defect in its gene causes osteosclerosis in humans and mice (GELB et al., 1996; SAFTIG et al., 1998). However, there is an autopsy case report concerning infantile osteopetrosis complicating neuronal ceroid-lipofuscinosis, in which generalized sclerosis and a thickening of cortical and spongy bones, the formation of mineralized cartilaginous tissues, and a narrowing of the marrow cavities were observed (TAKAHASHI et al., 1990). Moreover, osteoclasts around the thickened bone trabecules lack a ruffled border and clear zone along the cell membrane facing the bone matrix surface. Although it remains unknown whether cathepsin K was expressed in the patient, these lines of evidence highly suggest that TPP-I in osteoclasts may play a crucial role in bone resorption.

It has been shown that crinophagy is involved in the degradation of secretory granules in peptide hormone producing cells (MÖL et al., 1984; ORCI et al., 1984; WATANABE and UCHIYAMA, 1988; UCHIYAMA et al., 1990). By macroautophagy, part of the cytoplasm including unneeded secretory granules is sequestrated by the endoplasmic reticulum, forming crinophagosomes (autophagosomes) to which lysosomal enzymes are transported from the trans-Golgi network, mostly depending on MPRs. Localization of TPP-I in endocrine cells of the anterior pituitary, adrenal medulla, and pancreatic islets may play a role in the degradation of unnecessary secretory granules. In particular, specific localization of the enzyme in pancreatic islet D cells, in which cathepsin B has been shown to be localized (WATANABE et al., 1988), suggest that TPP-I may be involved in the degradation of somatostatin. Moreover, it is interesting that TPP-I immunoreactivity was intensely detected in steroid hormone-producing cells in the adrenal cortex and testis. At present, however, the reason for TPP-I is required in these cells remains unexplained. As for thyroid follicular epithelial cells, TPP-I immunoreactivity was intensely detected in those of mice but not in those of rats. Follicular epithelial cells contain lysosomal cysteine proteinases which are suggested to be involved in the production of thyroid hormones from reabsorbed colloid materials including thyroglobulin (UCHIYAMA et al., 1989a, b). Although it is not known whether TPP-I is present in rat follicular cells, it is possible to consider that the enzyme is involved in the degradation of thyroglobulin in the cells of mice.

In the kidney, localization of TPP-I differed between rats and mice. It has been shown that immunoreactivity for TPP-I or its activity is detected in epithelial cells of distal and collecting tubules (DIKOV et al., 2000; DU et al., 2001). This localization pattern of TPP-I in the rat kidney is confirmed by our present immunohistochemical results. In mice, however, its localization was widely detected in epithelial cells from the initial portion of proximal tubules up to the collecting tubes except for the loop of Henle. No positive staining was found in Bowman’s capsules and glomeruli. From these results, it is reasonable to assume that the localization of TPP-I in proximal tubules is involved in the degradation of reabsorbed proteins and peptides; however, the high expression of TPP-I in distal tubules and collecting tubes remains unexplained in rat and mice.

Finally, the present biochemical and immunohistochemical results suggest that TPP-I is precisely localized in lysosomes of tissue cells and widely distributed in various tissues of rats and mice, although its proteolytic activity does not always coincide with the tissue expression levels of the protein. This inconsistency may indicate the presence of an endogenous inhibitor TPP-I and another peptidase which cleaves Ala-Ala-Phe-MCA as a substrate.
REFERENCES


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