ORGANELLE LOCALIZATION OF RAT LIVER SERINE:PYRUVATE AMINOTRANSFERASE EXPRESSED IN TRANSFECTED COS-1 CELLS

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ABSTRACT

In rat liver where serine:pyruvate aminotransferase (SPT) is localized in both mitochondria and peroxisomes (SPT\(\text{m}\) and SPT\(\text{p}\), respectively), there are two types of SPT mRNAs with different sizes, and with in vitro translation, two immunoreactive products of different sizes (45 K and 43 K) are formed. SPT\(\text{m}\) has been shown to be synthesized from a larger mRNA as a precursor (45 K-translation product) which is specifically translocated into mitochondria with the concomitant processing to SPT\(\text{m}\) (43 K). In the present study, a truncated cDNA was constructed to encode the 43 K-product. When the cDNA encoding the 45 K-product was expressed in COS-1 cells, SPT immunoreactivity was almost specifically localized in mitochondria. In contrast, localization of SPT immunoreactivity was exclusively peroxisomal when the truncated cDNA was expressed. It was thus indicated that SPT\(\text{p}\) is synthesized from a smaller SPT mRNA as the 43 K-translation product. It is known that owing to transcription from different initiation sites, the smaller mRNA lacks a portion of 5'-terminal sequence of the larger mRNA which codes the mitochondrial targeting N-terminal extension peptide of the precursor. Therefore, the results of the present study indicate that the dual organelle localization of rat liver SPT results from transcription from two different initiation sites.

Serine:pyruvate aminotransferase (SPT, EC 2.6.1.51) is an enzyme whose organelle localization and hormone responsiveness differ with the animal species. Noguchi and Takada reported that in humans, monkeys, rabbits and guinea pigs, this enzyme is peroxisomal, while in cats and dogs, it is entirely mitochondrial (6, 16). In rat liver, there are two types of this enzyme in terms of organelle distribution and hormone responsiveness; one is a mitochondrial matrix enzyme which is markedly induced on the administration of glucagon (1, 5, 15, 16) or insulin (3), and the other is a glucagon-insensitive peroxisomal enzyme (5, 15–17).

In rat liver, the SPT gene appears to be single based on Southern blot analysis of genomic DNA (8), but there are two types of SPT mRNAs with different sizes (3, 12), and with in vitro translation two immunoreactive products of different sizes are formed (3, 9). With regards to mRNAs, one is an mRNA of approximately 1,900 nucleotides (1,900 nt-mRNA), the formation of which is greatly increased by glucagon or insulin, and the other an apparently hormone-insensitive 1,700 nt-mRNA. The 1,900 nt- and 1,700 nt-mRNAs differ from each other in that approximately 70 nucleotides of the 5'-terminal sequence of the former are lacking in the latter due to transcription from different initiation sites in exon 1. With regards to the translation products, one is a 45 K-product and the other is a 43 K-product. The 45 K-species has been shown to be formed from the 1,900 nt-mRNA in...
Fig. 1  cDNA fragments of pRspt321 that were expressed in cultured COS-1 cells. pRspt321 is a full-length cDNA encoding the 45 K-precursor of SPTm (preSPTm) (13). Narrow and wide boxes indicate the 5'- or 3'-untranslated region and the coding region, respectively. Hatched box shows the sequence encoding the mitochondrial targeting N-terminal extension peptide of preSPTm. The first nucleotide of the ATG triplet encoding the initiation Met of preSPTm is the numbered 1. Asterisks represent first, second, and third ATG codons. The first and third ATG triplets encode the initiation Met of preSPTm and SPTp, respectively. The numbers in parentheses indicate the 3'-terminal nucleotides generated on enzymic cleavage.

response to glucagon or insulin stimulation (3, 9, 11). This translation product is the precursor of SPTm (preSPTm), which is approximately 2 K larger than the mature subunit and is specifically transported into mitochondria with the concomitant processing to mature SPTm (9–11). Thus the hormone-responsive 1,900 nt-mRNA and 45 K-translation product were shown to be involved in the synthesis of SPTm. The remaining hormone-insensitive 1,700 nt-mRNA and 43 K-translation product have been assumed to be involved in the synthesis of SPTp. In the present study, a truncated SPT cDNA was constructed to encode the 43 K-translation product, and the truncated cDNA as well as the cDNA encoding the 45 K-product was expressed in cultured monkey kidney COS-1 cells, followed by immunocytochemical examination of the organelle distributions of the expression products.

MATERIALS AND METHODS

Materials

pSVL, an SV40 late promoter expression vector, was obtained from Pharmacia LKB Biotechnology (Sweden), pAM19, a plasmid vector with SP6 and T7 promoters, from Amershams (U.K.), restriction enzymes and other modifying enzymes from Takara Shuzo (Kyoto, Japan), Nippon Gene (Toyama, Japan), or Toyobo (Osaka, Japan), nitrocellulose filter from Toyo Roshi (Tokyo, Japan). COS-1 cells were kindly provided by Japanese Cancer Research Resources Bank (Tokyo, Japan).

Construction of cDNA Expression Vectors

A Rsal-Scal fragment (spanning bases +60–+1,476) of pRspt321 (a full length cDNA for rat preSPTm) (13) (Fig. 1) was introduced into HindII and SmaI sites of pAM19. The cloning sites were confirmed by sequencing with SP6 and T7 promoter primers and Sequenase (United States Biochemical, U.S.A.), and the in vitro transcription-translation product of the plasmid was confirmed to be that of 43 K. The insert was then cleaved off at Sphl and KpnI sites located 10 nucleotides to the 5'-direction from the Rsal site and 2 nucleotides to the 3'-direction from the Scal site, respectively. The Sphl-KpnI fragment was then blunted with T4 DNA polymerase and inserted into pSVL which had been cleaved with Xbal and blunted with Klenow fragment. The Rsal-Scal fragment of pRspt321 (spanning bases −36–+1,476) (Fig. 1) was directly introduced into pSVL at the blunt Xbal site.

Transfection of Plasmid DNAs to COS-1 Cells by Electroporation

COS-1 cells were grown to 70–80% confluence on 10-cm dishes in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum at 37°C. The cells were harvested with tryp-
sin and trypsin was inactivated with bovine serum. Cells were washed twice in ice-cold HEPES-buffered saline (140 mM NaCl, 5 mM KCl, 0.75 mM Na₂HPO₄, 25 mM HEPES, pH 7.4), and then resuspended in the same medium at a concentration of 2x10⁵ cells/ml. Plasmid DNAs were purified by subjecting once to CsCl gradient centrifugation before use. Twenty μg of purified plasmid DNA and 0.5 ml of cell suspension were placed in a 0.4-cm Gene Pulser cuvette (Bio-Rad Laboratories, U.S.A.). After 5 min at 0°C, the cells were pulsed once (25 μF, voltage setting at 1,000 V) at room temperature, and then returned to ice and incubated for 10 min. The transfected cells were diluted to 15 ml in DMEM supplemented with fetal calf serum, plated on 3 dishes (6-cm size) and allowed to grow at 37°C for 60 h with a medium change at 12 h.

Western Blot Analysis
COS-1 cells were washed three times with 5 ml each of cold Ca²⁺-, and Mg²⁺-free phosphate-buffered saline (PBS(-)), and incubated for 20 min on ice with 0.5 ml of lysis buffer consisting of 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml aprotinin. The lysate was scraped and transferred to a chilled microfuge tube and centrifuged at 12,000 g at 4°C for 10 min. The supernatant was stored at -70°C. The protein concentration of the supernatant was measured with BCA protein assay reagent (PIERCE, U.S.A.), and 30 μg of protein were subjected to SDS-10% polyacrylamide gel electrophoresis. After electrophoresis, the gel was soaked in 25 mM Tris/192 mM glycine/20% (v/v) methanol for 30 min, followed by electrophoretic transfer (2 mA/cm², 2 h) to a nitrocellulose membrane. The membrane was incubated at room temperature for 1 h in blocking buffer consisting of 5% (w/v) nonfat dried milk and 0.05% Tween 20 in PBS, and then washed three times for 15 min each with 0.05% Tween 20 in PBS. The membrane was placed in diluted rabbit anti-rat SPT serum (1:1,000) in blocking buffer at 4°C for 3 h, followed by washing three times for 15 min each with 0.05% Tween 20 in PBS. The membrane was incubated at room temperature for 30 min with horseradish peroxidase-coupled Fab' of goat anti-rabbit IgG (5 μg/ml) in blocking buffer. After washing three times for 15 min each with 0.05% Tween 20 in PBS, the membrane was placed in freshly prepared 3,3′-diaminobenzidine (DAB) substrate solution consisting of 0.5 mg/ml DAB, 0.02% (w/v) CoCl₂, 10 mM Tris-HCl (pH 7.6) and 0.03% H₂O₂.

Immunocytochemical Staining of SPT
COS-1 cells harvested 60 h after transfection were washed with PBS and fixed in a freshly prepared fixative consisting of 4% paraformaldehyde, 0.02% glutaraldehyde, and 0.15 M cacodylate buffer (pH 7.4) for 1 h at room temperature. Fixed cells were treated with 0.1 M lysine in 0.15 M cacodylate buffer (pH 7.4) for 15 min and then treated with 0.1% Triton X-100 in PBS at 4°C for 15 min. This was followed by overnight incubation with rabbit anti-rat SPT (50 μg/ml). Cells were then incubated with horseradish peroxidase-coupled Fab' of goat anti-rabbit IgG (5 μg/ml) for 30 min and fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4). Peroxidase activity was visualized with 0.05% (w/v) DAB/0.01% H₂O₂ in 0.1 M Tris-HCl.
(pH 7.2). Cells were post-osmicated and embedded in Epon. Semi-thin sections (1 μm thick) were cut, mounted on glass slide, and examined by a Nikon light microscope (Nikon, Tokyo). Ultra-thin sections were contrasted with uranyl acetate and lead citrate, and examined in a Hitachi electron microscope H600 at an accelerating voltage of 75 kV.

**Determination of Expression Rate**

After the transfection and immunocytochemical staining, 500 COS-1 cells were counted at 7 different areas. The percentage of DAB reaction-positive cells to the total number of counted cells was calculated.

**RESULTS**

**Expression of SPT in Cultured COS-1 Cells**

Upon Western blot analysis of COS-1 cells transfected with *Stul-Scal* cDNA fragment of pRsp321 (Fig. 1), a faint band of 45 K was observed in addition to a major 43 K band (Fig. 2, lane 2). The results are in accord with our previous observation with cultured rat primary hepatocytes that the newly synthesized preSPTm of 45 K is rapidly transported into mitochondria with processing to mature SPTm, resulting in a low steady state concentration of precursor in the cytosol (10). Thus the *Stul-Scal* fragment was shown to encode preSPTm. In contrast, when COS-1 cells were transfected with *Rsal-Scal* fragment of pRsp321 (Fig. 1) only the 43 K band was observed (Fig. 2, lane 3), indicating that the truncated cDNA fragment encodes directly the 43 K product, as expected. Determination of the expression rate showed that 16.2% and 16.8% of the total number of cells were expressing the SPT immunoreactivity when COS-1 cells were transfected with *Stul-Scal* fragment and *Rsal-Scal* fragment, respectively.

**Immunocytochemical Staining of SPT in Transfected COS-1 Cells**

Light microscopic observation showed that when COS-1 cells were transfected with *Stul-Scal* fragment encoding the 45 K-product, DAB reaction deposits were localized in the cytoplasmic granules which exhibited a rod-like profile (Fig. 3a). These positive granules were located in juxtanuclear region and extended to the cell periphery. The cell nuclei were devoid of reaction deposits. In contrast, when cells were transfected with the *Rsal-Scal* fragment encoding the 43 K-product, numerous fine discrete granules were stained (Fig. 3b). The stained granules were located throughout the cytoplasm. Frequently, the cytoplasm was diffusely stained, whereas the nuclei were negative. Untransfected COS-1 cells had no stained granules (Fig. 3c).

We also examined the immunoenzyme reaction by electron microscopy. In cells transfected with
the *StuI-Scal* fragment, most of stained granules were identified as mitochondria from morphological characteristics (Fig. 4a). In some cases, smaller granules were also positively stained, although the number was very small. When cells were transfected with the *Rsal-Scal* fragment, only the small granules were stained, and mitochondria were negative (Fig. 4b). These small granules exhibited various shapes as round, ovoid, and tubular. Since these shapes are characteristic of microperoxisomes (7), the stained small granules are inferred to be peroxisomes. Diffuse cytoplasmic staining was observed around these small granules. In untransfected COS-1 cells, neither mitochondria nor peroxisomes were stained (Fig. 4c). It is thus conclusively demonstrated that 43 K-product and 45 K-product formed from respective cDNA fragments are destined to be transported into peroxisomes and mitochondria, respectively.

**DISCUSSION**

In our previous studies, two kinds of mRNAs with different sizes were shown to be produced from a single rat SPT gene due to transcription from different initiation sites in exon 1 (8). When the first nucleotide, A, of the ATG triplet encoding the initiation Met of preSPTm is numbered 1, a larger mRNA (1,900 nt) was shown to be transcribed from nucleotide position −47 or −48, and in this mRNA, the first Met codon encountered was that at +1 to +3, indicating that the 1,900 nt-mRNA directs preSPTm, which consists of the mitochondrial targeting N-terminal extension peptide of 22 amino acids and mature SPTm of 392 amino acids. The N-terminal peptide was cleaved off, when preSPTm was transported into mitochondria, at a site between Asn-22 and Met-23, generating SPTm whose N-terminal amino acid is Met (Fig. 1). On the other hand, a smaller mRNA (1,700 nt) was transcribed from a position around +19 to +22, and in this mRNA the first Met codon encountered (+67 to +69) was that corresponding to N-terminal Met of mature SPTm (8, 10, 14). Thus, the translation product from the smaller mRNA should have same amino acid sequence as that of mature SPTm. Therefore, the hormone-insensitive 43 K-translation product observed in our previous studies has been assumed to be formed from the smaller mRNA and directed into peroxisomes by intramolecular peroxisomal targeting signal(s) (8). In the translation of transcripts from *StuI-Scal* and *Rsal-Scal* fragments used in the present study, the first Met codon encountered should be that at +1.
to +3 and +67 to +69, respectively (Fig. 1). If the above assumption is correct, the expression products of \textit{Sul1-Scal} and \textit{Ras1-Scal} fragments should have been translocated into mitochondria and peroxisomes, respectively, in the transfected COS-1 cells. In this study, SPT immunoreactivity was indeed localized only in small cytoplasmic granules when COS-1 cells were transfected with the \textit{Ras1-Scal} fragment (Fig. 2b). In addition, immunoelectron microscopic observation showed that mitochondria were negative for SPT, whereas many small granules were heavily stained (Fig. 4b). Based on morphological criteria, these granules were identical as the so-called microperoxisomes which have been reported in many cells and tissues (7). It was thus demonstrated that 43-K product synthesized in COS-1 cells by transfection of corresponding cDNA is imported into peroxisomes and not into mitochondria. On the other hand, when COS-1 cells were transfected with \textit{Sul1-Scal} fragment, SPT immunoreactivity was localized in two types of cytoplasmic granules, as revealed by immunoelectron microscopy (Fig. 4a). Positive granules were mostly mitochondria, but the immunoreactivity was also detected in a small number of smaller granules which seemed to belong to microperoxisomes. In an \textit{in vitro} transcription-translation of pRsp321 (a full-length cDNA encoding the 45 K product), preSPTm of 45 K was the predominant product, but a small amount of 43 K product was also formed, and this product appeared to derive from translation from the third AUG codon corresponding to N-terminal Met of mature SPT (unpublished data). The sequence around the second AUG codon does not satisfy the minimum requirement for the efficient translation initiation proposed by Kozak (2). Therefore, the results of the transfaction of COS-1 cells with \textit{Sul1-Scal} fragment of pRsp321 were interpreted to mean that predominantly formed preSPTm of 45 K was exclusively transported into mitochondria, but the 43 K product was also formed artificially in small amounts and was transported into peroxisomes. Since no SPT immunoreactivity was observed in untransfected COS-1 cells, it was evident that the immunoreactivity of the transfected cells was due to SPT newly synthesized and transported into the organelles.

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