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Tissue Distribution of Placenta-Type 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase

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Several isozymes of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase have been characterized from mammalian tissues and, based on tissue origin, they are classified as liver, skeletal muscle, heart, testis, and placenta isozymes. In this paper, we examined the tissue distribution of placenta-type isozyme in rat tissues at the levels of transcription and translation. Analysis by Northern blotting showed that placenta, brain, testis, liver, kidney, and skeletal muscle expressed mRNA of placenta-type isozyme. Western blot analysis of fractions from POROS-HQ column chromatography of extracts from various rat tissues showed that proteins of placenta-type isozyme are expressed in placenta, brain, testis, liver, spleen, heart and lung, but not in kidney and skeletal muscle. An immunohistochemical study showed that, in liver, placenta-type isozyme is localized in Kupffer cells. These results indicate that isozymes of this particular enzyme may occur in particular cell types within each tissue.

The bifunctional enzyme 6-phosphofructo-2-kinase [EC 2.7.1.105]/fructose-2,6-bisphosphatase [EC 3.1.3.46] (PFK-2/F2,6BPase) catalyzes both the synthesis and degradation of fructose 2,6-bisphosphate (F2,6BP), which is the most powerful activator of phosphofructokinase (1), a key regulatory enzyme of glycolysis. PFK-2/F2,6BPase is a homodimeric protein that has two distinct catalytic domains on each subunit, one for kinase activity and the other for phosphatase activity. In mammals, several isozymes of PFK-2/F2,6BPase have been identified. Based on the differences in their tissue distribution, molecular mass, immunogenecity, their response to phosphorylation by protein kinases and relative PFK-2 and F2,6BPase activities and, the identified tissue origin, they are classified as liver, skeletal muscle, heart and testis isozymes. Three genes encode these isozymes: Gene A encodes the liver and skeletal muscle isozymes (2), gene B encodes the heart isozyme (3) and an unidentified gene encodes the testis isozyme. In addition to these isozymes, we cloned a new cDNA of an isozyme from human placenta (4), named placenta-type PFK-2/F2,6BPase, expressed and characterized this enzyme (5, 6). The study of the placenta-type PFK-2/F2,6BPase is significant because: 1) the PFK-2 activity is relatively high compared to other isozymes, but the F2,6BPase activity is extremely low, and the ratio of the activity of PFK-2 *versus* F2,6BPase is the highest among the known isozymes; thus, 2) placenta-type PFK-2/F2,6BPase favors the maintenance of a high F2,6BP level, and as a result, glycolytic rate in cells containing placenta-type PFK-2/F2,6BPase may be kept at a high level; and 3) both cAMP-dependent protein kinase (protein kinase A) and protein kinase C phosphorylate HP2K with activation of PFK-2, similar to bovine heart isozyme (7). Furthermore, the gene encoding the placenta-type PFK-2/F2,6BPase was found to be different from the above three genes, which are localized in chromosome 10p14-p15 (8). Following our reports, Watanabe et al. reported that there are multiple forms of PFK-2/F2,6BPase mRNAs in rat brain that are generated by alternative splicing (9).

Nucleotide and amino acid sequence of one of these mRNAs (RB2K6) is 88 and 97% identical, respectively, to that of placenta-type isozyme, indicating that mRNA similar to that of placenta-type isozyme exists in rat brain. Further, these results indicate that placenta-type isozyme is present not only in placenta but also in other tissues. These observations led us to look for

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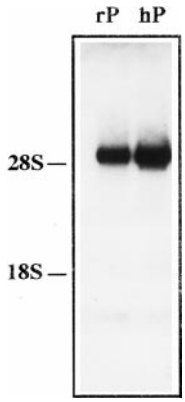

FIG. 1. Northern blot analysis of RNAs from human and rat placentas. Poly(A)$^+$ RNAs (2 μg) prepared from human (hP) and rat (rP) placentas were fractionated on a 1.5% formaldehyde-agarose gel and hybridized with a ^{32}P-labeled HP2K cDNA-specific probe as described in METHODS.

tissues expressing placenta-type isozyme. In this study, first we ascertained the expression of mRNA and protein of placenta-type isozyme in rat placenta. Next we attempted to find tissues expressing the placenta-type isozyme at the mRNA and protein levels. Histochemical study of the protein of placenta-type isozyme in rat liver, one of the tissues expressing the placenta-type isozyme determined in this experiment, was investigated.

MATERIALS AND METHODS

Animals. Male (180-220 g) and pregnant Wistar rats (18 days post coitus) were obtained from Ohtsubo Animals Co. (Nagasaki). The animals were killed by decapitation, and the tissues were removed and quickly frozen in liquid N_2 and stored at −80°C until needed. Clodronate treated rats were prepared by intraperitoneal injection of clodronate-encapsulated liposomes as described in (10).

Enzymes. Recombinant human placenta-type (HP2K) and rat testis-type (RT2K) PFK-2/F2,6BPases were purified as described previously (5, 11). Pure rat liver-type (RL2K) and bovine heart-type (BH2K) PFK-2/F2,6BPases were generous gifts from Dr. Kosaku Uyeda.

Antiserum against placenta-type and rat testis-type isozymes. Antibodies against human placenta-type PFK-2/F2,6BPase (anti-HP2K antiserum) and rat testis-type PFK-2/F2,6BPase (anti-RT antiserum) were raised in white rabbits by subcutaneous injections of 0.1 mg (0.25 ml) of pure enzyme with an equal volume of Freund's complete adjuvant for the first immunization and incomplete adjuvant for subsequent immunizations. Each animal was injected five times in 100 sites at intervals of two weeks. The animals were bled from the carotid artery seven days after the final immunization, and the IgG fraction (anti-HP2K IgG and anti-RT2K IgG) was prepared by Protein A Sepharose column, respectively, and stored at −80°C until needed.

Northern blot analysis. For Northern blot analysis, poly(A)$^+$ RNAs were prepared from total RNAs using oligotex-dT 30 (Takara). Poly(A)$^+$ RNAs (2 μg) were fractionated on a 1.5% formaldehyde-agarose gel, transferred to BIODYNE B nylon membrane filter (Pall), and fixed with Spectrolinker (Funakoshi). The filter was hybridized with the ^{32}P-labeled probe in 5×SSC containing 50% formamide, 5×Denhardt's solution, 0.05 M sodium phosphate, 0.2% sodium dodecyl sulfate (SDS), and 0.1 mg/ml denatured salmon sperm DNA at 37°C for 36 hours and washed in 2×SSC containing 0.1% SDS at 48°C. A ^{32}P-labeled (12) 587-bp fragment corresponding to nucleotides 178-764 of human placental PFK-2/F2,6BPase cDNA (4) produced by PCR, was used as the probe. The filter was then exposed to Kodak XAR film for 36 hours at room temperature.

Western blot analysis. Frozen tissues (1.5 g) were homogenized in ice-cold buffer A (50 mM Tris-phosphate buffer, pH 8.0, containing 10 mM dithiothreitol, 1 mM EDTA, 1 mM EGTA, 0.02% phenylmethanesulfonyl fluoride, 2 mM benzamidine and 10 U/ml aprotinin) (1:2 w/v) using a Ultra Turrax homogenizer (IKA-WERK). The homogenates were centrifuged at 20,000 × g for 30 minutes at 4°C. Polyethylene glycol 6000 was added to the resultant supernatant solutions to bring it to a final concentration of 3% and after 30 min the precipitate was removed by centrifugation. Sufficient polyethylene glycol was then added to the supernatant solution to bring it to a final concentration of 20%. After 30 min, the precipitate was collected by centrifugation and dissolved in an adequate volume of buffer A and used as samples (PEG fractions) for Western blot analysis. For Western blot analysis (13), PEG-fractions were subjected to POROS-HQ column (Perseptive Biosystems) chromatography. Proteins were eluted with a linear gradient of NaCl (0 to 0.5 M) using buffers of 50 mM Tris-PO_4 (pH 8.0), 2 mM DTT and 50 mM Tris-PO_4 (pH 8.0), 2 mM DTT, 1 M NaCl at a flow rate of 2.5 ml/min and 0.5 ml fractions were collected. An aliquot (20 μl) of each fraction was subjected to a 12% SDS-PAGE, performed according to the method of Laemmli (14) under reducing condition. After the proteins in the gel were transferred onto a nitrocellulose filter (BioTrace NT, Gelman Sci.), the filter was incubated with anti-HP2K IgG (5.4 μg IgG/ml), followed by detection with an ECL Western blot detection system (Amersham). Protein determinations were carried out by the method of Bradford (15) using a Protein Assay kit from Bio-Rad with bovine serum albumin as the standard.

Immunohistochemical study. Normal and clodronate-treated male Wistar rats (200 g) were anesthetized with pentobarbital, perfused transcardially with 100 ml of 10 mM phosphate-buffered saline, pH 7.4, (PBS) containing 0.1% heparine-Na and 0.1% $Na_2S_2O_5$ for 1 min. Subsequently, they were fixed by perfusion with 300 ml of 4% paraformaldehyde and 0.1% $Na_2S_2O_5$ in 0.1 M phosphate buffer (pH 7.4) for 5 min at room temperature. The livers were quickly excised, post-fixed overnight at 4°C in the same fixative, soaked in 30% sucrose in PBS and frozen in O.C.T. compound (Miles) for cryostat sectioning. The sections (4-μm) were treated with TBS (50

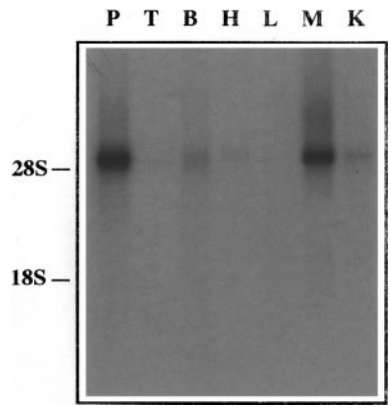

FIG. 2. Northern blot analysis of RNAs from rat tissues. Poly(A)$^+$ RNAs (2 μg) prepared from rat placenta (P), testis (T), brain (B), Heart (H), liver (L), skeletal muscle (M), and kidney (K) were fractionated and hybridized in the same manner as described in the legend to Fig. 1.

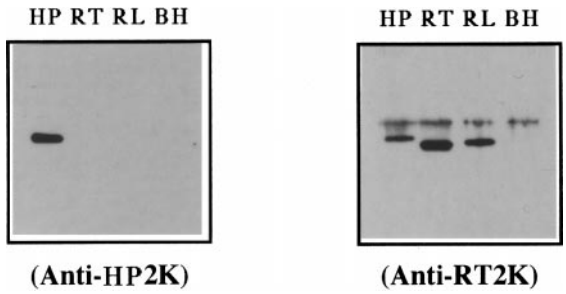

FIG. 3. Specificity of anti-HP2K antibody. Each purified protein of each isozyme (40 ng) of human placenta (HP), rat testis (RT), rat liver (RL), and bovine heart (BH) was subjected to SDS-PAGE and followed by Western blot analysis using anti-HP2K IgG (Anti-HP2K) and anti-RT2K IgG (Anti-RT2K) as primary antibodies.

mM Tris-HCl, pH 7.4, and 0.15 M NaCl) containing 0.25% bovine serum albumin, 10% normal goat serum and 0.1% saponin for 1 h at room temperature, and then incubated at 4°C for overnight with primary anti-HP2K IgG (14 μg IgG/ml) diluted in TBS containing 0.25% bovine serum albumin, 1% normal goat serum and 0.1% saponin. The sections were then incubated for 1 h at room temperature with a goat anti-rabbit IgG/Fab′ labeled with horse radish peroxidase. After rinsing with TBS, the site of the antigen-antibody reaction was seen for 10 min with diaminobenzidine and H_2O_2 (16).

RESULTS

Detection of placenta-type mRNA of PFK-2/F2,6BPase in rat placenta and other tissues. In human placenta, a 5.4 kb mRNA encodes the placenta-type PFK-2/F2,6BPase (HP2K) (4). To see if a similar placenta-type mRNA is expressed in rat placenta, Northern blot analysis was performed using ^{32}P-labeled 587-bp fragment of HP2K cDNA as a probe. As shown in Fig. 1, a single band of almost the same sized mRNA as HP2K-mRNA was detected in rat placenta, indicating that placenta-type PFK-2/F2,6BPase isozymes expressed in the human and rat are encoded by similar mRNA. The nucleotide sequences of several cDNA fragments corresponding to coding regions of HP2K amplified by RT-PCR from rat placenta total RNA as a template were highly homologous (>95% identical) to those of HP2K cDNA (data not shown).

The expression of placenta-type mRNA in various tissues of rat was determined by Northern blot analysis using the same 587-bp cDNA fragment as the probe. As shown in Fig. 2, all tissues examined contained placenta-type mRNA.

Specificity of antibody. Antibodies were raised against the placenta-type PFK-2/F2,6BPase (HP2K). The specificity of anti-HP2K antibody was examined by Western blot analysis. As shown in Fig. 3, this antibody reacts with HP2K but not with testis-type (rat), liver-type (rat), and heart-type (bovine) isozymes, indicating that the anti-HP2K antibody is specific for detecting protein of HP2K-type (placenta-type) isozyme under similar conditions. On the other hand, antibody raised against rat testis isozyme showed broad specificity.

Detection of PFK-2/F2,6BPase placenta-type isozyme in rat placenta and other tissues. Figure 4 shows Western blot analysis of fractions from POROS-HQ column chromatography of rat placental extracts. A single band of 58,000 daltons, which is also the molecular weight of the HP2K subunit (4), was detected in fractions numbered 37 to 42, indicating that the protein of HP2K-type (placenta-type) isozyme is expressed in rat placenta.

The expression of protein of placenta-type isozyme in various tissues of rat was determined by POROS-HQ column chromatography followed by Western blot analysis (Fig. 5). A single band of 58,000 daltons was detected in fractions from brain, heart, testis, liver, spleen and lung, but not from kidney and skeletal muscle.

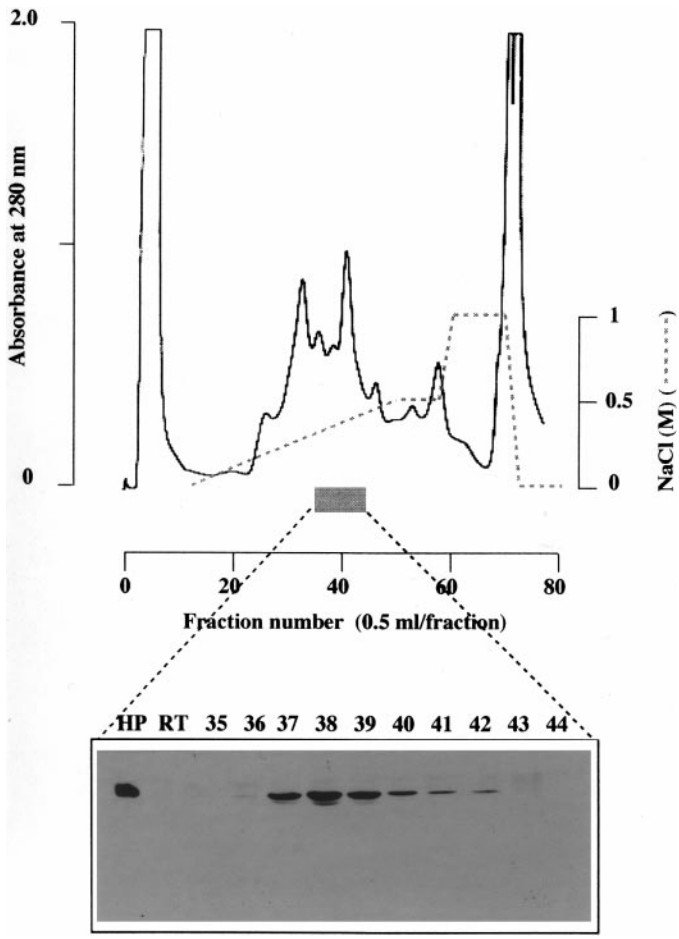

FIG. 4. Western blot analysis of rat placental cell extracts after POROS-HQ column chromatography. Proteins in rat placental cell extracts were separated by POROS-HQ column chromatography, and an aliquot of each fraction was analyzed by Western blotting as described in METHODS. HP and RT indicate 40 ng of marker HP2K and RT2K, respectively.

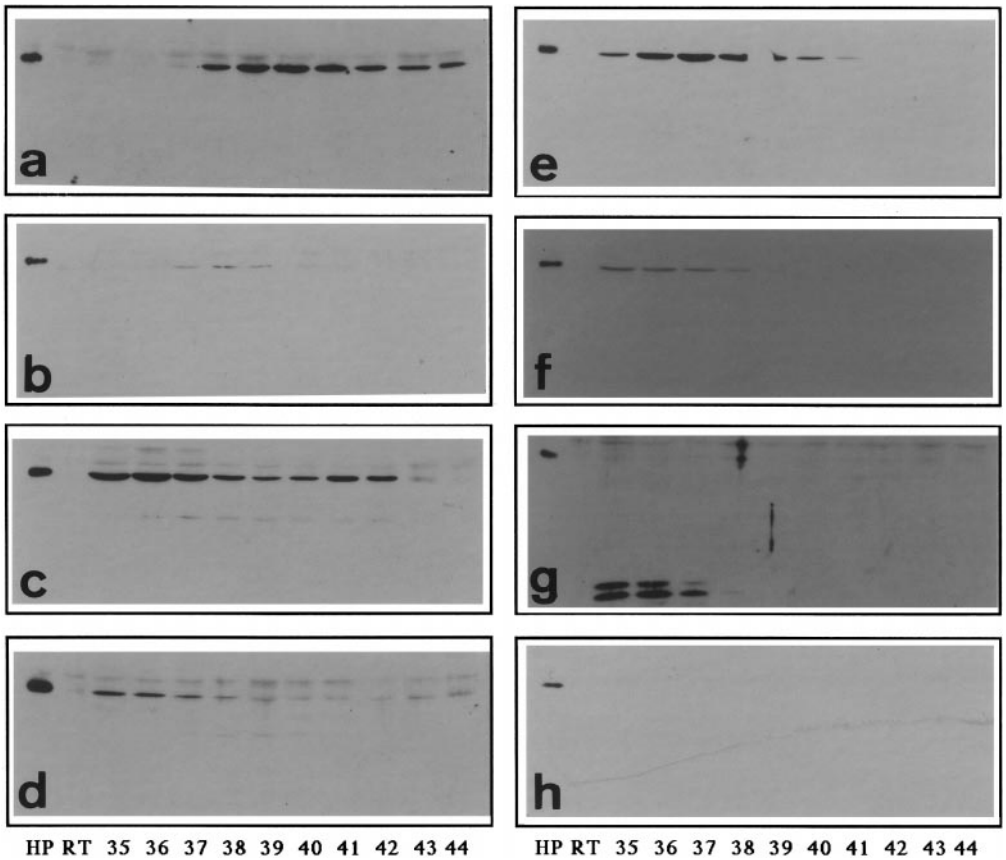

FIG. 5. Western blot analysis of cell extracts from rat tissues after POROS-HQ column chromatography. Proteins in cell extracts from brain (a), heart (b), testis (c), liver (d), spleen (e), lung (f), kidney (g), and skeletal muscle (h) were separated by POROS-HQ column chromatography and analyzed by Western blotting in the same manner as described in the legend of Fig. 4. Numbers indicate fraction numbers of POROS-HQ column chromatography.

Immunohistochemistry. Immunohistochemical study of rat liver, one of the tissues expressing protein of the HP2K-type isozyme as detected above, was performed. As shown in Fig. 6, only certain cells (Kupffer cells) but not parenchymal cells, were stained with anti-HP2K antibody. Absorption of this antibody by excess HP2K completely abolished the immunostaining. Furthermore, no immunostained cells were detected in livers from rats treated with clodronate (dichloromethylene diphosphonate), a macrophage and Kupffer cell eliminating agent (10). These results indicate that Kupffer cells express the placenta-type isozyme.

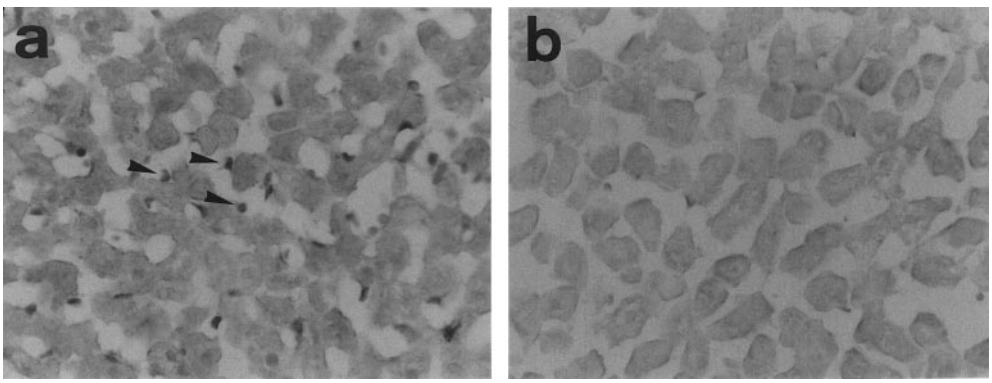

FIG. 6. Immunostaining of rat liver with anti-HP2K antibody. Livers from naive (a) and clodronate-treated (b) rats were processed for immunostaining as described in METHODS. Arrow heads indicate immunostained Kupffer cells. × 200.

DISCUSSION

The current study clearly indicates that both human and rat placentas express a common PFK-2/F2,6BPase mRNA and its protein, which is called to be placenta-type isozyme. Evidences of the expression of placenta-type isozyme at the mRNA and protein levels in various tissues (brain, heart, testis, liver, spleen and lung) besides placenta were also obtained. Two apparent isoforms of placenta-type isozyme were found on the basis of the position of elution on POROS-HQ column chromatography, one eluted in fractions numbered 35 to 39, and the other in fractions numbered 37 to 42 (Fig. 5). The differences in the position of elution may be caused by phosphorylation or as yet unidentified modification of the protein of placenta-type isozyme in various tissues. In the kidney, proteins of much smaller molecular weights than that of the protein subunit of placenta-type isozyme were detected by Western blot analysis, indicating that a very fast rate of degradation of proteins of this isozyme may be occurring in the kidney. Oddly, in the skeletal muscle, although the level of expressing of placenta-type isozyme mRNA appears is seems to be high enough to express its protein, no immunoreactive bands with molecular mass with 58,000 daltons was observed. These results may indicate that the protein of the placenta-type isozyme in skeletal muscle is unstable and susceptible to proteases or the mRNA of the placenta-type isozyme in the skeletal muscle is untranslatable because the mRNA is unstable or not functional by translatable, or the mRNA is not transported to the proper location in the cells to be translated. 3′ untranslated region of mRNA is known to provide translocation signals (17) and to be responsible for stabilization of mRNA and the regulation of translation. Thus, comparative studies of the nucleotide sequence of 3′ untranslated regions in placenta-type mRNAs from various tissues may provide some insight into the mechanisms of the translocation and regulation of translation of mRNA.

Thus far several isozymes of PFK-2/F2,6BPase are classified as liver, skeletal muscle, heart, testis and placenta isozymes based on the identified tissue origin. In particular, protein of liver-type isozyme was purified as the major isozyme form of PFK-2/F2,6BPase from rat liver (18), and this isozyme is believe to be present in parenchymal cells. Thus, it could to be presumed that other isozymes present in the liver are provably present in nonparenchymal cells. Immunohistochemical studies performed as part of this study proved that placenta-type isozyme is expressed in the Kupffer cells of the liver. This is the first report of immunochemical study on PFK-2/F2,6BPase isozyme. The presence of placenta-type isozyme in Kupffer cells suggests a high rate of glycolysis (glucose utilization) in these cells. Meszaros et al. reported that a several-fold enhanced rate of glucose utilization by the livers of fasting rats is due predominantly to nonparenchymal cells and endotoxin enhance the rate of glucose utilization by Kupffer cells (19). Enhanced glucose metabolism of Kupffer cells may be part of the hepatic immune response. It may be worthwhile to determine the relationship between functions of Kupffer cells and regulation of placenta-type isozyme activity consequently the regulation of glycolysis in these cells.

In conclusion, data obtained in this study suggest that PFK-2/F2,6BPase isozymes may occur in particular cell types within each tissue and characterization of the isozyme at the cellular level may provide useful information about the relationship between the cell function and the regulation of glycolysis of the cell.

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