Hydrolysis of Synthetic Substrate, L-Pyroglutamyl p-Nitroanilide is Catalyzed Solely by Pyroglutamyl Aminopeptidase I in Rat Liver Cytosol

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Pyroglutamyl aminopeptidase I (PAP-I) is a cytosolic cysteine peptidase, which hydrolytically removes the L-pyroglutamate residue from the amino terminus of endogenous proteins and peptides. L-Pyroglutamyl p-nitroanilide serves as the synthetic substrate of this enzyme, while there is a possibility of other hydrodases being involved in the hydrolysis of this xenobiotic substrate. We cloned a full-length cDNA encoding rat PAP-I from a rat liver cDNA library and expressed this cDNA in Escherichia coli to obtain a recombinant PAP-I as a single protein. The cDNA encoded a sequence of 209 amino acids with a calculated molecular weight of 22913 Da. The homology of the deduced amino acid sequence of rat PAP-I was 98.6 and 94.3% to mouse and human PAP-I, respectively. The biochemical properties of the recombinant rat PAP-I were almost identical to those of the recombinant mouse and human PAP-I and the purified rat liver cytosolic PAP-I in terms of the molecular weight, subunit structure, affinity to the substrate, inhibitor profile and pH optimum. Immunoblot analysis using an antibody raised against recombinant rat PAP-I showed that rat PAP-I is present almost exclusively in the cytosolic fraction of the rat liver. Moreover, the hydrolyzing activity for l-pyroglutamyl p-nitroanilide in rat liver cytosolic fraction was completely inhibited by the antibody, strongly suggesting that this xenobiotic substrate is hydrolyzed solely by PAP-I.

Key words cysteine peptidase; cytosolic enzyme; pyroglutamyl aminopeptidase I; rat liver

Pyroglutamyl aminopeptidase I (PAP-I, EC 3.4.19.3) is a typical cytosolic cysteine peptidase, displaying ubiquitous tissue distribution, and hydrolytically removes the L-pyroglutamate (L-pGlu) residue from the amino terminus of L-pGlu-proteins and peptides such as thyrotropin-releasing hormone (TRH), luliberin (LH-RH) and neurotensin.1–3 Cyclation of the N-terminal l-glutamic acid, namely the formation of L-pGlu, allows the peptides to have a longer biological half-life than other peptides of similar size, since the majority of peptides are not capable of hydrolyzing the L-pGlu-X bond. The physiological role of PAP-I currently remains unclear, but seems to be widely involved in the metabolism of L-pGlu-containing bioactive peptides including TRH and LH-RH.4,5

Gene encoding human PAP-I has been recently cloned and expressed in Spodoptera frugiperda cells for characterization by Dando et al.6 They demonstrated that the recombinant human PAP-I was enzymatically active and had properties similar to those of the naturally occurring mammalian enzymes reported previously.7–9 However, the enzyme in rats, the species most frequently used in drug developmental experiments, has not been completely investigated yet. L-pGlu p-nitroanilide (L-pGlu-pNA) has been used as the synthetic substrate of PAP-I. Recently we found that PAP-I is involved in the hydrolysis of other xenobiotic compounds having L-pGlu or L-pGlu-related structure, now under development in our company. Due to a xenobiotic nature of these compounds including L-pGlu-pNA, however, there was a possibility that other unknown hydrolases also catalyze the hydrolysis of these substrates. To exclude this possibility, it is necessary to conduct the inhibition experiment using an antibody against pure PAP-I.

In the present study, we cloned the rat PAP-I gene from a rat liver cDNA library, and expressed and obtained the functional form of rat PAP-I in Escherichia coli as a single protein at the first time. Using this preparation, we compared the biochemical properties with those of recombinant mouse and human PAP-I and the purified rat liver cytosolic PAP-I. An antibody was raised in rabbits against the purified recombinant rat PAP-I and the contribution of PAP-I to the hydrolysis of L-pGlu-pNA in rat liver was examined.

MATERIALS AND METHODS

Chemicals and Materials Bestatin, TRH, phenylmethyisulfonfonylfluoride (PMSF), N-ethylmaleimide (NEM), l-trans-epoxysuccinyl-leucylamido-(4-guanidinobutane) (E-64), p-nitroanilide (pNA), p-chloromercuribenzoate (PCMB), 1,10-phenanthroline, phenacetin, bovine serum albumin, rabbit IgG, L-pGlu-pNA, L-Leu-pNA, L-Ala-pNA and L-Pro-pNA were obtained from Sigma Chemical Co. Disopropyl fluorophosphate (DFP) was from Wako Pure Chemical Industries, Ltd. Human liver microsomes and cytosols were purchased from the International Institute for the Advancement of Medicine (IIAM). All other reagents and solvents used were of commercially available, guaranteed grade.

cDNA Cloning The total RNA from rat liver was prepared using ISOGEN (Nippon Gene Co., Ltd.). To obtain the cDNA sequence encoding rat PAP-I by a RT-PCR method, a sense primer, PAPR1 (5′-ATGGACGACGCCGGCAGAGGC-3′), and an antisense primer, PAPR2 (5′-CACGCAGCCT-GAGGCGGCGG-3′), were designed according to the conserved region of the cDNA sequence of mouse PAP-I (AJ278829) and human PAP-I (AJ278828). RT-PCR was performed with the template of the total RNA using ProSTAR™ First Strand RT-PCR Kit (Stratagene). The PCR cycle consisted of 0.5 min denaturation at 94 °C, 0.5 min annealing at 54 °C, and 1 min extension at 72 °C for 40 cycles. The PCR
product was radiolabeled with [α-32P]dCTP using Rediprime™ II (Amersham Biosciences). A rat liver AZAPII cDNA library (Stratagene) was screened with the plaque hybridization method. The nucleotide sequence of rat PAP-I cDNA has been deposited in the DDBJ/EMBL/GenBank™ databases under Accession No. AB098134.

**Construction of Bacterial Expression Vector and Expression of PAP-I in E. coli** The total RNA from mouse liver was prepared as described above for rat liver. Human liver Poly A+ RNA was purchased from Clontech Laboratories Inc. The full-length clones were amplified by RT-PCR as described above using the primers PAPR3 (5'-GGATCCATGAGCAGCCCGGAAAGC-3') and PAPR4 (5'-TCAGAGCTTGGCAGCAG-3') for rat PAP-I, PAPM1 (5'-GGATCCATGAGCAGCCCGGAAAGC-3') and PAPM2 (5'-TCAGAGCTTGGCAGCAG-3') for mouse PAP-I, and PAPH1 (5'-GGATCCATGAGCAGCCCGGAAAGC-3') and PAPH2 (5'-TCAGAGCTTGGCAGCAG-3') for human PAP-I. The PCR products were subcloned into a pGEM®-T Easy Vector (Promega Corporation). The full-length clones were excised following BamHI/EcoRI digestion and subcloned into pGEX-6P-3 (Amersham Biosciences).

By infecting E. coli strain JM109 with each vector, rat, mouse and human PAP-Is were expressed and obtained each as a single protein using GST gene fusion systems (Amersham Biosciences) according to the manufacturer's instructions. The proteins obtained (recombinant PAP-I) were pure as judged by SDS-PAGE. The enzymes obtained were dissolved in phosphate buffered saline containing 2 mM DTT as judged by SDS-P AGE. The enzymes obtained were distilled. The proteins obtained (recombinant PAP-I) were pure as a single protein using GST gene fusion systems (Amersham Biosciences). which had been equilibrated with CHT2-I, 2 ml, Bio-Rad), which had been equilibrated with volumes of 10 mM potassium phosphate buffer, pH 7.4, containing 2 mM DTT and 10% glycerol (hereafter referred to as buffer A). This fraction was subjected to further purification by the 2nd CHT2-I column chromatography as above. The enzyme fractions were combined, concentrated and stored frozen at −80°C until used.

**Measurement of PAP-I Activity** Potassium phosphate buffer (10 mM, pH 7.4) containing 1 mM dithiothreitol (DTT) and an appropriate amount of each enzyme source (45 μl) was preincubated at 37°C for 2 min, and the reaction was started by the addition of 5 μl of 200 μM L-pGlu-pNA (final concentration: 20 μM). After incubation of the mixture for 5—20 min at 37°C, the reaction was stopped by the addition of acetonitrile containing 0.25 μM of phenacetin as the internal standard for determination of pNA by LC tandem mass spectrometry (LC/MS/MS). The mixture was centrifuged at 20000 g for 2 min at 4°C and the supernatant was stored at 4°C until analysis. In a separate experiment it was confirmed that the pNA produced was stable during the testing period.

The optimum pH for the enzyme was determined at the substrate concentration of 20 μM over a pH range of 5—9 using 100 mM potassium phosphate buffer for pH 5—7.4 and 100 mM Tris-Cl for pH 7.4—9.

A SCIEX API-III Plus (Perkin Elmer Japan Co., Ltd.) connected to an LC-10A system (Shimadzu Corp.) was used as the LC/MS/MS system for measurement of pNA. The column used was a CAPCELL PAK C18 UG120Å (2.0 mm I.D.×150 mm, Shiseido Co., Ltd., Tokyo, Japan). A mixture of acetonitrile and water (75:25, v/v) containing 1 mM ammonium acetate was used as the mobile phase. Protonated molecular ion, [M+H]⁺, (pNA: m/z=139, phenacetin: m/z=180.1) was selected as the precursor ion, and the product ion (pNA: m/z=122, phenacetin: m/z=110) was monitored. The detection limit of pNA was 0.2 μM.

**Additional Peptidase Assay** The rate of enzymatic hydrolysis of L-Leu-pNA, L-Ala-pNA and L-Pro-pNA (final concentrations: 1 mM in 50 mM potassium phosphate buffer, pH 7), known substrates of many peptidases, was assayed at room temperature after addition of the enzyme solution by colorimetric determination of pNA at 410 nm (ε=8800).

**Protein Determination** Protein concentrations were determined according to the dye-complex method reported by Bradford(11) or according to Lowry's method(2) using DC Protein Assay Kit (Bio-Rad). Bovine serum albumin was used as a standard.

**Sodium Dodecylsulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)** SDS-PAGE was performed on 12.5% or 10—20% gel according to the method of Laemmli. (13) Protein bands were stained with SYPRO® Ruby.
protein gel stain (Molecular Probes Inc.).

**Protein Identification** The protein bands were excised from the gel stained with SYPRO® Ruby. In-gel digestion of the protein using sequencing grade modified trypsin (Promega) was performed essentially according to the method by Shevchenko et al. The resultant proteolytic peptides were analyzed by nano-LC/MS/MS to collect the peptide sequence information. A Q-TOF 2 hybrid mass spectrometer (Micromass) equipped with a nano-electrospray ion source (nano-ESI) was used as the nano-LC/MS/MS system.

**Subcellular Fractionation of Rat and Mouse Tissue Homogenates** Male F344/DuCrj rats and male C57BL/6J mice were sacrificed and the excised tissues were homogenized in 10 mM potassium phosphate buffer (pH 7.4) containing 1.15% potassium chloride. Subcellular fractionation of the homogenates was performed according to the method of Tanaka et al.

**Immunoblot Analysis** The polyclonal antibody against the rat PAP-I recombinant protein purified from E. coli was raised in rabbits according to Nakamura et al. The antisera was further purified by passing through a Protein A column (NGK Insulators, Ltd.), and the IgG fraction was obtained. SDS-PAGE of denatured samples (7 μg protein) was performed on 10—20% gels. Immunoelectrophoretic (Western) blot analysis was carried out with polyvinylidene fluoride (PVDF) membranes (Immobilon, Millipore), and the protein was detected by the enhanced chemiluminescence detection method (Phototope-HRP Western Blot Detection Kit, Cell Signaling Technology, Inc.).

**RESULTS**

**cDNA Cloning and Expression of Rat, Mouse and Human PAP-Is** We cloned cDNA encoding rat PAP-I by screening the rat liver cDNA library using a probe which was prepared by RT-PCR. The rat PAP-I cDNA was 939 bp in length with 627 bp comprising the open reading frame. The predicted translation product of the rat PAP-I cDNA encodes a protein of 209 amino acids with a calculated molecular weight of 22913 Da (Fig. 1).

The amino acid sequence was highly conserved among rat, mouse and human PAP-Is. The homology of the deduced amino acid sequence of rat PAP-I was 98.6% to mouse PAP-I and 94.3% to human PAP-I (Fig. 1). The amino acid sequence between rat and human PAP-Is was mainly different in a part of the C-terminal amino acid residues.

Mouse and human PAP-I cDNAs were prepared by PCR cloning based on the respective cDNA information, and these cDNAs as well as rat PAP-I cDNA were each inserted into a bacterial expression vector to express the enzyme proteins in E. coli. The yields were 7—9 mg PAP-I culture. The molecular weights of the rat, mouse and human PAP-I expressed in E. coli were calculated to be approximately 25 kDa by both SDS-PAGE and gel filtration chromatography, showing that the rat, mouse and human PAP-Is are all monomeric enzymes.

**Purification of Rat Liver PAP-I** Using anion-exchange, gel-filtration, 1st hydroxylapatite and 2nd hydroxylapatite column chromatographies, PAP-I was purified 1762-fold from rat liver homogenate with an overall yield of 1% (Table 1). This purified fraction did not show any hydrolyzing activities toward L-Leu-pNA, L-Ala-pNA or L-Pro-pNA, general substrates for many other known peptidases, indicating that the enzyme fraction was free from other peptidases.

**SYPRO® Ruby gel staining of the purified enzyme after SDS-PAGE showed two protein bands. Both the elution profile of the activity and the density of the bands on SDS-
PAGE in the last chromatography indicated that the upper band is PAP-I. The molecular weight of the purified PAP-I was 23.4 kDa by gel filtration chromatography and was 25 kDa by SDS-PAGE, indicating that rat liver PAP-I exists as a monomer. This molecular weight is comparable to recombinant PAP-Is and the values previously reported for the enzymes purified from various mammalian tissues.17–20

Amino Acid Sequence Analysis of Peptide Fragments for Identification of Rat Liver PAP-I

In-gel digestion of the two protein bands in the purified PAP-I preparation was performed, and the peptide fragments were analyzed by nano-LC/ESI-MS/MS. The peptide sequences obtained from the upper band (LGGLDSVLDHVYEIPVEYQTVQR, LI-PALWEK and SAFVHVPPPLGKPYNADQLGR) were identified to the sequence of the rat PAP-I, demonstrating that the upper protein band was PAP-I.

The lower protein band whose molecular weight was estimated to be 24 kDa by SDS-PAGE was identified to be a protein found in the EST database at NCBI (H32616) with unknown function. There was no amino acid sequence homology between rat PAP-I and the H32616 protein but difficulty in separating PAP-I from this protein in column chromatography may be due to a strong affinity of the protein to PAP-I leading to a possible complex formation. However, investigating the fractions containing two protein bands in each chromatography in detail, there was a tendency of separation of PAP-I from this protein, suggesting that the two proteins merely possess a very similar affinity to each column and the complex formation is unlikely (data not shown).

Characterization of Purified and Recombinant PAP-Is

The $K_m$ value for the purified rat liver PAP-I in hydrolyzing $\text{l-pGlu-pNA}$ was 0.030 mM (Table 2). The $K_m$ values for recombinant rat, mouse and human PAP-Is expressed in E. coli were 0.054, 0.038 and 0.038 mM, respectively, being very similar to that of the purified rat liver PAP-I. The $V_{\text{max}}$ values for recombinant rat, mouse and human PAP-Is were 17.7, 8.0 and 10.8 $\mu$mol/min/mgP, respectively, and 5.4 $\mu$mol/min/mgP for the purified rat liver PAP-I.

The effects of various peptidase inhibitors (1 mM) on the activities of the purified rat liver PAP-I and recombinant rat, mouse and human PAP-Is were investigated at a substrate ($\text{l-pGlu-pNA}$) concentration of 20 $\mu$m. As shown in Table 3, the inhibition profiles were almost the same among the four PAP-I preparations. Typical serine peptidase inhibitors (DFP and PMSF) and chelating agents (EDTA and 1,10-phenanthroline) did not inhibit the enzyme activity at all. The leucine aminopeptidase inhibitor (bestatin) also had no effect. On the other hand, typical inhibitors of thiol-dependent enzymes (NEM and PCMB) completely inhibited the activities of the four PAP-I preparations. However, a more selective inhibitor of cysteine peptidases of the papain family (E-64) did not inhibit the enzyme. TRH, a PAP-I substrate, showed strong inhibition, decreasing the enzyme activity to approximately 10%. Zn$^{2+}$ and Co$^{2+}$, known inhibitors of PAP-I, were both inhibitory, but Mg$^{2+}$ and Mn$^{2+}$ were not. Similar results were obtained at an inhibitor concentration of 0.2 mM (data not shown).

The optimum pHs for recombinant rat, mouse and human PAP-Is were 8, 8.5 and 8.5, respectively, and 8.5 for the purified rat liver PAP-I.

Subcellular Localization of PAP-I in Rat Liver and Its Distribution in Rat Tissues

The fractions (7–10%) of the rat liver cytosolic, mitochondrial, microsomal and nuclear fractions were separated and analyzed for PAP-I activity using $\text{l-pGlu-pNA}$ as the substrate. The PAP-I activities in the cytosolic, mitochondrial, microsomal and nuclear fractions were 0.006, 0.012, 0.438 and 0.004 nmol/min/mgP, respectively, showing that the PAP-I activity was localized in the microsomal fraction. The PAP-I activities in the nuclear fraction were lower than those in other fractions.

Table 2. Hydrolytic Activities of Purified Rat Liver PAP-I and Recombinant Rat, Mouse and Human PAP-I toward l-pGlu-pNA

<table>
<thead>
<tr>
<th>Species</th>
<th>$K_m$ (mM)</th>
<th>$V_{\text{max}}$ (μmol/min/mgP)</th>
<th>$V_{\text{max}}/K_m$ (ml/min/mgP)</th>
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<tbody>
<tr>
<td>Purified PAP-I</td>
<td>0.030</td>
<td>5.4</td>
<td>176.0</td>
</tr>
<tr>
<td>Recombinant PAP-I</td>
<td>0.054</td>
<td>17.7</td>
<td>327.7</td>
</tr>
<tr>
<td>Mouse</td>
<td>0.038</td>
<td>8.0</td>
<td>209.9</td>
</tr>
<tr>
<td>Human</td>
<td>0.038</td>
<td>10.8</td>
<td>280.9</td>
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$K_m$ and $V_{\text{max}}$ values were determined via Eadie–Hofstee plots.

Table 3. Effects of Various Inhibitors on the Activities of Purified Rat Liver PAP-I and Recombinant Rat, Mouse and Human PAP-I

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<thead>
<tr>
<th>Inhibitor</th>
<th>Relative activity (%)</th>
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<tr>
<td>DFP</td>
<td>98</td>
</tr>
<tr>
<td>PMSF</td>
<td>92</td>
</tr>
<tr>
<td>NEM</td>
<td>0</td>
</tr>
<tr>
<td>PCMB</td>
<td>0</td>
</tr>
<tr>
<td>EDTA</td>
<td>101</td>
</tr>
<tr>
<td>Bestatin</td>
<td>94</td>
</tr>
<tr>
<td>E-64</td>
<td>94</td>
</tr>
<tr>
<td>TRH</td>
<td>4</td>
</tr>
<tr>
<td>MgCl$_2$</td>
<td>99</td>
</tr>
<tr>
<td>MnCl$_2$</td>
<td>95</td>
</tr>
<tr>
<td>ZnCl$_2$</td>
<td>0</td>
</tr>
<tr>
<td>CoCl$_2$</td>
<td>0</td>
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</table>

Following preincubation with the above inhibitors for 0.5—1 h on ice, residual PAP-I activity was assayed, as described in Materials and Methods, in the presence of a) 1 mM DTT and b) 0.4 μM DTT. The final concentration of each inhibitor was 1 mM. Activity is expressed as a percentage of the control activity (100%). 1,10-PNT: 1,10-phenanthroline.

MW (kDa)

<table>
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<th>MW (kDa)</th>
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<tbody>
<tr>
<td>31</td>
</tr>
<tr>
<td>21.5</td>
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</tbody>
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Fig. 2. Immunoblotting Analysis of Rat, Mouse and Human Liver Subcellular Fractions

The fractions (7 μg protein) were separated on a 10–20% polyacrylamide SDS gel and then subjected to immunoelectrophoretic blot analysis with an antibody against recombinant rat PAP-I. Lanes 1—5: rat liver, Lanes 6—7: mouse liver, Lanes 8—9: human liver; Lane 1, whole homogenate; Lane 2, nuclear fraction; Lane 3, mitochondrial fraction; Lanes 4, 6, 8, cytosolic fraction; Lanes 5, 7, 9, microsomal fraction.

Distribution in Rat Tissues

In the rat liver, the PAP-I activities, assayed using l-pGlu-pNA as the substrate, in the nuclear, mitochondrial, cytosolic and microsomal fractions were 0.006, 0.012, 0.438 and 0.004 nmol/min/mgP, respectively, showing that the PAP-I activity was localized in the cytosolic fraction. The PAP-I activities in the cytosolic fractions from the kidneys, testis, aorta, brain, lungs, heart, small intestine and muscle were 1.317, 0.061, 0.021, 0.062, 0.120, 0.031, 0.030 and 0.019 nmol/min/mgP, respectively. The results indicated that the kidneys and liver showed relatively high activities compared with the other tissues and organs.
There was no activity in the plasma.

**Immunoblotting Analysis of Rat, Mouse and Human Liver Subcellular Fractions by Antibody** The whole homogenate and the nuclear, mitochondrial, cytosolic and microsomal fractions of rat liver were analyzed by immunoblotting using an antibody raised against recombinant rat PAP-I in rabbits (Fig. 2). An immunoreactive band with an expected size of 25 kDa was detected most intensively in the whole liver homogenate and the cytosolic fraction. The immunoreactive band was also found in the cytosolic fractions of the mouse and human liver (Fig. 2).

**Effects of Antibody on pGlu-pNA Hydrolyzing Activity** The effects of the antibody on the hydrolysis of pGlu-pNA (20 μM) were examined in rat, mouse and human liver cytosols (Table 4). The control hydrolyzing activities toward pGlu-pNA in rat, mouse and human liver cytosols were 0.46, 0.32 and 0.20 nmol/min/mgP, respectively. The hydrolyzing activities in rat and mouse liver cytosols were completely inhibited by the addition of the antibody at a concentration of 1 mgP/ml. The control IgG did not affect the hydrolyzing activities at all.

**DISCUSSION**

We obtained the cDNA encoding rat PAP-I from the rat liver cDNA library using primers designed according to the conserved region of the cDNA sequence of the mouse and human PAP-Is in GenBank™. The rat PAP-I cDNA encoded a protein of 209 amino acids with a calculated molecular weight of 22913 Da. The homology of the deduced amino acid sequence of recombinant rat PAP-I, indicating that PAP-I is solely involved in the hydrolysis of the amide bond of pGlu-pNA in rat liver cytosol. Note, however, that there still remains the possibility of the existence of the enzymes which crossreact with the antibody against recombinant rat PAP-I, as the antibody used was the IgG fraction, not the purified antibody.

The terminal pGlu residue is thought to donate a more or less xenobiotic nature to the peptidase leading to resistance to hydrolysis by various peptidases. Since PAP-I recognizes only the pGlu structure, ignoring the rest of the amino acid sequence, we hypothesized that PAP-I, as a xenobiotics-metabolizing enzyme, will hydrolyze other pGlu-containing xenobiotics with a low molecular weight. Among candidate compounds having this chemical structure, we have found several xenobiotic PAP-I substrates as will be reported elsewhere. It will be also interesting to investigate the xenobiotic compounds having glutamyl moiety instead of the pGlu moiety to understand the importance of pGlu structure as the substrate of PAP-I, and future studies should be awaited.

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REFERENCES


