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MOLECULAR CLONING OF HUMAN CYTOSOLIC PURINE 5'-NUCLEOTIDASE

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SUMMARY: The cDNA of human cytosolic purine 5'-nucleotidase (EC 3.1.3.5), which is the supposed regulatory allosteric enzyme of purine nucleotide degradation, has been cloned from a human placenta cDNA library. The predicted open reading frame encodes a protein of 561 amino acids with a molecular mass of 64,966 Da. The deduced amino acid sequence exhibits 95% identity with the sequence of the B type subunit of chicken cytosolic purine 5'-nucleotidase. Northern blot analysis of human placenta poly(A)$^+$RNA revealed a single band of 3.6 kilobases using the cloned cDNA.

Purine 5'-nucleotidase (EC 3.1.3.5) is one of soluble nucleotidases including pyrimidine 5'-nucleotidase (1), deoxyribonucleotidase (2), and AMP-selective 5'-nucleotidase (3,4). Purine 5'-nucleotidase preferentially hydrolyzes IMP and other purine nucleotides, and is allosterically activated by various compounds including ATP (5,6). The enzyme is exclusively located in the cytoplasmic matrix of cells (7,8), and may have a critical role in the maintenance of a constant composition of intracellular purine/pyrimidine nucleotides in cooperation with other nucleotidases as described above (1,9).

To clarify the structure of this enzyme, we have isolated cDNA clones encoding cytosolic purine 5'-nucleotidase from the chicken liver cDNA library, and demonstrated the unexpected existence of two distinct but closely related types of cDNAs designated as types A and B (10). The encoded A- and B-type proteins are similar throughout a central core of 472 amino acids sharing 79% sequence identity (10). In this study, using degenerate oligonucleotides based on identical portions of amino acid sequences between A- and B-type subunits of the chicken enzyme, we isolated human cytosolic purine 5'-nucleotidase cDNA clones with polymerase chain reaction (PCR) (11), and report its determined nucleotide and amino acid sequences as the first success of molecular cloning among the mammalian cytosolic nucleotidases.

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EXPERIMENTAL PROCEDURES

Cloning of Human Cytosolic Purine 5'-Nucleotidase Using Degenerate Primers

From deduced amino acid sequences of the chicken A- and B-type subunits (10), two peptide sequences were selected: KCFGFDMD (A, amino acids 39-46; B, 47-54) and YPNKFIQR (A, amino acids 129-136; B, 137-144). Two degenerate oligonucleotides were synthesized on an Applied Biosystems 381A DNA synthesizer: sense primer 65, CATAGAATTCAA(AG)TG(CT)TT(CT)GG(AGCT)TT(CT)GA(CT)ATGGA corresponding to KCFGFDMD plus an *Eco*RI restriction site; antisense primer 66, CATAGGATCCC(GT)(CT)TG(AG)AT(AG)AA(CT)TT(AG)TT(AGT)GG(AG)TA corresponding to YPNKFIQR plus a *Bam*HI site. The first strand cDNAs were synthesized with oligo(dT) as a primer and 1 μg of human placenta poly(A)$^+$RNA (Clontech) by using a cDNA synthesis kit (Pharmacia), precipitated with ethanol, and dissolved in 30 μl of 10 m**M** Tris/HCl, pH 8.0, and 1 m**M** EDTA (cDNA pool). PCR amplification was carried out in a total volume of 100 μl containing 6 μl of cDNA pool, 4 μg each of degenerate primers 65 and 66, 10 m**M** Tris/HCl, pH 8.3, 50 m**M** KCl, 1.5 m**M** $MgCl_2$, 0.01% gelatin; 150 μ**M** each dATP, dGTP, dCTP, dTTP, and 2.5 units of *Thermus aquaticus* DNA polymerase (Perkin-Elmer Cetus), under conditions of denaturation at 94°C for 1 min, annealing at 37°C for 1 min for the first two cycles, and at 48°C for 1 min for the next 30 cycles, and extension at 72°C for 2 min. The amplified DNAs were digested with *Eco*RI and *Bam*HI, and subcloned into pBluescript II plasmid (Stratagene). Several clones containing an expected length (0.3 kilobase (kb)) of inserts were randomly selected, and their sequences were determined by the dideoxy chain termination method (12). The plasmid ph12 was revealed to contain an insert highly homologous to chicken cytosolic purine 5'-nucleotidase.

Screening of λ Phage Library

A phage λgt11 human placenta cDNA library (1.0×10^6 independent clones; Clontech) was screened. The plasmid ph12 was radiolabeled with [α-^{32}P]dCTP (~111 TBq/mmol; New England Nuclear) by a random primer DNA labeling kit (Takara), and used as a hybridization probe. Plaque hybidization and washing of the filter were performed as previously described (13). One positively hybridized plaque was purified, subcloned into pBluescript (ph14), and sequenced.

Rapid Amplification of a cDNA End to Determine 5' Region

The plasmid ph14 lacked the translation initiation site. PCR amplification of 5' ends was carried out in a total volume of 100 μl as described above with 1×10^8 plaque forming units of the human placenta cDNA library as a template using 200 ng each of gt11 *Eco*RI site primer (forward, GGTGGCGACGACTCCTGGAGCCCG; New England Biolabs) and antisense primer 203, CCGGATCCAAATCCATGTGCACAAACCAAGAGG corresponding to nucleotides (nt) 351-375 plus a *Bam*HI site, under conditions of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min for 30 cycles. The amplified DNAs were digested with *Eco*RI and *Bam*HI, and subcloned into pBluescript. Positive clones were selected with PCR using internal sequences, and one (ph28) of them was sequenced.

Northern Blot Analysis

Total RNA was isolated from human placenta by the guanidium isothiocyanate/CsCl method, and poly(A)$^+$RNA was separated from the total RNA using Oligotex -dT30 (Roche)(14). Total and poly(A)$^+$RNAs were electrophoresed in a 1% agarose/2.2 **M** formaldehyde gel, and blotted onto a nitrocellulose filter. The conditions for hybridization and washing of the filter were the same as described above for screening the library.

RESULTS AND DISCUSSION

We have isolated two types of cDNAs, referred to as A and B, coding for chicken cytosolic purine 5'-nucleotidase (10). The encoded amino acid sequences of A and B

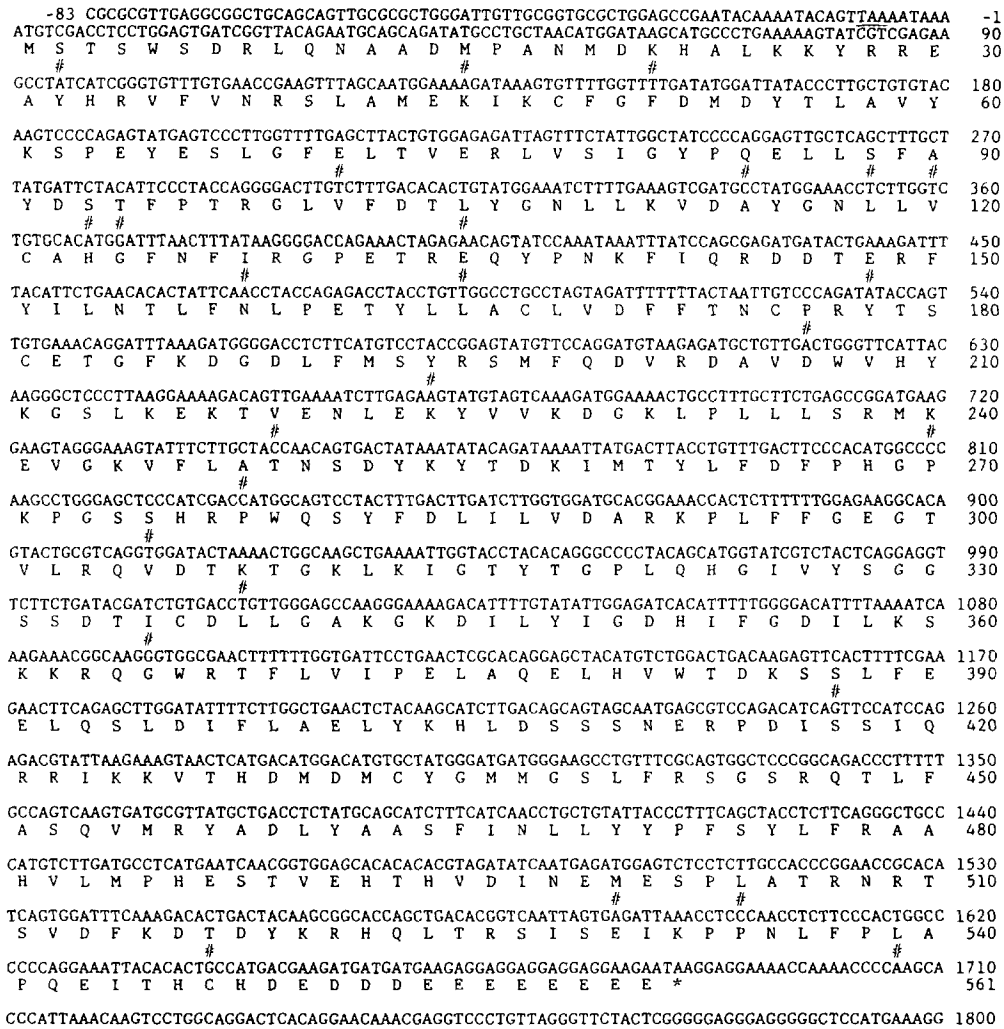

Figure 1. Nucleotide sequence of human cytosolic purine 5'-nucleotidase cDNA and the deduced amino acid sequence. The deduced amino acid sequence is shown below the nucleotide sequence in single letter codes. Numbers in the right margin, nucleotides from the predicted initiation codon, and amino acids beginning with 1 for the predicted N terminus; asterik, in-frame termination codon; underline, in-frame termination codon 5' upstream (nt -9 to -7) in a noncoding region; amino acids undermarked with # (26 out of 561 amino acids), not identical in the sequence with the B type protein of chicken cytosolic purine 5'-nucleotidase. The nucleotide sequence reported in this paper has been submitted to the GSDB, DDBJ, EMBL, and NCBI nucleotide sequence databases with accession number D38524

subunits show 79% identity in the internal 472 residues, but no significant homology in the N- and C-terminal regions (10). We selected two peptide sequences common to both of the A and B proteins, and synthesized degenerate oligonucleotides. Using the oligonucleotides, we amplified human cDNA fragments with PCR, and demonstrated that one clone (ph12) encoded an 82-amino acid sequence of 71% and 89% identity with the A and B proteins, respectively, between the two primers.

Approximately 1.05 x 10^6 plaques were screened using ph12 as a hybridization probe. Two positive phages (λh13 and -14) were obtained, but the two were revealed to be identical as a result of mapping with PCR. The sequence of the subcloned plasmid ph14 encompassed nt 142-3173, and lacked the translation initiation site. The initiation methionine codon and 5' noncoding region were obtained from PCR amplification of 1 x 10^8 plaque forming units of the human placenta cDNA library as a template. The sequence of the plasmid ph28 encompassed nt -83 to 350 excluding that of primer 203. The entire coding region of human cytosolic purine 5'-nucleotidase cDNA was covered by these two clones (ph14 and -28).

Fig. 1 shows the 3,256-basepair sequence of the cDNA clone encoding human cytosolic purine 5'-nucleotidase and the deduced 561-amino acid sequence. There is a single open reading frame starting at the first ATG (nt 1-3), and ending with a TAA at nt 1684-1686. In Northern blot analysis (Fig. 2), the size of human cytosolic purine 5'-nucleotidase mRNA was shown to be 3.6 kb.

The deduced amino acid sequence encoded by this open reading frame is a peptide chain of 561 amino acids with the calculated molecular mass of 64,966 Da. Human cytosolic purine 5'-nucleotidase was once highly purified from human placenta, and the subunit molecular mass was then shown to be 53 kDa upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis (15). This molecular mass of 53 kDa was probably a result of proteolysis during purification as proved in the case of the chicken liver enzyme (8,16).

The entire derived amino acid sequence of human cytosolic purine 5'-nucleotidase exhibited 95% identity (535 out of 561 amino acids identical) with that of the B type subunit of the chicken enzyme (Fig. 1), although the chicken B protein composed of 570 amino acids elongated nine more glutamic acid residues at its C-terminal region (10). The coding region of the human enzyme contains six peptide sequences analyzed in the lysyl endopeptidase-digests of purified chicken liver enzyme preparation (data not shown) (10). These findings strongly suggest that the isolated clone encodes human cytosolic purine 5'-nucleotidase, reconfirming previous immunological data that an anti-chicken liver enzyme antibody was effectively reactive with human placenta cytosolic purine 5'-nucleotidase (17).

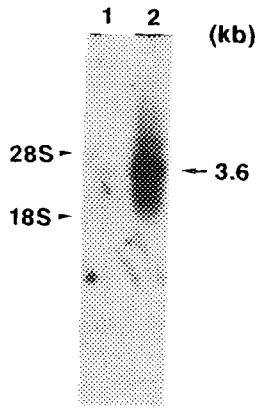

Figure 2. Northern blot analysis of human placenta RNAs. Total and poly(A)$^+$RNAs (10 μg in lane 1, and 10 μg in lane 2, respectively) from human placenta were electrophoresed. Positions of 28S (4.5 kb) and 18S (1.9 kb) rRNAs and a size of a band are indicated. An *Sac*I(nt 819-824)/*Pst*I(nt 1870-1875) fragment (1.05 kb) of the plasmid ph14 was used as a hybridization probe.

In chicken, two types of cDNAs encoding purine 5'-nucleotidase correspond to two separate genes (10). The evolutionary distance between the two genes was by limiting to the core regions of 472 amino acids calculated (18), resulting in a K_s value of 0.769. This finding suggests that the two genes diverged from the ancestor so long ago as impossible to assign the time of gene duplication, that the mammalian enzyme is expected to be composed of two distinct subunits as well as the avian enzyme. In this study, we cloned the B type cDNA from a human placenta library. Whether an A type gene of cytosolic purine 5'-nucleotidase is then expressed or not in the placenta remains to be clarified.

In the cytoplasmic matrix of cells, coexistent are several distinct cytosolic nucleotidases with overlapping specificities for nucleotide substrates in millimolar K_m values (1-6). On the other hand, micromolar K_m values possesses ecto-5'-nucleotidase on the external surface of the cell, the cDNAs of which have been already obtained, and its putative catalytic site was assigned (19,20). Purine 5'-nucleotidase is firstly cloned among the cytosolic nucleotidases, demonstrating to be unrelated to the primary structure of ecto-5'-nucleotidase. The catalytic site for cytosolic purine 5'-nucleotidase may differ from that of ecto-5'-nucleotidase.

Cytosolic purine 5'-nucleotidase is a regulatory allosteric enzyme of purine nucleotide degradation (5,6). Therefore, the abberation of this enzyme may be related to the urate production in hyperuricemia and gout. In fact, the exacerbated purine nucleotide degradation is a phenotype of human T lymphoblasts with elevated cytosolic purine 5'-nucleotidase activity (9). Cloned human cytosolic purine 5'-nucleotidase cDNA is an essential probe to screen gene abnormalities in patients with hyperuricemia and gout.

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