

The cDNA cloning of human placental ecto-ATP diphosphohydrolases I and II¹

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Abstract The cDNA clones of two isoforms (enzymes I and II) of human placental ecto-ATP diphosphohydrolases have been isolated based on the N-terminal amino acid (aa) sequence of the immunopurified 82 kDa protein and characterized. The cDNA clone encoding enzyme I consists of 2081 nucleotides and the predicted enzyme I consists of 517 aa residues. Enzyme I has a 5'-UTR and an N-terminal 11 aa sequence that differ from CD39, but the rest of the sequence is the same as CD39. The hydropathy plot indicated that enzyme I has two hydrophobic regions near the N- and C-termini of the molecule. In contrast, enzyme II consists of 1814 nucleotides and the predicted protein consists of 306 aa residues. The sequence of 1–1018 nucleotides of enzyme II is identical to that of enzyme I, but the 1019–1814 nucleotide sequence is different from both enzyme I and CD39. The hydropathy plot indicated that enzyme II has one hydrophobic region near the N-terminus, suggesting that enzyme II is also anchored to the cell membrane. It is, however, likely that some of enzyme II exists as a soluble form in plasma, possibly after proteolytic processing.

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Key words: Ecto-ATP diphosphohydrolase; Placenta; cDNA cloning

1. Introduction

Ecto-ATP diphosphohydrolases (ATPDases), or ecto-apyrases, are ubiquitous integral membrane proteins that play important roles in essential biological functions such as apoptosis, signal transduction, and hemostasis [1]. Ecto-ATPDases hydrolyze ATP and ADP to AMP in the presence of Ca²⁺ or Mg²⁺, and AMP is then converted to adenosine by 5'-nucleotidase [1]. ADP is a typical agonist of platelet aggregation, whereas adenosine is an inhibitor of platelet aggregation [2,3]. Ecto-ATPDases, therefore, function as strong inhibitors of platelet aggregation through their scavenging effects on endogenous ADP released from the activated platelets in association with adenosine [3].

CD39, initially identified in activated B-lymphocytes [4], has been demonstrated to be a vascular ecto-apyrase in humans from the following evidence. (1) The cDNA sequence of CD39 is partially homologous to that of soluble potato apyrase, and ATPDase activity appears in Epstein-Barr virus-transformed B-lymphocytes, which express CD39 on their cell surface [5,6]. (2) The partial internal amino acid (aa) sequences of human placental ecto-ATPDase closely match the deduced aa sequence of CD39 [7], and the enzyme activity also appears in CD39 cDNA-expressed COS cells [8]. (3) Anti-CD39 murine monoclonal antibody (mAb) immunoprecipitates the protein with the simultaneous disappearance of ATPDase activity from solubilized human umbilical vein endothelial cells (HUVECs) [9].

Human placental ecto-ATPDase has been initially reported to have a blocked N-terminus [7], but recently we have obtained N-terminal 30 residue aa sequence of the highly purified 82 kDa enzyme, prepared by an immunoadsorbent chromatography with a specific mAb (MK33, IgG1-κ) [10]. Interestingly, the first 11 residue sequence was different from that of CD39, but the following residues 12–30 and partial sequences derived from the internal regions were the same as those of CD39 [10]. This suggested that placental ecto-ATPDase is an isoform of CD39. More recently, CD39L1-L4 clones have been isolated from human cDNA libraries, but without extensive characterization. [11,12]. Further, the cDNA cloning of human brain ecto-ATPDase has been performed using polymerase chain reaction (PCR) in which query nucleotide sequences derived from the chicken smooth muscle ecto-ATPase cDNA were used [13]. These findings have suggested that human ecto-ATPDases consisted of a family of CD39s as indicated in non-human ecto-ATPDases [4,11–20].

Based on the protein sequence data obtained with the immunopurified placental ecto-ATPDase, we isolated two cDNA clones and examined the entire nucleotide sequences of the two. The protein deduced from enzyme I clone consisted of 517 aa residues and had structural features in accord with the immunopurified enzyme, but differed only in the N-terminal aa sequence from CD39. The other (enzyme II) was encoded with a nucleotide sequence highly homologous to that of the enzyme I clone, and consisted of 306 aa residues. Both enzymes I and II had the putative ATP-binding motif [20] and the predicted apyrase conserved regions [6] comparable with CD39. The hydropathic plots predicted that enzyme I had two putative transmembrane sequences in both the N- and C-terminal regions like CD39, but, unlike CD39, enzyme II had only one putative transmembrane sequence in the N-terminal region.

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¹ The novel cDNA sequences reported in this paper have been submitted to the EMBL Nucleotide Sequence Database on 26 February 1999 and are available under accession numbers AJ133133 and AJ133134.

2. Materials and methods

2.1. N-terminal and internal aa sequences of human placental ecto-ATPDases

The aa sequences of the N-terminal 30 residues of the immunopurified 82 kDa human placental ecto-ATPDase and of the peptides generated with *Achromobacter* protease (AP) I and separated by reversed-phase high performance liquid chromatography on a C18 column have already been determined [10] as follows: MKGTKDL-TSQQKESNVKTFxSKNILAILGF (N-terminus), FVQK (AP 7-1), DLTSQK (AP 7-2), GPGISK (AP 8-1), VTEMx (AP 8-2), TSYAGVK (AP 12), DQALWQK (AP 19), DYNYYTHSLxYQK (AP 28), YGIVLDAGGHTSLYIY (AP 29), TRWFIVPYET NNQETGETFGALDLGGAxTQVxF (AP 36) (where x indicates an unidentified residue).

2.2. Isolation and sequencing of the 5'-terminal partial cDNA nucleotide, N-300

Human placental cDNA library (Lot 102) was obtained from Takara Shuzo Co. (Shiga, Japan), and used as a template to conduct semi-nested PCR. Since the N-terminal 11–30 aa residue sequence of human placental ecto-ATPDase was completely identical with that of residues 5–23 of human CD39 [4], the first PCR was performed using two primers: the sense primer (T7) with the fixed nucleotide sequence of 5'-TAATACGACTATAGGG-3' corresponding to the flanking T7 promoter region of vector pAP3neo DNA, and the antisense primer (PL2) of the degenerated nucleotide sequence of 5'-(G/A/T/C)(G/C)(A/T)(A/G)AA(G/A/T/C)CC(G/A/T/C)A(A/G)(G/A/T)AT(G/A/T/C)GCA(G/A/T/C)A(A/G)(G/A/T)A-3' corresponding to the aa sequence ILAILGF in the N-terminal region. The reaction mixture including Ex-Taq polymerase (Takara Shuzo Co.), 1 µl of human placental cDNA library, 0.5 µM T7, and 0.5 µM PL2 was heated at 94°C for 5 min once, followed by thermal cycling at 94°C for 30 s, 55°C for 30 s, and 72°C for 90 s for 30 cycles, and finally one cycle at 72°C for 7 min. For the second semi-nested PCR, the two primers used were T7 (sense) and the degenerate primer, 5'-AA(G/A/T/C)GT(C/T)TT(G/A/T/C)AC(A/G)TT(G/A/T/C)(G/C)(A/T)(C/T)TC-3' (antisense, PL4) corresponding to the aa sequence ESNVKT in the N-terminal region. The second PCR amplified 1 µl of a 1:50 dilution of the first PCR product using Ex-Taq polymerase, 0.5 µM T7, and 0.5 µM PL4 with the same thermal cycling protocol as that used in the first PCR. After the second PCR, only one cDNA band of ~300 bp termed N-300 was obtained. The N-300 band was cut from the gel and purified by GeneClean II Kit (Bio 101, California, USA), and then it was subcloned using TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA) for nucleotide sequencing.

2.3. Isolation and sequencing of the residual and 3'-terminal partial cDNAs, C-1800 and C-1600

The 3'-terminal cDNA sequence of human placental ecto-ATPDase was determined by the semi-nested PCR using the same library described above. The first PCR was conducted using two fixed primers: 5'-TGAAGGGAACCAAGGACCTG-3' (sense, PL18, nucleotides 185–204 of N-300) which was determined as the nucleotide sequence corresponding to the aa sequence KGTKDL, specific to placental ecto-ATPDase but not to CD39 (see Section 3), and 5'-TAATACGACTACTATAGGG-3' (antisense, T3, corresponding to the sequence specific to the flanking T3 promoter region of the vector). The first PCR was performed using Ex-Taq polymerase, 1 µl of the human placental cDNA library, 0.5 µM T3, and 0.5 µM PL18. The reaction mixture was subjected to a thermal cycling process as already described. The second semi-nested PCR amplified 1 µl of a 1:50 dilution of the first PCR product using Taq polymerase, 0.5 µM T3 (antisense), and 0.5 µM PL19 (sense, 5'-GAAGGGAACCAAGGACCTGA-3', a gene-specific 186–205 nucleotide sequence of N-300). The thermal cycling protocol of the second PCR was the same as that used in the first PCR. After the second PCR, the two partial cDNA bands with different sizes of ~1800 bp and ~1600 bp, termed C-1800 and C-1600 fragments, were detected using agarose gel electrophoretic analysis. Both the partial cDNA bands were cut from the gel separately, and were then independently subcloned for nucleotide sequencing.

2.4. DNA sequencing and database analysis

DNA sequencing was performed with an automated ABI-Prism 310

Genetic Analyzer. Both the DNA and deduced protein sequences were compared with sequences in the GenBank, Swiss-Prot or PIR databases using BLAST programs [21].

2.5. Detection of enzyme I, II and CD39 in placenta and HUVEC cDNA libraries by PCR

To examine the tissue-specific localization of enzyme I, II and CD39, PCR was conducted using placenta and HUVEC cDNA libraries as the templates. For this, two nucleotide sequences of sense and antisense primers complementary to the determined N- and C-terminal aa sequences of the respective cDNAs were used as shown in Fig. 4.

The reaction mixture including Taq polymerase, 1 µl of human placenta or HUVEC λ-ZAP (kindly provided by Dr. Toshiyuki Miyata of the Research Institute, National Cardiovascular Center, Osaka, Japan) cDNA library, 0.5 µM sense primer, and 0.5 µM antisense primer was subjected to PCR using the same thermal cycling process. The amplified PCR products were separated on 1% agarose gel electrophoresis in the presence of ethidium bromide and visualized under UV light.

2.6. Northern analysis using enzyme II-specific probe in placenta

For placental mRNA analysis, Northern Territory mRNA blots, a charge-modified nylon membrane to which poly(A)⁺ RNA (2 µg per lane) from human placenta had been transferred, was used (Invitrogen, Carlsbad, CA, USA). The enzyme II-specific probe was generated from the placental cDNA library by PCR using two primers, PL43 (sense) and PL30 (antisense) as shown in Fig. 5. The PCR was performed using Ex-Taq polymerase, 1 µl of the human placental cDNA library, 0.5 µM PL43, and 0.5 µM PL30. The reaction mixture was heated at 94°C for 5 min once, then heated to 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s for 30 cycles, and finally one cycle at 72°C for 7 min. The amplified PCR products were separated by 1% agarose gel electrophoresis in the presence of ethidium bromide and visualized under UV light. The fragment (482 bp) for the enzyme II-specific probe was purified with the GeneClean II kit and labeled with BrightStar psoralen-biotin non-isotonic labeling kit (Ambion, Austin, TX, USA). Prehybridization and hybridization were carried out following the manufacturer's instructions of Northern Territory mRNA blots. And the detection procedure was carried out with BrightStar BioDetect non-isotonic detection kit (Ambion). The blot was exposed to a Kodak scientific imaging film (Eastman Kodak Company, Rochester, NY, USA) for 20 h.

3. Results and discussion

Nucleotide sequencing revealed that the 5'-terminal cDNA fragment (N-300) was composed of 239 bp, and the aa sequence deduced from the internal 184–237 nucleotide sequence (Fig. 1, shaded bar) completely matched the N-terminal 1–18 residue sequence (MKGTKDLTSQQKESNVKT) of human placental ecto-ATPDase [10], but differed from that of human CD39 [4]. The first 183 nucleotides appeared to encode 5'-UTR, because no significant propeptide cleavage site was found using the PSORT program [22], and in addition, this nucleotide sequence was also completely different from that of CD39. After determination of the nucleotide sequence of N-300, the cDNA sequence(s) encompassing the residual and 3'-terminal portions were determined using semi-nested PCR amplification, in which the first PCR was performed using the sense primer, PL18 (5'-TGAAGGGAACCAAGGACCTG-3', corresponding to nucleotides 185–204) and the antisense primer, T3 (5'-TAATACGACTACTATAGG G-3', specific to the flanking region of the vector), and the second PCR used PL19 (sense, 5'-GAAGGGAACCAAGGACCTGA-3', corresponding to nucleotides 186–205) and the antisense primer of T3 common to the first PCR. After the second PCR amplification, two distinct cDNA fragments, termed C-1800 and C-1600, were obtained, and the following

1 cccgggtgtacgtgtaaaattatgatcaaaatattgtatgcocttttctctattaacc
 61 tgcocttttttgcagcaggtgtgcaagtgaaactcagagggcaagggaagtttctcttg
 121 gccctccagttttgtgtgtgaacaggatccaagaagctgctgtctctctggaagct
 181 gcaatgaagggaacaaaggacgtgacacgaacagagaggtctaacgtgaagacattt
 M K G T K D L T S Q Q K E S N V K T F 19
 241 tgctccaaagaatactcagcgaactgtgcttctctctatcatagctgtgatctgttg
 C S K N I L A I L G F S S I I A V I A L 39
 301 cttgctgtgggtgtgaccagaaacaaagcattgocagaaaacgttaagtattgggtgtg
 L A V G L T Q N K A L P E N V K Y G I V 59
 361 ctggatcgggggttctctcacaacagtttatacatctataagtggccagcagaaaaggag
 L D A G S S H T S L Y I Y K W P A E K E 79
 421 aatgacacaggcgtgtgtgcaatgaagaatgaaggttaaggtcgtggaatctca
 N D T G V V H Q V E E C R V K G P G I S 99
 481 aaattttgtcagaagaataatgaatgagcattacgtgactgattgcatggaagagct
 K F V Q K V N E I G I Y L T D C M E R A 119
 541 aggggaagtgttccaaaggtccacagcacaacagcagcagcgtttacgtgggagccagcga
 R E V I P R S Q H Q E T P V Y L G A T A 139
 601 ggcatcgggtgtgctcaggtggaagtgaggtggcagacaggttctggatgtgtg
 G M R L L R M E S E E L A D R V L D V V 159
 661 gagaggagcctcagcaactaccccttgacttccaggggtgacaggtatcattactggccaa
 E R S L S N Y P F D F Q G A R I I T G Q 179
 721 gaggaaggtgcatgtggtgattactatcaactatctgtgggcaaatcagtcagaaa
 E E G A Y G W I T I N Y L G K F S Q K 199
 781 acaaggtgtgttcagcatagtcoccatatgaacccaataatcaggaacacgtttggagcttg
 T R W F S I V P Y E T N N Q E T F G A L 219
 841 gaccttgggggagcctctacacaaagtcactttgtaccccaaaacagactatcagagtc
 D L G G A S T Q V T F V P Q N Q T I E S 239
 901 ccagataatgctgtcgaatttgcctctatggcaggaactacaatgtctacacacatagc
 P D N A L Q F R L Y G K D Y N V Y T H S 259
 961 ttctgtgtctatgggaaggtcagcagcactctgacagaaactggcgaagacattcaggtt
 F L C Y G K D Q A L W Q K L A K D I Q V 279
 1021 gcaagtaataaattctcagggacccatgcttctcactggtatataagaaggtgagtaac
 A S N E I L R D P C F H P G Y K K V V N 299
 1081 gtaagtcacctttacagacccctcagcacaagagatttgatgacttctccatccag
 V S D L Y K T P C R F L Y G K D Y N V Y T H S 319
 1141 cagtttgaatccaggggtattgaaactatcaaatgcaatcagaacatcctggagctc
 Q F E I Q G I G N Y Q Q C H Q S I L E L 339
 1201 ttcaacaccagttactgcocttactcagcagtgcttcaatgggattttcttggccacca
 F N T S Y C P Y S Q C A F N G I F L P P 359
 1261 ctccaggggggtttgggacatttactgttactttgtgtagaagtttttaacttg
 L Q G D F G A F S A F Y F V M K F L N L 379
 1321 acatcagagaaagtctctcaggaaggtgactgagatgatgaaagttctgtgctcag
 T S E K V S Q E K V T E M M K K F C A Q 399
 1381 ccttggggaggagataaaacattctacgtggaaggaaggaagtaacgtgagtaaac
 P W E E I K T A G T V K E K Y L S E Y 419
 1441 tgcttttctgttactacattctctcctcctctcgaaggtatcattcagagctgat
 C F S G T Y I L S L L L Q G Y H F T A D 439
 1501 tctctggggacacatccatttctggcgaagatccagggcagcagcgggtggaatttg
 S W E H I H F I G K I Q G S D A G W T L 459
 1561 ggctacatgctgaacctgacacatgatccagcagtgagcaacattgtccacacactc
 G Y M L N L N L C C P A E Q P L S T P L 479
 1621 tcccaactccacattgtcttctcctcgttcttctcctcgttcttctcagagtgcc
 S H S T Y V F L M V L F S L V L F T V A 499
 1681 atcatagaggtgtgttattcttcaagccttcatattctggaagataggtatagcaa
 I I G L L I F H K P S Y F W K D M V O
 1741 aagcagctgaaatagctgggtgagtgaggaaaaatcgtccaggagacatttctctcc
 1801 atcgagctgttcaaggcactcctcctgtctcgtcgaaggcagctgttgagagtggaag
 1861 ctctcctgtgttcttactgaagccttcttcttggaggttattcaatccttctgctcag
 1921 acttcggcagatactgtcttctcaggttttctcagcagcagcacttctccttctgtac
 1981 ttgtgtgtgtataggttttaagacagcagcacttctcagcagcacttcttcttataaaga
 2041 acaattattgactttgtctagaaaaaataaataaataaataaataaataaataaataa

Fig. 1. The cDNA sequence and the deduced amino acid sequence of human placental ecto-ATPase (enzyme I). The entire cDNA sequence of enzyme I was determined by analysis of two partial cDNAs, N-300 (nucleotides 1–239) and C-1800 (nucleotides 186–2081) as described in Section 3. The shaded area indicates the N-terminal 30 aa sequence determined by protein sequencing [10]. The putative ATP-binding domain [6] is enclosed, and the putative apyrase conserved regions [20] are underlined. The potential *N*-glycosylation sites are indicated by asterisks. The position of the termination (stop) codon is indicated by a circle.

1 cccgggtgtacgtgtaaaattatgatcaaaatattgtatgcocttttctctattaacc
 61 tgcocttttttgcagcaggtgtgcaagtgaaactcagagggcaagggaagtttctcttg
 121 gccctccagttttgtgtgtgaacaggatccaagaagctgctgtctctctggaagct
 181 gcaatgaagggaacaaaggacgtgacacgaacagagaggtctaacgtgaagacattt
 M K G T K D L T S Q Q K E S N V K T F 19
 241 tgctccaaagaatactcagcgaactgtgcttctctctatcatagctgtgatctgttg
 C S K N I L A I L G F S S I I A V I A L 39
 301 cttgctgtgggtgtgaccagaaacaaagcattgocagaaaacgttaagtattgggtgtg
 L A V G L T Q N K A L P E N V K Y G I V 59
 361 ctggatcgggggttctctcacaacagtttatacatctataagtggccagcagaaaaggag
 L D A G S S H T S L Y I Y K W P A E K E 79
 421 aatgacacaggcgtgtgtgcaatgaagaatgaaggttaaggtcgtggaatctca
 N D T G V V H Q V E E C R V K G P G I S 99
 481 aaattttgtcagaagaataatgaatgagcattacgtgactgattgcatggaagagct
 K F V Q K V N E I G I Y L T D C M E R A 119
 541 aggggaagtgttccaaaggtccacagcacaacagcagcagcgtttacgtgggagccagcga
 R E V I P R S Q H Q E T P V Y L G A T A 139
 601 ggcatcgggtgtgctcaggtggaagtgaggtggcagacaggttctggatgtgtg
 G M R L L R M E S E E L A D R V L D V V 159
 661 gagaggagcctcagcaactaccccttgacttccaggggtgacaggtatcattactggccaa
 E R S L S N Y P F D F Q G A R I I T G Q 179
 721 gaggaaggtgcatgtggtgattactatcaactatctgtgggcaaatcagtcagaaa
 E E G A Y G W I T I N Y L G K F S Q K 199
 781 acaaggtgtgttcagcatagtcoccatatgaacccaataatcaggaacacgtttggagcttg
 T R W F S I V P Y E T N N Q E T F G A L 219
 841 gaccttgggggagcctctacacaaagtcactttgtaccccaaaacagactatcagagtc
 D L G G A S T Q V T F V P Q N Q T I E S 239
 901 ccagataatgctgtcgaatttgcctctatggcaggaactacaatgtctacacacatagc
 P D N A L Q F R L Y G K D Y N V Y T H S 259
 961 ttctgtgtctatgggaaggtcagcagcactctgacagaaactggcgaagacattcaggtCa
 F L C Y G K D Q A L W Q K L A K D I Q V A 279
 1021 agtataaactcaatccagacgtgcccctctcactctcagcactcagcagcccaactcctgt
 S I T Q S R P A P E T S A P P A P T S C 299
 1081 tggtttctgttcaaatcagtagtttctgtggaactgtgttattgtacttctccaaactct
 C F L F Q I Q O
 1141 tttaggcaggatattgaactacactctgttgacccctataaattgtttcagtcagacatc
 1201 cctcccatgtttgttactgcttctcactcagcagacacacagagatctctctcagga
 1261 aataatttctccctcctgtgggtgtgataatgaataatctgtgtagaatttctgtgt
 1321 ggagggggaattgcaatttataagctcttcttagacacactttttaaagctcctctctc
 1381 aaactacaaaaaaacaggtgttagaagacgttggaaggaagaaaaaaacacacac
 1441 actctcaggaagaaacacccgaatgatacactgacacactcgaactctgtctctgtcc
 1501 cagatgcaactgttttgaacggagaaaggttaaaacacacttttgagggtgaggtgggt
 1561 ggatcactgtgaggtcaggtgttgagacacgctggcgaacatggtgaaacacacatct
 1621 actaaaaatacaaaaaaagaaaaaataagctggcagcagcagcagcagcagcagcagc
 1681 agctacttgggaggtgagaaaaggagaatcacttgaactgggaggtggaggtgtcagtg
 1741 gcagagattggcgaactacacacacgctgggacagagcagagactcctcctcaaaag
 1801 aaaaaaaaaaaaaa

Fig. 2. The cDNA sequence and the deduced amino acid sequence of human placental ecto-ATPase (enzyme II). The entire cDNA sequence of enzyme II was determined by analysis of two partial cDNAs, N-300 (nucleotides 1–239) and C-1600 (nucleotides 186–1814) as described in Section 3. The putative ATP-binding domain [6] is enclosed, and the putative apyrase conserved regions [20] are underlined. Potential *N*-glycosylation sites are indicated by asterisks. The position of the termination (stop) codon is indicated by a circle. The shaded area indicates 28 aa residue sequences which cannot be identified in enzyme I.

subcloning and nucleotide sequencing revealed that C-1800 was composed of 1895 bp, whereas C-1600 was 1628 bp. The 5'-terminal first 54 nucleotide sequences were identical to each other and overlapped with the nucleotide sequence 187–239 of N-300. A stop codon was identified at nucleotides 1735–1737 (TAG) for C-1800, and at nucleotides 1101–1104 (TAG) for C-1600. To address whether a pair of N-300 and C-1800 or N-300 and C-1600 came from the same cDNAs, 5'-terminal nucleotide sequences were determined by PCR using two sets of primers each of which was specific to enzyme I and II. A pair of primers T7 (sense) and PL24 (antisense, shown in Fig. 4) for enzyme I and of primers T7 (sense) and PL32 (antisense, shown in Fig. 4) for enzyme II were used. The nucleotide sequencing of these two PCR products confirmed

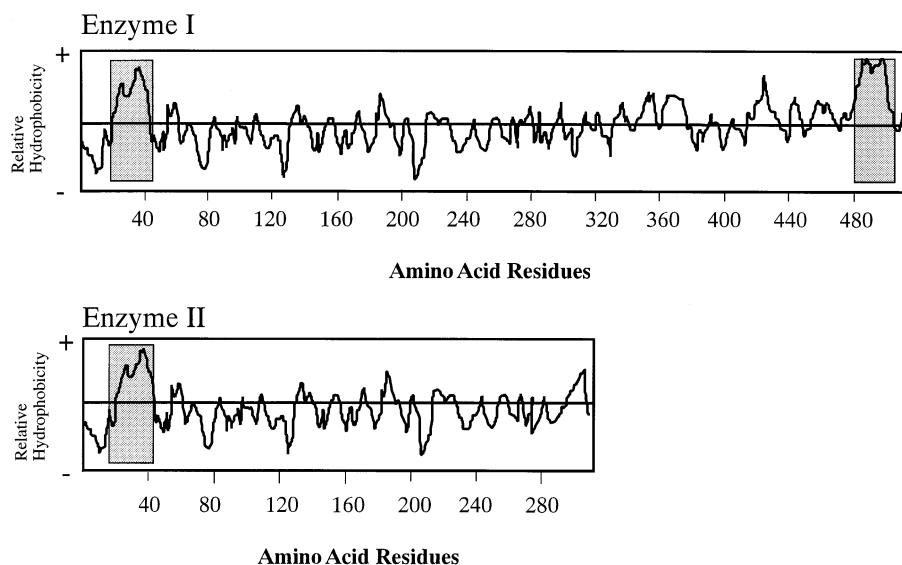


Fig. 3. Hydropathy plot of human placental ecto-ATPDases (enzymes) I and II. Hydropathy plots using the Sosui program indicate that enzyme I has two hydrophobic regions near the N- (aa residues 22–44) and C-termini (aa residues 485–507), and enzyme II has only one hydrophobic region near the N-terminus (aa residues 22–44). The hydrophobic regions, which may be potential membrane-spanning regions, are enclosed.

that each set of N-300 and C-1800 or N-300 and C-1600 originated from similar but independent cDNAs (data not shown). The full-length cDNAs and the deduced aa sequences of two isoforms of human placental ecto-ATPDases (enzyme I and II) are shown in Figs. 1 and 2. The cDNA of enzyme I consisted of 2081 nucleotides which include 183 bp of 5'-UTR, 1551 bp of ORF, a stop codon and 344 bp of 3'-UTR, and the aa residues were predicted to be 517. In com-

parison of the aa sequences, enzyme I only differed from CD39 in the N-terminal region, indicating that they were generated by alternative splicing at the pre-mRNA level. An entirely different structure in the 5'-UTR cDNA of enzyme I and CD39 also supports this speculation. The molecular mass of enzyme I was calculated to be 58 706 Da based on the backbone aa sequence. It contained six potential *N*-glycosylation sites, and the *pI* was estimated to be 6.29 by ExpASY

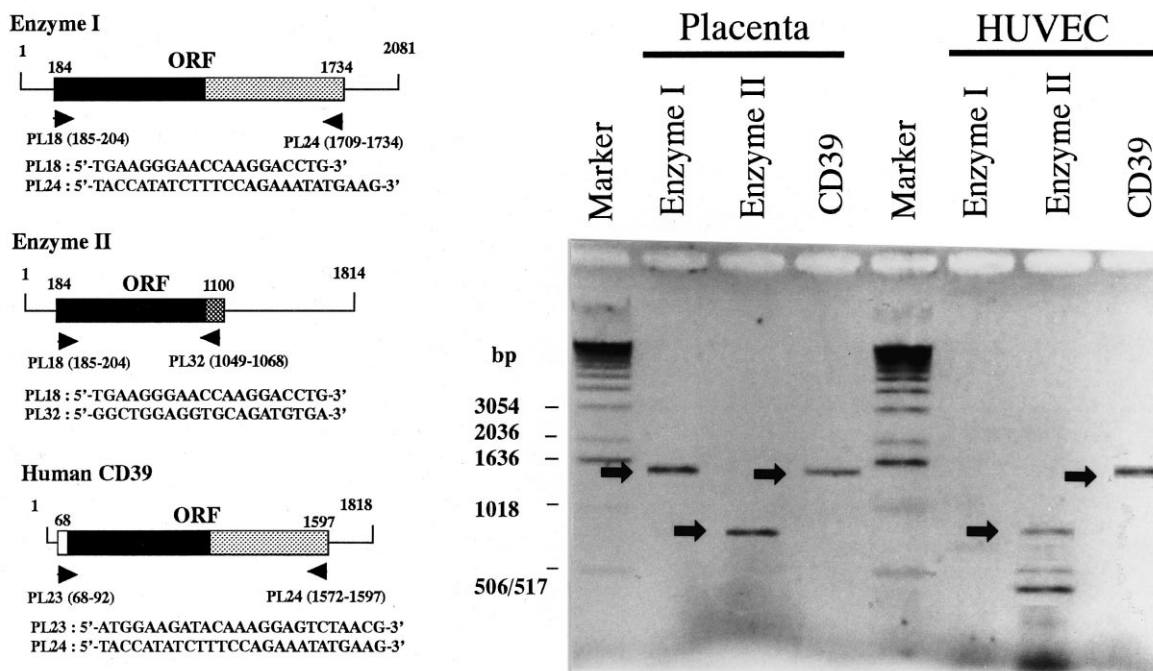


Fig. 4. Agarose gel analysis of PCR products with a pair of primers specific to placental ecto-ATPDases (enzymes) I, II, and CD39, using placenta and HUVEC cDNA libraries. In the left panel, the primer design used in this study is shown. The black bars indicate identical regions in the three enzymes. From the placenta cDNA library, each single band with ~1550 bp for enzyme I, ~884 bp for enzyme II, or ~1530 bp of CD39 was detected. From the HUVEC library, one band with ~1530 bp of CD39 was detected, but no band relevant to enzyme I was observed. Further, for enzyme II some bands were also detected in addition to the ~884 bp band. The expected bands are shown by the arrows.

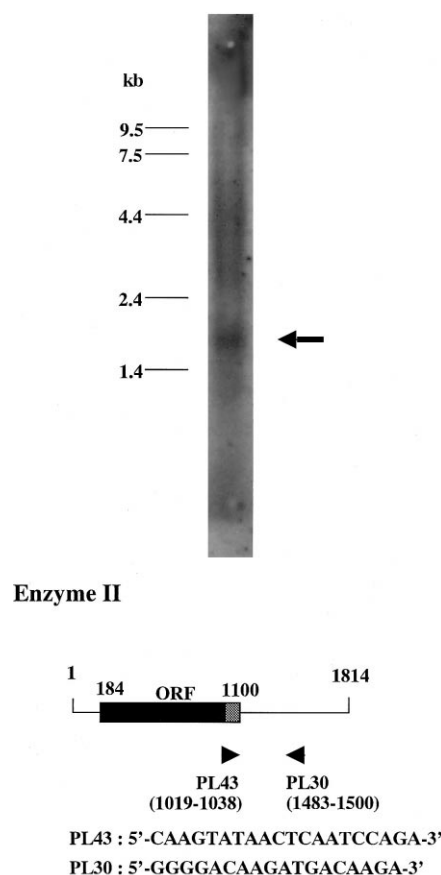


Fig. 5. Expression of enzyme II mRNA in placenta. Identification of mRNA for enzyme II in human placenta was undertaken by Northern analysis (upper panel). Poly(A)⁺ RNA (2 µg/lane) from human placenta was hybridized with the enzyme II-specific probe. We found several messages for enzyme II but the size of one of them (arrow) was identical to enzyme II (approximately 1800 bp). The lower panel shows the primer design used in generating the enzyme II-specific probe (482 bp).

server [23]. These structural features were in accord with those of the immunopurified placental ecto-ATPase [10]. Furthermore, the hydropathy plot using the SOSUI program [24] indicated that enzyme I had two hydrophobic regions in the molecule (Fig. 3, top), suggesting that enzyme I was anchored to the cell surface of placental syncytiotrophoblasts in the N- and C-terminal regions, in a manner similar to CD39.

In contrast, enzyme II consisted of 1814 nucleotides which included 183 bp of 5'-UTR, 918 bp of ORF, a stop codon and 710 bp of 3'-UTR. Thus, the deduced enzyme II consisted of 306 residues with a molecular mass of 34 175 Da. It had an estimated *pI* of 6.45, and contained two potential *N*-glycosylation sites by ExPASy server. The sequence of nucleotides 1–1018 of enzyme II is totally identical to that of enzyme I, but the 1019–1814 nucleotide sequence was quite different from both enzyme I and CD39. The predicted C-terminal aa sequence of enzyme II is ASITQSRPAPFTSAPPAPTSC-CFLFQIQ, and no homologous protein to this sequence was found by BLAST search. Hydropathy plot indicated that enzyme II had one hydrophobic region near the N-terminus, which may be the transmembrane domain (Fig. 3, bottom).

We have previously shown that placental ecto-ATPase localizes on microvillous syncytiotrophoblasts and umbilical

veins but not on umbilical arteries using the specific murine mAb MK33. However, this distribution was indistinguishable by the use of anti-human CD39 mAb [10]. Since the entire cDNA sequences of placental ecto-ATPDases were elucidated here, we examined the tissue specificity of enzyme I, II, and CD39 by PCR using placenta and HUVEC cDNA libraries as the templates. A pair of primers specific to the respective cDNAs are shown on the left of Fig. 4. When placenta cDNA was used as a template, the results were clear because each PCR product gave one band with the estimated size of nucleotides (Fig. 4). In addition, when HUVEC cDNA was used, a PCR band representing CD39 was clearly detected, but that of enzyme I was missing. More interestingly, in the HUVEC cDNA library, several PCR bands, including enzyme II seen in the placenta cDNA library, were detected (Fig. 4). Taking these findings together, both enzyme I and CD39 appear to be membrane-bound proteins and structurally similar to each other, but they localize separately.

Of particular interest is enzyme II, because this enzyme is not immunopurified by mAb MK33, but the protein sequence deduced from its cDNA indicates the presence of a putative ATP-binding domain (aa residues 59–65) and 'apyrase conserved regions' (aa residues 63–68, 131–132, 137–142, 178, 182–183, 190, 220, and 222–227) common to enzyme I and CD39. To demonstrate the evidence for the existence of enzyme II, we analyzed mRNA purified from human placenta by hybridization with the enzyme II-specific probe (482 bp). We found a message for enzyme II in approximately 1800 bp, identical to enzyme II in size, and several other messages in a high bp region. Several different mRNA transcripts reacting with the enzyme II-specific probe seem to be multiple RNA transcripts probably by alternative splicing. Similar results of Northern analysis in CD39 were reported by Kaczmarek et al. [8].

One of the reasons why enzyme II is not immunopurified may be explained by a lack of the epitope of mAb MK33, because this mAb was raised against enzyme I. Another possibility may be the much more reduced expression of enzyme II than enzyme I in placental microvilli. A speculation that enzyme II may be proteolytically more cleavable than enzyme I is also possible, and thus it may exist as soluble ATPase in plasma during normal pregnancy [25,26]. Since we have shown that the placental ecto-ATPase functions as a potent inhibitor not only for agonist-induced platelet aggregation but also for shear-induced platelet aggregation [10], the enzyme(s) particularly in its soluble form may participate in anti-thrombotic functions in normal pregnancy with a hypercoagulable state.

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