Molecular Cloning and Characterization of Expressed Human Ecto-Nucleoside Triphosphate Diphosphohydrolase 8 (E-NTPDase 8) and Its Soluble Extracellular Domain†

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ABSTRACT: An ecto-nucleoside triphosphate diphosphohydrolase (ecto-NTPDase) has been cloned from human liver RNA by RT-PCR. The 1.5 kb cDNA codes for a protein of 495 amino acids. Sequence analysis indicated that it is most closely related to a chicken ecto-ATPDase previously cloned in our laboratory [Knowles et al. (2002) *Eur. J. Biochem. 269*, 2373−2382] and a mouse homologue that has been designated as E-NTPDase 8 [Bigonnesses et al. (2004) *Biochemistry 43*, 5511−5519]. The human E-NTPDase 8 has similar topology as the avian and mouse E-NTPDase 8 but has fewer potential N-glycosylation sites and only two amino acid residues in the cytoplasm at its C-terminus. Despite 52% identity in primary structures, enzymatic properties of human E-NTPDase 8 expressed in HEK293 cells differ from that of the chicken E-NTPDase 8. In contrast to the chicken E-NTPDase 8, the human E-NTPDase 8 hydrolyzes MgADP poorly and is inhibited by several detergents as well as benzyl alcohol; the latter attribute may be related to weaker interaction of the transmembranous domains of the human E-NTPDase 8. To demonstrate that inhibition by detergents is mediated by the transmembranous domains, a recombinant pSecTag2 plasmid containing the extracellular domain (ECD) of the human E-NTPDase 8 was constructed. The soluble human E-NTPDase 8 which was secreted into the culture media of transfected HEK293 cells was purified by ammonium sulfate fractionation and nickel affinity chromatography. Besides becoming resistant to detergent inhibition, the soluble human E-NTPDase 8 ECD displays greater activity with Ca nucleotide substrates, an increased affinity for ATP, different pH dependence, and a decreased sensitivity to azide inhibition when compared to the membrane-bound enzyme. These differences may result from the different conformations that the ECD assume without or with constraints exerted by the transmembranous domains. These results indicate that the transmembranous domains are important in regulating enzyme activity as well as in determining the structure of human E-NTPDase 8.

The ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDase)[1] family is comprised of proteins that have nucleoside triphosphate (NTP) hydrolase activities. Some of them are also able to hydrolyze nucleoside diphosphates (NDP). Eight subfamilies can be distinguished by the extent of sequence homology. Members of four of the subfamilies (E-NTPDase 1, 2, 3, and 8) are cell surface proteins, i.e., ectoenzymes, whereas members of the other subfamilies are located in intracellular membranes or secreted (see review in ref *1*). The latter also display a preference for NDP as substrates (*2*−*8*).

The cell surface E-NTPDases (or ecto-NTPDases) have similar topology. They have a large extracellular domain and are anchored to the plasma membrane by two transmembranous domains, one each at the N- and C-termini with short intracytoplasmic domains. E-NTPDase 2 (also called ecto-ATPase) is a nucleoside triphosphatase and hydrolyzes NDP poorly (*9*, *10*), whereas E-NTPDases 1, 3, and 8 (also called ecto-ATP-diphosphohydrolase or ecto-ATPDase) hydrolyze both NTPs and NDPs. NTPDase 1 (also called CD39), the first E-NTPDase cloned (*11*) and characterized (*12*), is distributed mostly in cells of the vascular system and has been referred to as vascular ATP diphosphohydrolase (*13*). E-NTPDase 3 was cloned from a human brain cDNA library (*14*). Northern blot analysis indicated, however, that its transcript is also abundant in placenta, pancreas, spleen, and prostate (*15*). A sequence that codes for an ecto-ATPDase that differs from E-NTPDase 1 and 3 in the extent of sequence homology and tissue distribution was first obtained from two partial cDNA sequences cloned from a chicken oviduct cDNA library (*16*). A full-length cDNA was later obtained by RT-PCR cloning using chicken liver RNA (*17*). The enzymatic properties of the expressed chicken ecto-ATPDase in HeLa cells are similar to those of the native enzymes purified from chicken oviduct (*18*) and liver (*17*). More recently, a mouse ecto-ATPDase which is predominantly expressed in the mouse liver was cloned and expressed

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[1] Abbreviations: E-NTPDase, ecto-nucleoside triphosphate diphosphohydrolase; ecto-ATPDase, ecto-ATP-diphosphohydrolase; ACR, apyrase conserved regions; CR, conserved regions; ECD, extracellular domain; TMD, transmembranous domain; Mops, 3-(*N*-morpholino)-propanesulfonic acid; NP-40, Nonidet P-40; RT-PCR, reverse tanscription polymerase chain reaction; NBT/BCIP, nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate.

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and has been designated as E-NTPDase 8 (*19*). Among the ecto-ATPDases, the mouse E-NTPDase 8 is most closely related to the chicken oviduct and liver ecto-ATPDase with 53% amino acid sequence identity (*19*). We report here the molecular identification and characterization of a third member of the E-NTPDase 8 subfamily, a human E-NTPDase 8. While it has 52% sequence identity to the chicken E-NTPDase 8, it differs from the latter with respect to detergent inhibition and ADP hydrolysis. We have also generated a soluble enzyme by expressing the extracellular domain of human E-NTPDase 8. The biochemical properties of the soluble human E-NTPDase 8 differ markedly from the membrane-bound enzyme with respect to resistance to detergent inhibition, substrate utilization, effects of pH and temperature, and inhibition by azide. These findings, together with previously published results from our laboratory (*20*), demonstrate the importance of the transmembranous domains in determining the structure and regulating the activity of membrane-bound E-NTPDases.

MATERIALS AND METHODS

Materials. Human liver total RNA and PCR Ready cDNA from human liver were purchased from Ambion. The Access Quick RT-PCR kit, pGEM-T-Easy vector, and goat anti-mouse Ig conjugated with alkaline phosphatase were obtained from Promega. pcDNA3, the pcDNA3.1/V5-His topo cloning kit, pSecTag2, *Taq* polymerase, Platinum PCR Supermix, lipofectamine, anti-V5, and anti-His monoclonal antibodies were obtained from Invitrogen. *Pfu* Turbo DNA polymerase was purchased from Strategene. Restriction enzymes and PNGase-F were purchased from New England Biolabs. NBT/BCIP tablets for color development of alkaline phosphatase reaction of immunoblots were obtained from Roche. [γ-^{32}P]-ATP was purchased from PerkinElmer. All other biochemicals were obtained from Sigma Biochemical Co. Primers for PCR and sequencing were synthesized by the SDSU Microchemical Core Facility. DNA sequencing was done by the same facility.

Molecular Cloning of the Human E-NTPDase 8. Two oligonucleotides that anneal to the 5′- and 3′-ends of the deduced coding region of a sequence homologous to human E-NTPDase 8 (BC050293) were designed (forward primer, 5′-ATGGGGCTGTCCCGGAAG-3′, and reverse primer, 5′-CTAGTCCTGCAACCAGAAGAG-3′) and used as primers in RT-PCR. The 50 μL RT-PCR reaction mixture contained 0.5 μg of human liver total RNA and 0.02 μM forward and reverse primers, in addition to other components supplied in the Access Quick RT-PCR kit. AMV reverse transcriptase (5 units) was added to initiate the reaction. PCR conditions were as follows: 48 °C for 45 s, followed by 95 °C for 2 min and then 10 cycles of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min. This was followed by 25 cycles of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min with an addition of 5 s at the 72 °C extension step for each of the 25 cycles, after which the PCR reaction ends with 7 min at 72 °C. Two PCR products, a 1.7 and a 1.5 kb DNA fragment, were obtained and introduced into pGEM-T-Easy by TA cloning. Sequencing of the insert in the recombinant plasmids indicated that the 1.5 kb DNA fragment contained the coding sequence of human E-NTPDase 8 whereas the 1.7 kb DNA fragment contained two introns in addition to the coding sequence. The 1.5 kb insert was excised from the recombinant pGEM-T-Easy plasmid by *Eco*RI digestion and ligated to the pcDNA3 vector that was also digested with *Eco*RI and treated with calf intestinal phosphatase. The recombinant pcDNA3 plasmid was used to transform DH5α *Escherichia coli* cells, and DNA was isolated from several colonies. Orientation of the 1.5 kb DNA insert in pcDNA3 was determined by restriction enzyme mapping. Colonies containing insert DNA with the correct orientation were propagated, and DNA was isolated.

For the purpose of obtaining a His- and V5-tagged human E-NTPDase 8, a 1.5 kb cDNA without the stop codon was obtained by using the 1.5 kb PCR product described above as the template, the same forward primer, and a reverse primer lacking the stop codon (5′-GTCCTGCAACCAGAAGAGCTG-3′) in a PCR reaction using *Taq* polymerase with an annealing temperature of 55 °C. The ~1.6 kb PCR product was ligated to the pcDNA3.1/V5-His vector by TA cloning. After transformation, the bacterial colony containing insert with the correct orientation was propagated and DNA isolated.

Molecular Cloning of the Extracellular Domain (ECD) of Human E-NTPDase 8. A 1.3 kb DNA which codes for the extracellular domain of human E-NTPDase 8 (amino acid residues 31−471) was obtained by using the full-length human E-NTPDase 8 cDNA in pcDNA3 as the template. The forward primer, GCATACTGCAAGCTT*GAGGCCACCAGC*, contains a *Hin*dIII site (underlined) upstream of the sequence that anneals to nucleotide 91−102 (italicized) of human E-NTPDase 8 and a reverse primer, GCTCGAATTCGCGGCCGC*TTTGGCCACC*, that contains a *Not*I site (underlined) downstream of nucleotide 1404−1413 (italicized) of human E-NTPDase 8. PCR reaction was carried out using *Pfu* Turbo DNA polymerase in the following thermal cycling program: 4.5 min at 94 °C, followed by 35 cycles of 0.45 min at 94 °C, 1 min at 60 °C, and 3 min at 72 °C, and ending with 10 min at 72 °C. The PCR product obtained was digested with *Hin*dIII and *Not*I and ligated to pSecTag2 that had been digested with the same restriction enzymes. After transformation in DH5α cells, the bacterial colony containing plasmid DNA with correctly orientated insert was propagated and DNA isolated.

Expression of Human E-NTPDase 8 and Its ECD. Human embryonic kidney 293 (HEK293) cells were used for transfection of human E-NTPDase 8 in pcDNA3 or pcDNA3.1/V5-His and human E-NTPDase 8 ECD in pSecTag2 using lipofectamine as described previously (*10*). Cells transfected with human E-NTPDase 8 cDNA were harvested 48−72 h after transfection for ATPase activity determination. Stably transfected cells were established as described previously (*10*, *20*) by geniticin selection (300 μg of geniticin/mL of media). HEK293 cells stably transfected with human E-NTPDase 8 ECD in pSecTag2 were established by selecting and propagating cells that were resistant to zeocin (200 μg/mL).

Purification of Soluble Human E-NTPDase 8 ECD. HEK293 cells stably transfected with the human E-NTPDase 8 ECD in pSecTag2 were grown in DMEM containing 5% fetal calf serum and 5% newborn calf serum. Cells harvested from one 10 cm confluent plate were distributed into three plates. These cells usually reach confluency after 4 days with change of media after 2 days. Media collected at midgrowth and confluency were combined and used for purification of

the human E-NTPDase 8 ECD. Pooled media (100−140 mL) were subjected to ammonium sulfate fractionation. Approximately 80% of the total activity and 20% of the protein were recovered in the fraction that was precipitated between 45% and 70% ammonium sulfate saturation. The precipitate was dissolved in 50 mM Tris-HCl, pH 8, and applied to a His.bind column with a bed volume of 2−2.5 mL. The column was washed and eluted according to the manufacturer's instruction except the concentration of imidazole in the elution buffer was reduced from 1.0 to 0.5 M. Approximately 40% of total activity applied to the column was eluted together with several unrelated proteins by the wash buffer which contains 60 mM imidazole in 20 mM Tris-HCl, pH 7.9, and 0.5 M NaCl. The remaining 60% of ATPase activity with little contaminating proteins was recovered in the second and third 1 mL fraction after elution with 0.5 M imidazole in 20 mM Tris-HCl, pH 7.9, and 0.5 M NaCl. The soluble human E-NTPDase 8 activity was stable for several weeks when stored at 0 °C.

Nuclotide Hydrolase Activitiy Determination. For determination of ATPase activity of intact cells, the harvested cells were washed and resuspended in isotonic buffer (0.1 M NaCl, 0.01 M KCl, and 25 mM Tris-HCl, pH 7.5), and aliquots (50−100 μg of cell protein) were used for ATPase determination. Enzymatic properties of the human and chicken E-NTPDase 8 were characterized using membrane preparations enriched in plasma membranes prepared from stably transfected HEK293 cells as previously described (*10*). Specific ATPase activities in membrane preparations from different batches of stably transfected cells varied from 1.3 to 3.2 μmol min^{-1} mg^{-1} at pH 7.5. There was little loss of enzyme activity when membranes were stored at −20 °C for several months. Unless indicated otherwise, standard ATPase assays were carried out in a reaction mixture (0.5 mL) containing 25 mM Tris-HCl, pH 7.5, 2.5 mM $MgCl_2$, and 2.5 mM ATP for 10 min at 37 °C. Phosphate released was determined colorimetrically as described previously (*18*). In determining the K_m for MgATP and CaATP of human E-NTPDase 8 and its ECD and ATPase activity of the ECD, [γ-^{32}P]ATP (∼1000 cpm/nmol) was used as the substrate. [^{32}P]P_i released was separated from radioactive ATP by isobutyl alcohol−benzene extraction after complexing with ammonium molybdate (*21*). All assays were carried out in duplicate. Values are given as mean ± standard deviations.

Cross-Linking and Deglycosylation of Human E-NTPDase 8. Cross-linking of membrane proteins by 10 mM glutaraldehyde was carried out as previously described (*10*, *20*). Deglycosylation by PNGase F was carried out using membranes containing human and chicken E-NTPDase 8 according to the manufacturer's instructions.

SDS−PAGE and Western Blot Analysis. SDS−PAGE and Western blot analysis were carried out as described previously (*10*). Anti-V5 (2000-fold dilution) and anti-His (2500-fold dilution) were used as the primary antibodies, and goat anti-mouse Ig conjugated to alkaline phosphatase was used as the secondary antibody (5000-fold dilution). Immunoreactive protein bands were detected after reaction with the alkaline phosphatase substrate, NBT/BCIP.

RESULTS

Molecular Cloning and Identification of the Human E-NTPDase 8. We initially attempted RT-PCR cloning of human E-NTPDase 8 using human cDNA reverse transcribed from human liver RNA as template and various pairs of primers with the nucleotide sequence corresponding to apyrase conserved regions (ACR) of the chicken E-NTPDase 8. These attempts were unsuccessful. Subsequently, Blast search using the amino acid sequence of chicken E-NTPDase 8 revealed a homologous human sequence (BC050293, released April 2003), which was cloned from a human testes cDNA library. Translation of the 2851 nucleotide sequence in three different frames indicated that a contiguous coding sequence containing the hallmark ACR sequences of E-NTPDases could be pieced together from sequences translated in frame 3 and frame 1. In addition to the 3′-untranslated region (nt 1−551) and 5′-untranslated region (nt 2287−2851), the coding sequence is disrupted by two introns (nt 949−1077 and 1237−1352). Besides the five ACR, the amino acid sequence deduced from the 1.5 kb coding sequence revealed the presence of four other conserved regions (CR), ten conserved cysteine residues, and two transmembranous domains that are characteristic of other cell surface E-NTPDases (*9*, *11*, *14*, *17*, *19*).

The IMAGE clone containing the 2.85 kb DNA (BC050293) was purchased. A 1.7 kb DNA fragment corresponding to the coding sequence and containing the two introns was obtained by PCR using primers corresponding to the 5′- and 3′-ends of the coding region. The 1.7 kb DNA (AY903953) was inserted first into pCR2.1 by TA cloning and then excised and introduced into the mammalian expression vector, pcDNA3. However, cells tranfected by the recombinant pcDNA3 plasmid containing the 1.7 kb cDNA did not express any ectonucleotidase activity. This result indicated that the transfected cells were not able to excise the two introns to yield the 1.5 kb cDNA that codes for a functional protein. Subsequently, both 1.7 and 1.5 kb DNA were detected in RT-PCR reactions using human liver RNA as the template and the same PCR primers as described above. The 1.5 kb cDNA was amplified and inserted into pcDNA3 and the pcDNA3.1/V5-His vector as described in Materials and Methods. HEK293 cells transiently transfected with these plasmids express an ATPase activity that is 5−10-fold greater than HEK293 cells transfected with empty vectors.

The nucleotide sequence of human E-NTPDase 8 (AY903954) and the deduced amino acid sequence are shown in Figure 1. The 495 amino acid sequence shares 52% identity and 66% similarity with that of chicken E-NTPDase 8 (AF426405) (*17*) and 67% identity and 77% similarity with that of mouse E-NTPDase 8 (AY364442) (*19*). As expected, there is greater nucleotide homology (∼80%) throughout the entire coding sequences of human and mouse E-NTPDase 8, whereas extensive homology between human and chicken E-NTPDase 8 nucleotide sequences is limited to only four regions, i.e., in ACR2, ACR5, and CR3 and between CR4 and ACR5.

While the deduced amino acid sequence of human E-NTPDase 8 contains similar ACR and CR and the same conserved cysteine residues as the chicken and mouse E-NTPDase 8, it has only seven potential N-glycosylation sites compared to eight in mouse E-NTPDase 8 and twelve in chicken E-NTPDase 8. Additionally, transmembranous domains predicted by the TMHMM and TMPred programs indicate that it has only two amino acid residues in the

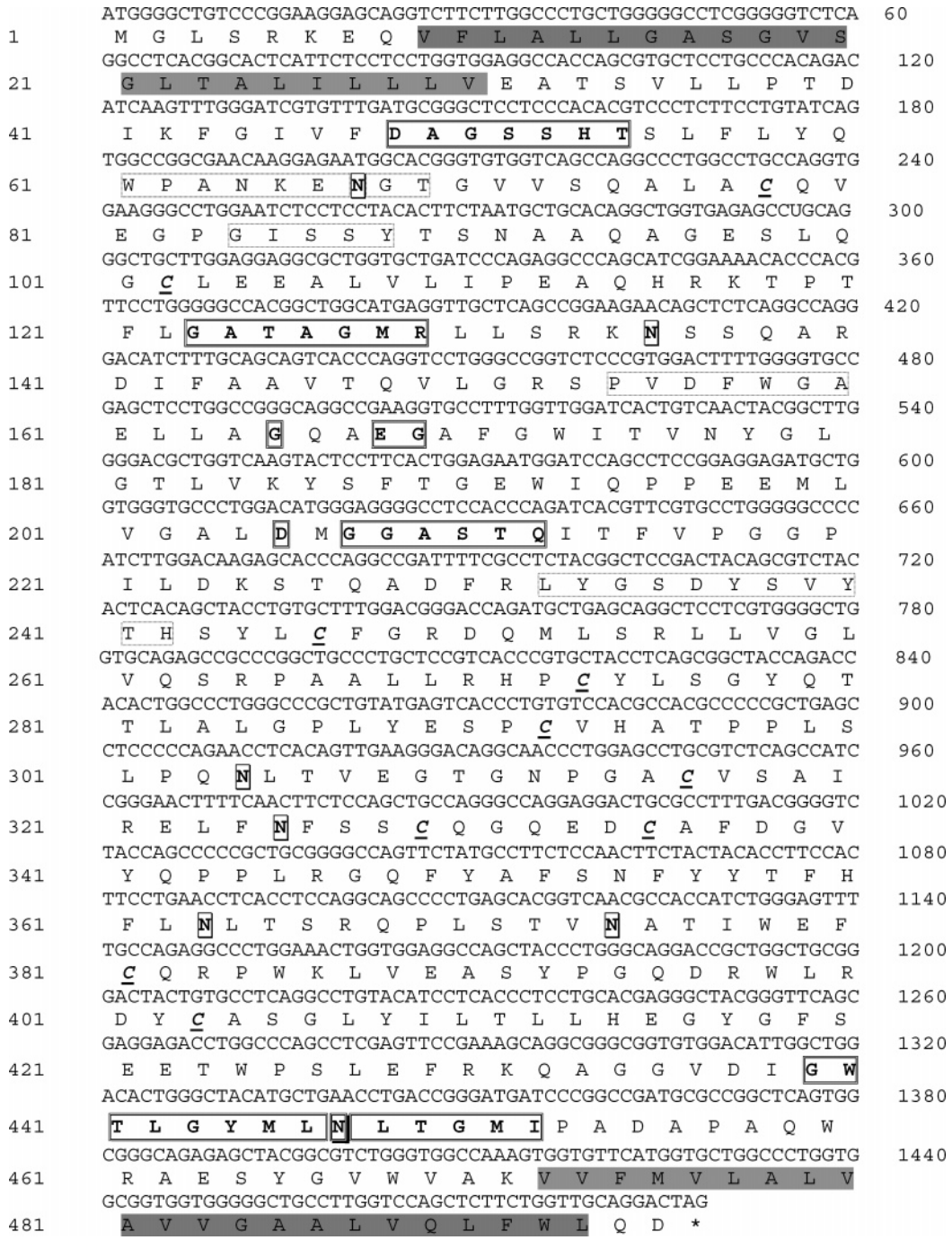

FIGURE 1: Nucleotide and deduced primary sequences of human E-NTPDase 8. Nucleotide numbers are on the right side and amino acid residue numbers are on the left side of the figure. The transmembranous domains of the protein at the N- and C-terminus are shaded. The five apyrase conserved regions (ACR) are in bold and in double-lined boxes. The four conserved regions (CR) common to all cell surface E-NTPDases are in boxes bordered by broken lines. The ten conserved cysteine residues (in italic and bold) are underlined. Asparagine residues in N-glycosylation consensus sequences, NX(T/S), are in individual boxes.

intracytoplasmic domain at the C-terminus (Figure 1) compared to seven in both chicken and mouse E-NTPDase 8 (*17*, *19*).

Blast search revealed several sequences that are related to the human E-NTPDase 8. A 1.7 kb sequence (AY430414) contains the 1.5 kb coding sequence of E-NTPDase 8 as well as 3′- and 5′-untranslated sequences. Another human sequence (AY359088 or NM_198585) cloned in the secreted protein discovery initiative encodes a protein that has nearly identical sequence to the human E-NTPDase 8; however, it lacks 27 amino acids (corresponding to aa 351−387) in the C-terminal half of the protein. A rat sequence (XM_231041) annotated as similar to blood group A glycosyltransferase I contains additional nucleotides in the middle of the coding sequence. A partial mouse E-NTPDase 8 sequence (BC031143) lacks the N-terminal 197 amino acids.

We transfected HEK293 cells with the 1.5 kb human E-NTPDase 8 cDNA in pcDNA3.1/V5-His which would allow detection of the expressed protein using anti-V5 antibody. Western blot analysis indicated that the expressed protein has a lower molecular mass (∼90 kDa) (Figure 2, lane 4) than the mature chicken E-NTPDase 8 (∼100 kDa) (Figure 2, lane 2) despite the added molecular mass of ∼5 kDa from the V5-His tag. The lower molecular mass of the

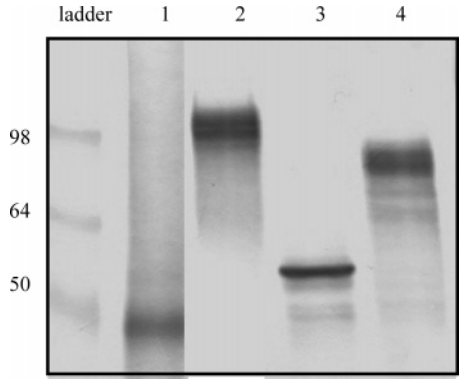

FIGURE 2: Molecular masses of expressed human and chicken E-NTPDase 8 without and with deglycosylation. Membranes were prepared from HEK293 cells stably transfected with human E-NTPDase 8 cDNA (in pcDNA3.1/V5-His) and chicken E-NTPDase 8 cDNA (in pcDNA3). The membranes (50 μg) were denatured and either untreated or treated with PNGase F as described in Materials and Methods overnight at 37 °C. Aliquots of membrane proteins (15 μg) were used for Western blot analysis. After separation by SDS−PAGE, the proteins were transferred to PVDF membrane, and the blot was probed with anti-V5 at 2000-fold dilution (for human E-NTPDase 8) or monoclonal antibody, MC18, at 400-fold dilution (for chicken E-NTPDase 8). Because the epitope in chicken E-NTPDase 8 recognized by MC 18 is lost upon reduction of the sample, β-mercaptoethanol was eliminated from sample buffers. Lanes: ladder, SeeBlue (Invitrogen) prestained standards; lane 1, deglycosylated chicken E-NTPDase 8; 2, untreated chicken E-NTPDase 8; 3, deglycosylated human E-NTPDase 8; 4, untreated human E-NTPDase 8.

human E-NTPDase 8 is expected from its fewer potential N-glycosylation sites. The estimated molecular mass of expressed human E-NTPDase 8 is ~85 kDa. After deglycosylation by PNGase-F, the human (495 amino acids) and chicken E-NTPDase 8 (493 amino acids) polypeptides should have nearly identical molecular mass, ~54 kDa. However, the mobility of the deglycosylated chicken E-NTPDase 8 (Figure 2, lane 1) indicated a protein with a molecular mass of ~50 kDa according to the "SeeBlue" ladder (Figure 2). Nevertheless, the greater molecular mass of the deglycosylated human E-NTPDase 8 polypeptide due to the V5-His tag is clearly shown by its slower mobility (Figure 2, lane 3).

ATP and ADP Hydrolysis by Human E-NTPDase 8. E-NTPDase 8, as well as E-NTPDases 1 and 3, is an ecto-ATPDase that hydrolyzes both NTP and NDP. We determined the ATP and ADP hydrolysis activities of human E-NTPDase 8 in the presence of either Mg^{2+} or Ca^{2+} in buffers of different pH using membranes prepared from HEK293 cells stably transfected with human E-NTPDase 8 cDNA. Figure 3A shows that MgATP, MgADP, and CaADP hydrolyses have pH optima in the range of pH 6−7 whereas CaATP hydrolysis has a more alkaline pH optima in the range of pH 8−9 where CaATPase activity also exceeds MgATPase activity. Substantial ATP hydrolysis activity remains even at pH 10 with either Mg or Ca. In contrast, ADP hydrolysis activity diminishes at pH higher than 7. CaADP activity is greater than MgADPase activity at all pH values.

The pH−activity curves of human E-NTPDase 8 and its utilization of the different nucleotide substrates differ from that obtained with purified chicken E-NTPDase 8 from liver (*17*) and oviduct (*18*, *22*). A comparison of ADP hydrolysis by the expressed human and chicken E-NTPDase 8 with either Mg or Ca shows that the MgADPase/MgATPase ratio of the human E-NTPDase 8 is significantly lower than that obtained with the chicken E-NTPDase 8 at pH 7.5 (Table 1). The ratio is only increased slightly at pH 6.4, which increases the ADPase activity of chicken E-NTPDase 8 significantly (*17*) as seen by the higher MgADPase/MgATPase ratio of chicken E-NTPDase 8. The inability of the human E-NTPDase 8 to hydrolyze MgADP at neutral pH is reminiscent of E-NTPDase 2, which hydrolyzes NDP poorly under most conditions (*9*, *10*, *23*). The CaADPase/CaATPase ratios of the human E-NTPDase 8 are greater than the MgADPase/MgATPase ratios at both pH 7.5 and pH 6.4 and are similar to the values obtained with the chicken E-NTPDase 8.

The K_m values for MgATP and MgADP of expressed human E-NTPDase 8 at pH 7.5 are 226 ± 2.8 and 127 ± 18 μM, respectively, and 135 ± 12 and 177 ± 26 μM for CaATP and CaADP. The K_m values for ATP are similar to that for chicken E-NTPDase 8, but K_m values for ADP are 1 order of magnitude lower than that for chicken E-NTPDase 8, which is in the range of 3.9−5.3 mM (*17*, *18*, *22*). Despite the lower K_m for ADP, contribution to the amount of P_i produced when ATP is used as a substrate is minimal for two reasons: (i) the amount of ADP produced during the ATP hydrolysis reaction is well below the K_m values, and (ii) V_{max} of ADP hydrolysis by the enzyme is less than 20% of the V_{max} for ATP hydrolysis (data not shown).

Similar to chicken E-NTPDase 8 (*17*, *20*, *22*) and other ecto-ATPDases, the expressed human E-NTPDase 8 is inhibited by high concentrations (5−10 mM) of azide with the greatest inhibition (80−90%) obtained at pH 6.4 using MgADP as the substrate (see Table 5).

Effect of Membrane Perturbation on Human E-NTPDase 8 Activity. In addition to different preferences for nucleotide substrates, the most striking difference between the human and chicken E-NTPDase 8 is inhibition of the former, but not the latter, by detergents. The chicken E-NTPDase 8 is not inhibited by NP-40 (*20*) and can be purified after solubilization from membranes by 5% NP-40 (*17*, *18*). Other detergents commonly used in membrane solubilization also have little effect on the chicken E-NTPDase 8 activity at 0.1% (Table 2). While this low concentration of detergents is insufficient to solubilize membrane proteins, NP-40, Triton X-100, and dodecyl β-D-maltoside inhibit the human E-NTPDase 8 by 80−90%. Octyl glucoside, which has a significantly higher critical micelle concentration than dodecyl β-D-maltoside, inhibits human E-NTPDase 8 by ~50% at 0.1%. Inhibition of human E-NTPDase 8 activity by NP-40 is substantially reduced if the membranes are pretreated by glutaraldehyde, a chemical cross-linking agent, so that ~80% activity remains at 0.2% NP-40 (Figure 4A). Inhibition by NP-40 is also reduced if the membranes are treated with a bifunctional cross-linking agent, disuccinimidyl suberate, but it is less effective than glutaraldehyde (data not shown).

The human and chicken E-NTPDase 8 also show different responses to the membrane fluidizing agent, benzyl alcohol (*24*). The chicken E-NTPDase 8 is slightly stimulated at all benzyl alcohol concentrations tested whereas human E-NTPDase 8 activity is inhibited (Figure 4B). Nevertheless, ~60% of human E-NTPDase 8 activity still remains at 0.5%

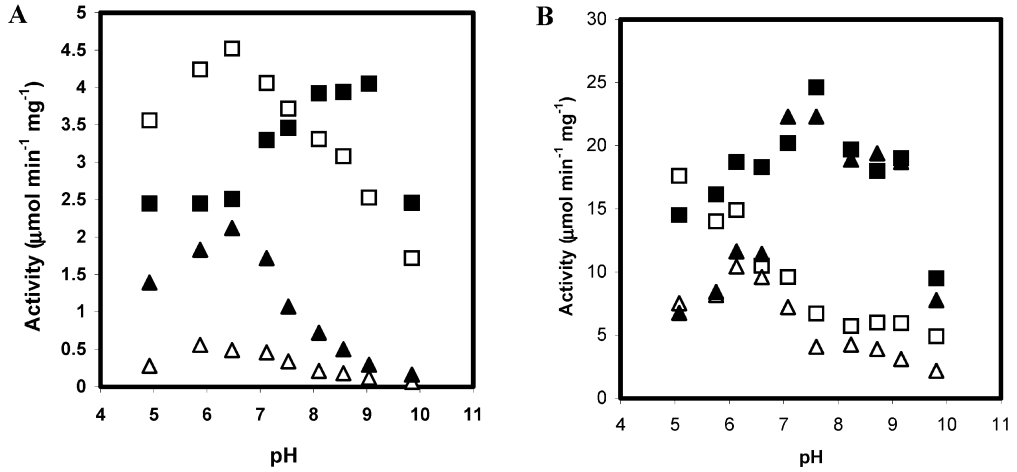

FIGURE 3: Effect of pH on nucleotidase activity of human E-NTPDase 8 and its ECD. (A) The nucleotidase activity of expressed human E-NTPDase 8 was determined with MgATP (□), CaATP (■), MgADP (△), or CaADP (▲) in a 0.5 mL reaction mixture containing 25 mM buffer, 2.5 mM substrates, and 15 μg of membrane protein. The reaction time was 10 min at 37 °C. The following buffers were used: acetate (pH 4.8), imidzole (pH 6−7), Tris-HCl (pH 7−9), and glycine (pH 9.8). (B) The same experiment was conducted with 0.5 μg of partially purified human E-NTPDase 8 ECD. Data shown were from one of three separate experiments.

Table 1: ADPase/ATPase Ratios of Human and Chicken E-NTPDase 8 Obtained with Mg^{2+} and Ca^{2+} Nucleotides at pH 7.5 and 6.4[a]

	MgADPase/MgATPase		CaADPase/CaATPase	
	pH 7.5	pH 6.4	pH 7.5	pH 6.4
human E-NTPDase 8	0.07 ± 0.038	0.132 ± 0.017	0.59 ± 0.034	0.97 ± 0.016
chicken E-NTPDase 8	0.16 ± 0.066	0.80 ± 0.046	0.31 ± 0.06	1.36 ± 0.064

[a] ATPase and ADPase activities were determined in reaction mixtures containing either 25 mM Tris-HCl (pH 7.5) or 25 mM Mops (pH 6.4) with 2.5 mM Mg or Ca nucleotide using 5−15 μg of membrane proteins. Membranes were prepared from HEK293 cells stably transfected with human E-NTPDase cDNA or chicken E-NTPDase 8 cDNA in pcDNA3. The reaction time was 10 min at 37 °C ($n = 3$). MgADPase/MgATPase ratios of human E-NTPDase 8 are significantly lower than that of chicken E-NTPDase 8 at both pH 7.5 and pH 6.4 whereas CaADPase/CaATPase ratios of the two enzymes are comparable.

Table 2: Effect of Various Detergents on the MgATPase Activities of Human and Chicken E-NTPDase 8[a]

detergent	human E-NTPDase 8	chicken E-NTPDase 8
none	100	100
NP-40	9.14 ± 0.06	118.6 ± 4.6
Triton X-100	17.9 ± 5.83	126.6 ± 0.8
deoxycholate	95.1 ± 8.30	101.2 ± 3.3
Chaps	80.5 ± 8.44	99.8 ± 3.8
dodecyl β-D-maltoside	21.0 ± 2.13	125.0 ± 7.3
octyl glucoside	46.4 ± 1.31	102.8 ± 1.5
digitonin	102.4 ± 2.97	120.2 ± 5.2

[a] ATPase activity was determined in reaction mixtures containing 25 mM Tris-HCl, pH 7.5, 2.5 mM $MgCl_2$, and 2.5 mM ATP using 5−15 μg of membrane proteins in the absence and presence of 0.1% of the indicated detergents ($n = 2$). The 100% values for the human E-NTPDase 8 are 2.59 and 2.92 μmol min^{-1} mg^{-1} in the two experiments, and the 100% values for chicken E-NTPDase 8 are 6.1 and 8.0 μmol min^{-1} mg^{-1} in the two experiments. Chicken E-NTPDase 8 is not inhibited by any of the detergents whereas human E-NTPDase 8 is inhibited by NP-40, Triton X-100, dodecyl β-D-maltoside, and octyl glucoside.

benzyl alcohol. The response of the human E-NTPDase 8 to benzyl alcohol is thus intermediate between the more stable chicken E-NTPDase 8 and the labile human E-NTPDase 2 (*10*, *20*), which is inhibited more than 80% by most of the detergents shown in Table 2 and by 0.5% benzyl alcohol (data not shown). Inhibition of the human E-NTPDase 8 activity by benzyl alcohol is also reduced when the membranes are pretreated by glutaraldehyde (data not shown).

The ATPase activity of human E-NTPDase 8 increases in the range of 25−37 °C, and activity is maintained at higher temperature up to 55 °C (Figure 5A). The response of human E-NTPDase 8 to temperature is also intermediate between that of human E-NTPDase 2 and chicken E-NTPDase 8. The activity of human E-NTPDase 2 declines after 37 °C (*10*), and activity at 55 °C is ∼50% of that at 37 °C (*20*). In contrast, the activity of chicken E-NTPDase 8 increases up to 60 °C, and activity at 55 °C is ∼200% of that at 37 °C (*20*).

Transcription of E-NTPDase Genes in Human Liver. It has been shown previously by Northern blot analysis that, of the five human E-NTPDase genes [*ntpd1* (*CD39*), *ntpd2* (*CD39L1*), *ntpd3* (*CD39L3*), *ntpd5* (*CD39L4*), and *ntpd6* (*CD39L2*)] cloned at the time, *ntpd5* is strongly expressed in human liver (*15*). Subsequently, it was reported that *ntpd5* encodes a nucleoside diphosphatase (E-NTPDase 5/CD39L4) that is secreted (*5*, *25*). To compare the extent of transcription of *ntpd8* and *ntpd5/CD39L4* in human liver, we conducted PCR reactions using human liver cDNA as the template and primers that anneal to the 5′- and 3′-ends of full-length cDNA of human E-NTPDase 2, 5, and 8. The results in Figure 6 show that transcripts of E-NTPDase 8 (Figure 6, lane 2) and E-NTPDase 5 (Figure 6, lane 3) were detected in the human liver cDNA whereas the transcript of E-NTPDase 2 was not detectable (Figure 6, lane 4). Interestingly, the major E-NTPDase 8 species detected in human liver cDNA was that of the 1.7 kb cDNA, in amounts exceeding the 1.5 kb cDNA which codes for the functional E-NTPDase 8 (see control in Figure 6, lane 1). This result agrees with that

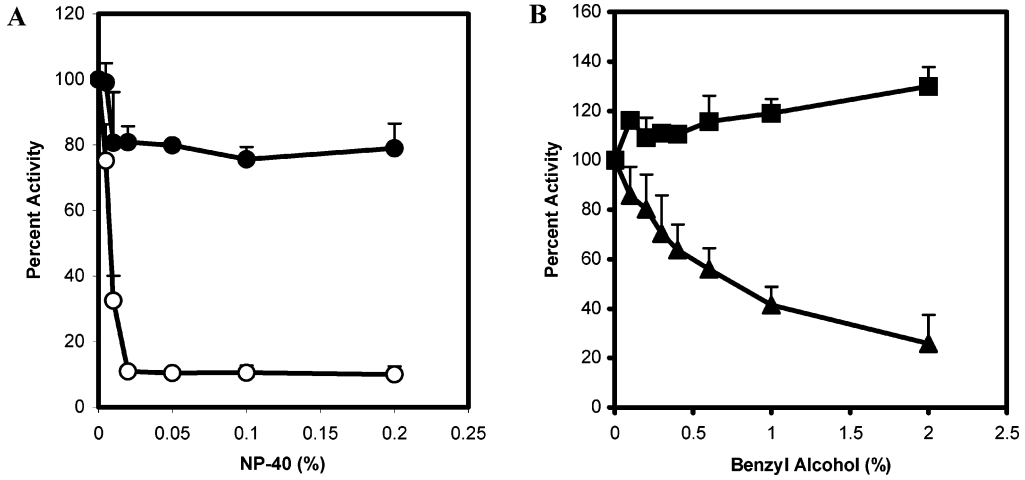

FIGURE 4: Effect of NP-40 and benzyl alcohol on MgATPase activity of human E-NTPDase 8. (A) Membranes prepared from HEK293 cells stably transfected with human E-NTPDase 8 were untreated (○) or treated with glutaraldehyde (●) as described in Materials and Methods. Aliquots of the membranes (19 μg) were used for determination of MgATPase activity in the absence or presence of the indicated concentrations of NP-40. (B) MgATPase activities of membranes (4.4−14.8 μg) prepared from HEK293 cells stably transfected with chicken E-NTPDase 8 cDNA (■) or human E-NTPDase 8 (▲) were determined in the absence or presence of the indicated concentrations of benzyl alcohol. The reaction time was 10 min at 37 °C. 100% values for human E-NTPDase 8 activity are in the range of 1.64−3.75 μmol min^{-1} mg^{-1}. 100% values for chicken E-NTPDase 8 activity are in the range of 4−8.5 μmol min^{-1} mg^{-1}. Human E-NTPDase 8 ATPase activity is inhibited by both NP-40 and benzyl alcohol whereas chicken E-NTPDase 8 ATPase activity is not. Inhibition of human E-NTPDase 8 activity by NP-40 is abolished if the enzyme is cross-linked by glutaraldehyde before incubation with NP-40.

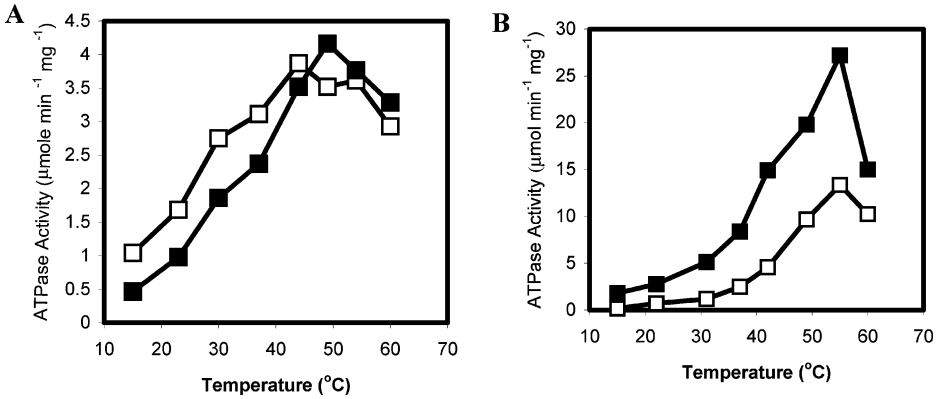

FIGURE 5: Effect of temperature on the ATPase activity of human E-NTPDase 8 and its ECD. (A) Reaction mixtures containing 25 mM Tris-HCl, pH 7.5, and 2.5 mM MgATP (□) or 2.5 mM CaATP (■) were brought to the indicated temperatures in water baths for 5 min. Reactions was initiated by the addition of 15 μg of membrane protein and terminated after 10 min by the addition of trichloroacetic acid. (B) Experimental conditions were similar to that described in (A) using soluble human E-NTPDase 8 ECD. ATPase assays were carried out in a 0.25 mL reaction mixture containing 25 mM Tris-HCl, pH 7.5, and 1 mM MgATP or 1 mM CaATP with 0.4 μg of protein. Data shown were from one of three separate experiments.

obtained with RT-PCR using human liver total RNA as described above and indicates that human liver cells accumulate significant amounts of an E-NTPDase 8 transcript that is not properly spliced.

Biochemical Characterization of the Extracellular Domain of Human E-NTPDase 8. We hypothesized that the inhibitory effects of detergents and benzyl alcohol on the human E-NTPDase 8 are mediated by its transmembranous domains. To provide evidence for this hypothesis, we constructed a recombinant plasmid by inserting a DNA fragment that codes for the extracellular domain (ECD) of human E-NTPDase 8 into the pSecTag2 vector which has an Ig_κ leader sequence so that the expressed protein, which also contains a His tag at its C-terminus, is secreted. HEK293 cells transfected by the recombinant plasmid did not display any cell surface ATP hydrolysis activity above that of the mock-transfected cells whereas ATPase activity was detected in the culture media. The secreted and soluble ATPase was purified by ammonium sulfate fractionation followed by affinity chromatography on a nickel (His.bind) column. The purified ECD have a specific activity of 12−30 μmol min^{-1} (mg of protein)$^{-1}$ with CaATP as the substrate at pH 7.5. An overall purification of ∼2000−5000-fold was obtained in four separate purification experiments. The marked purification was seen in the stained SDS−polyacrylamide gel in Figure 7A (compare lane 3 with lane 1). Diffuse protein bands in the range of 70−90 kDa were seen in the fraction eluted from the nickel column by 0.5 M imidzole (Figure 7A, lane 3). The significant purification obtained was also seen in the Western blot using anti-His antibody. Only a weak signal was seen in the ammonium sulfate fraction (Figure 7B, lane 1) compared to the strong signal obtained with the purified protein (Figure 7B, lane 3). Some of the human E-NTPDase 8 ECD was eluted by the wash buffer which contains 60 mM imidazole as indicated by both activity determination and Western blot analysis (Figure 7B, lane 2); however, the majority of the protein eluted is unrelated (Figure 7A, lane 2). The molecular mass of the ECD as indicated by Western blot analysis

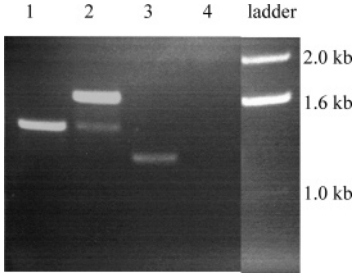

FIGURE 6: Determination of the amounts of transcript of human E-NTPDase 2, 5, and 8 in human liver cDNA. PCR reactions were conducted in a 50 μL reaction volume using Platinum PCR Supermix (Invitrogen) with 0.1 μg of human E-NTPDase 8 cDNA in pcDNA3 (lane 1) or 1 μg of PCR Ready cDNA from human liver (lanes 2−4). An annealing temperature of 60 °C was used in thermal cycling. An aliquot of the sample (20 μL) was analyzed by gel electrophoresis on 1% agarose. Lanes: 1 and 2, PCR primers annealing to the 5′- and 3′-ends of human NTPDase 8 cDNA (accession number AY903954) were used in PCR reaction; 3, PCR primers annealing to the 5′- and 3′-ends of the coding region of human E-NTPDase 5 cDNA (accession number AF039918) were used; 4, PCR primers annealing to the 5′- and 3′-ends of human E-NTPDase 2 (accession number AF144748) were used. The amount of unspliced 1.7 kb human E-NTPDase 8 cDNA exceeds that of the coding 1.5 kb cDNA. The cDNA of human E-NTPDase 5, which codes for an ADPase, is also present in human liver cDNA, but the cDNA of human E-NTPDase 2, which codes for an ecto-ATPase normally present in brain and muscle tissues, is not detectable under the experimental condition.

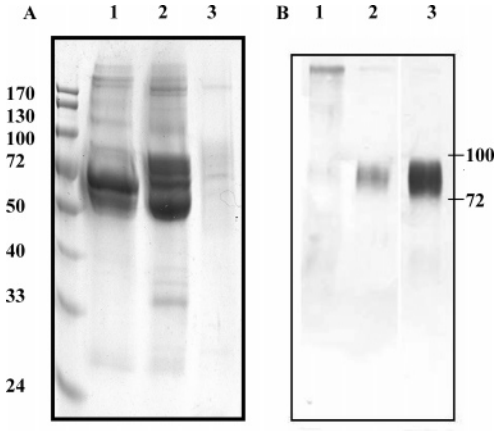

FIGURE 7: SDS−PAGE and Western blot analysis of partially purified human E-NTPDase 8 ECD. (A) SDS−PAGE analysis of partially purified human E-NTPDase 8 ECD. The gel was stained by Coomassie blue after gel electrophoresis. Lanes: 1, soluble proteins (20 μg) that were secreted into the culture media and precipitated by 45−70% saturation ammonium sulfate; 2, proteins (10 μg) eluted from the His.bind column by 60 mM imidazole; 3, proteins (0.3 μg) eluted by 0.5 M imidazole. (B) Western blot analysis of purified human E-NTPDase 8 ECD. Lanes: 1, proteins precipitated by 45−70% ammonium sulfate (25 μg); 2, proteins eluted from the His.bind column by 60 mM imidazole (25.6 μg); 3, proteins eluted by 0.5 M imidazole (0.3 μg). The immunoblot was treated by anti-His antibody as the primary antibody and goat anti-mouse conjugated with alkaline phosphatase as the secondary antibody. The immunoreactive protein has a molecular mass of ~80 kDa.

corresponds to the expected molecular mass (~80 kDa) of a protein, which has 64 fewer amino acid residues than the full-length enzyme but contains 21 extra amino acids due to the Myc and His epitopes.

The enzymatic properties of the soluble human E-NTPDase 8 ECD differ significantly from the membrane-bound form. In contrast to the membrane-bound human E-NTPDase 8 (Figure 4 and Table 2), its ECD is no longer inhibited by NP-40, dodecyl β-D-maltoside, or benzyl alcohol (Table 3), providing definitive evidence that the inhibitory effects of these reagents on human NTPDase 8 are mediated by its TMD. Unexpectedly, the human E-NTPDase 8 ECD displays greater activity with Ca nucleotides as the substrates. Whereas the CaATPase activity of the membrane-bound human E-NTPDase 8 is slightly lower than its MgATPase at neutral and more acidic pH (Figure 3A), the CaATPase activity of the ECD is ~4-fold of its MgATPase activity at pH 7.5 (Table 4). Similarly, ~4 times higher activity was obtained with CaUTP than with MgUTP (Table 4). ADP and UDP hydrolysis are also greater with Ca^{2+} than with Mg^{2+}. Despite the lower MgATPase activity, the K_m for MgATP of human E-NTPDase 8 ECD is 5.1 ± 0.4 μM, which is almost 50-fold lower than that obtained with the membrane-bound enzyme. The K_m for CaATP is also decreased, however, by only 4-fold to 39 ± 7 μM.

Figure 5B further shows that MgATPase activity of the human E-NTPDase 8 ECD is lower than CaATPase activity at all temperatures. In addition, the sharp increase of activity between 37 and 55 °C differs from that observed with the membrane-bound E-NTPDase 8 (Figure 5A). The CaATPase activity of the human E-NTPDase 8 ECD is also higher than its MgATPase activity in buffers of pH more alkaline than 6 (Figure 3B). MgATPase activity is comparable to CaATPase activity at pH below 6 but decreases markedly between

Table 3: Lack of Inhibitory Effect of NP-40, Dodecyl β-D-Maltoside, and Benzyl Alcohol on the ATPase Activity of Soluble Human E-NTPDase 8 ECD[a]

addition	MgATPase	CaATPase
none	100	100
NP-40 (0.1%)	130.7 ± 40.7	137.0 ± 19.1
dodecyl β-D-maltoside (0.1%)	144.2 ± 13.3	127.8 ± 21.9
benzyl alcohol (1%)	105.6 ± 23.6	95.9 ± 14.8

[a] MgATPase and CaATPase activities of human E-NTPDase 8 ECD (2.5−4 μg) were determined in a 0.25 mL reaction mixture containing 25 mM Tris-HCl, pH 7.5, and 1 mM MgATP or 1 mM CaATP in the absence or presence of the indicated concentrations of NP-40, dodecyl β-D-maltoside, and benzyl alcohol for 15 min at 37 °C ($n = 3$). [^{32}P]P_i released from [γ-^{32}P]ATP was determined as described in Materials and Methods. ATPase activity of soluble human E-NTPDase 8 is not inhibited by NP-40, dodecyl β-D-maltoside, or benzyl alcohol.

Table 4: Hydrolysis of ATP, ADP, UTP, and UDP in the Presence of Mg^{2+} and Ca^{2+} by Human E-NTPDase 8 and Its Soluble ECD[a]

substrate	human E-NTPDase 8 (μmol min^{-1} mg^{-1})	human E-NTPDase 8 ECD (μmol min^{-1} mg^{-1})
MgATP	3.55 ± 0.23	2.5 ± 0.42
CaATP	2.89 ± 0.09	11.47 ±1.47
MgADP	0.30 ± 0.001	2.5 ± 0.00
CaADP	1.23 ± 0.12	10.52 ± 1.12
MgUTP	3.65 ± 0.51	2.07 ± 0.43
CaUTP	5.67 ± 1.10	9.22 ± 0.34
MgUDP	0.37 ± 0.02	1.64 ± 0.26
CaUDP	0.81 ± 0.28	6.29 ± 0.95

[a] Membranes prepared from HEK293 cells stably transfected with human E-NTPDase 8 (4.3 μg) or human E-NTPDase 8 ECD (2.3 μg) were assayed in a 0.25 mL reaction mixture containing 25 mM Tris-HCl, pH 7.5, and the indicated substrates (1 mM) for 10−20 min at 37 °C. The amount of P_i released was determined colorimetrically. Data were obtained in two separate experiments using the same membrane preparation containing human E-NTPDase 8 or the soluble human E-NTPDase 8.

Table 5: Effect of Azide on the ADPase Activities of Human E-NTPDase 8 and Its Soluble ECD[a]

azide (mM)	human E-NTPDase 8	human E-NTPDase 8 ECD
0	100	100
2	28.0 ± 1.8	75.2 ± 18.7
5	14.7 ± 1.56	48.8 ± 4.7
10	10.3 ± 0.9	33.1 ± 7.5

[a] Activities of human E-NTPDase 8 (21 μg) and its ECD (2.3 μg) were determined in a 0.25 mL reaction mixture containing 25 mM Mops, pH 6.4,1 mM MgADP, and the indicated concentrations of azide at 37 °C. The reaction time was 10 min. P_i released was determined colorimetrically ($n = 3$). 100% values of human E-NTPDase ADPase activity are in the range of 0.36−0.53 μmol min^{-1} mg^{-1}. 100% values of human E-NTPDase 8 ECD ADPase activity are in the range of 4.3−5.0 μmol min^{-1} mg^{-1}. The soluble human E-NTPDase 8 is less sensitive to azide inhibition than the membrane-bound enzyme.

pH 5 and pH 8, whereas CaATPase activity increases in the same pH range. The reduction of MgATPase activity with increasing pH differs from that observed with the membrane-bound enzyme, the MgATPase activity of which decreases significantly only at pH higher than 8 (Figure 3A). Figure 3B also shows that the MgADPase/MgATPase ratios of the ECD are approximately 1 at most pH values in contrast to the low ratios (≤0.1) obtained with the membrane-bound E-NTPDase 8 (Figure 3A). Despite a higher ADPase/ATPase ratio, the soluble human E-NTPDase 8 is more resistant to azide inhibition. ADP hydrolysis by the membrane-bound enzyme at pH 6.4 is inhibited ∼70% by 2 mM and ∼85% by 5 mM azide, whereas the soluble enzyme is inhibited by only ∼25% and ∼50% at the respective azide concentrations (Table 5).

In summary, the removal of the TMD of human E-NTPDase 8 has markedly altered its enzymatic properties. In addition to becoming insensitive to inactivation by detergents and benzyl alcohol, the soluble enzyme displays higher activity in the presence of Ca^{2+}, altered K_m values for ATP, higher activity with ADP, but loss of inhibition by azide. In addition, the CaATPase and MgATPase activities of the soluble and membrane-bound human E-NTPDase show marked difference with respect to the effect of temperature and pH.

DISCUSSION

An ATPase that is associated with the bile canaliculi of rat hepatocytes was detected in many earlier studies using cytochemical staining (*26*−*28*). Subsequent determination of ATP hydrolysis activity in rat liver plasma membrane preparations enriched in either the bile canaliculi or sinusoid also indicated that the former has a higher ATPase activity than the latter and that the activity is the manifestation of a cell surface ATPase which also hydrolyzes ADP, i.e., an ecto-ATPDase (*29*). The first ecto-ATPDase of this type to be purified to homogeneity was obtained from chicken liver and oviduct (*17*, *18*). The localization of the chicken liver ecto-ATPDase at the bile canaliculi was confirmed by immunolocalization (*17*). The chicken ecto-ATPDase has been cloned (*16*, *17*) and is a member of the ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDase) family (*1*), which is composed of several subfamilies based on sequence homology. The primary structure and tissue distribution of the chicken ecto-ATPDase distinguish it from two other cell surface ecto-ATPDases, i.e., CD39 (E-NTPDase 1) (*11*, *12*) and CD39L3/HB6 (E-NTPDase 3) (*14*, *15*). A mammalian homologue of chicken liver and oviduct ecto-ATPDase, assigned to the subfamily of E-NTPDase 8, was recently cloned from mouse liver, expressed, and characterized (*19*).

Earlier studies using cytochemical staining also showed that the cell surface ATPase activity of carcinogen-induced hepatoma is altered with respect to magnitude, distribution, and biochemical properties when compared to that on normal rat hepatocytes (refs *28*, *30*, and *31* and summarized in ref *32*). Molecular reagents are needed to determine if a similar process occurs in human liver cancer development. We have shown previously that the cell surface ATPase of a human hepatoma cell line, Li-7A (*21*), and small cell lung carcinoma (*33*) have the properties of an ecto-ATPase with little activity toward ADP. This was corroborated by molecular cloning of the ecto-ATPase/E-NTPDase 2 cDNA from the two human tumors (*10*). Ecto-ATPases are normally present in the muscle and brain (*34*−*38*). In the liver, its distribution is limited to the portal fibroblasts, but not on the hepatocytes (*39*), and is therefore a minor component of the liver ecto-nucleotidase repertoire. This paper reports for the first time the cloning and expression of the major E- NTPDase of human liver, an E-NTPDase 8.

While the human and chicken E-NTPDase 8 are homologous, there are several important differences with respect to their enzymatic properties. (1) The human E-NTPDase 8 has markedly reduced activity toward MgADP (Table 1 and Figure 3). (2) The human and chicken E-NTPDase 8 have different pH−activity curves of MgATP hydrolysis (compare Figure 3 in this paper and Figure 2 in ref *17*). (3) The human E-NTPDase 8 has higher CaATPase activity than MgATPase activity at pH higher than 7.5 (compare Figure 3 in this paper and Figure 1A in ref *22*). (4) The human E-NTPDase 8 is inhibited by several detergents and benzyl alcohol, which have no effect on chicken E-NTPDase 8 activity (Table 3 and Figure 4B). The last observation indicates that the chicken E-NTPDase 8 is most likely unique and is thus far the only membrane-bound E-NTPDase that is resistant to detergent inhibition. We recently demonstrated that the inhibitory effect of detergents on human ecto-ATPase/E-NTPDase 2 is mediated by its TMD, since the chicken E-NTPDase 8 becomes inhibited by NP-40 if either of its TMD at the N- or C-terminus is exchanged with the corresponding region of the human ecto-ATPase/E-NTPDase 2 (*20*). Conversely, the human ecto-ATPase/E-NTPDase 2 becomes resistant to detergent inhibition if both of its TMD are exchanged with those of chicken E-NTPDase 8 (W.-C. Chiang and A. F. Knowles, EB2006 meeting abstracts). We have proposed that the different detergent sensitivities of the chicken E-NTPDase 8 and human E-NTPDase 2 are due to different strengths of interaction of the respective pairs of TMD which have different amino acid sequences (*20*). An examination of the sequences in the two TMD of the human E-NTPDase 8 indicates that there are fewer polar amino acids than those in the chicken E-NTPDase 8 and, like human E-NTPDase 2, may have weaker interhelical interaction. The amino acid sequences of mouse and human E-NTPDase 8 TMD are more similar; however, the effect of detergents on the expressed mouse E-NTPDase was not determined. Compared to human E-NTPDase 8, the mouse E-NTPDase is more active with CaATP than MgATP as the substrate at

pH 7.4. While the pH−activity of CaADP hydrolysis is quite similar to that of the human E-NTPDase 8 with a pH optimum of ∼6.5, the CaATPase activity of mouse E-NTPDase 8 declines more rapidly at pH >8 (*19*).

To demonstrate that the TMD of human E-NTPDase-8 are responsible for mediating detergent inhibition, we constructed and expressed a soluble enzyme composed only of the extracellular domain. Similar soluble E-NTPDases have been generated from both human and rat CD39/E-NTPDase 1 (*40*, *41*). In contrast to the wild-type rat CD39, its soluble form is no longer inhibited by Triton X-100 (*41*). We found this also to be the case with the ECD of human E-NTPDase 8 (Table 3). More interestingly, the biochemical properties of the ECD differ significantly from those of the wild-type enzyme. Our results indicated that removal of the TMD has converted the enzyme to a CaATPase (Table 4 and Figure 3B). Furthermore, ADP (and UDP) hydrolysis activity of the ECD is increased relative to ATP (and UTP) hydrolysis so that NDPase/NTPase ratios are higher (∼1) than that obtained with the membrane-bound enzyme (∼0.1−0.4) (Table 4). The ECD also shows an increase of affinity for the substrates, a difference in response to pH, and a decrease of sensitivity to azide inhibition. These results strongly suggest that the conformation of the active site in the ECD differs from that in the membrane-bound enzyme and lends further support to the proposal that the TMD of E-NTPDases play important roles in regulating enzyme catalysis (*10*, *20*, *41*−*43*).

In addition to the alteration of enzyme properties, the presence of the TMD appears to be also important for the membrane-bound E-NTPDases to attain their maximal activity. It was reported previously that CaATPase activities of purified soluble human and rat CD39/E-NTPDase 1 are ∼4 μmol min^{-1} mg^{-1} (*40*, *44*). Although the partially purified human soluble E-NTPDase 8 displays a higher CaATPase activity, i.e., 12−30 μmol min^{-1} mg^{-1}, it is still less than 5% of the specific activities (∼1000 μmol min^{-1} mg^{-1}) of membrane-bound ecto-ATPase/E-NTPDase 2 purified from rabbit T-tubule (*45*) and chicken gizzard (*35*) and E-NTPDase 8 purified from chicken liver and oviduct (*17*, *18*). Taken together, these results suggest that the TMD of E-NTPDases are important structural determinants in promoting more efficient catalysis. On the other hand, while the ECD may have a conformation that differs from the "native" conformation in the wild-type enzyme, it is more amenable to structural determination than the membrane-bound enzyme and should provide useful information as to the causes of different catalytic properties from the wild-type protein.

The function of E-NTPDase 8 in liver remains to be elucidated. It is generally accepted that E-NTPDases are involved in regulation of purinergic signaling by removing the ligands of purinergic receptors, i.e., ATP, UTP, ADP, and UDP. The effect of extracelluar ATP on liver cells is well documented (*46*−*49*). The distribution of E-NTPDase 8 in the bile canaliculi (*17*) suggests that it may be important in regulating ATP-induced biliary secretion. Two other E-NTPDases, i.e., CD39/E-NTPDase 1 and CD39L3/E-NTPDase 3, although having similar catalytic activity as E-NTPDase 8 in that they hydrolyze both ATP and ADP, are expressed at low levels in the liver (*15*). CD39L4/E-NTPDase5, which is highly expressed in liver according to Northern blot analysis (*15*), is primarily an NDPase that is usually secreted (*50*). We showed by PCR that the functional 1.5 kb E-NTPDase 8 cDNA is present at a level similar to that of CD39L4/E-NTPDase 5 (Figure 6). Interestingly, the 1.7 kb cDNA of human E-NTPDase 8, which still contains two introns and is part of the 2.8 kb cDNA (BC050293), is present in larger amounts than the 1.5 kb form. Blast search in the human genome revealed that the human E-NTPDase 8 gene (NT_08756.1) is located on chromosome 9. The ∼7.7 kb genomic DNA sequence of human E-NTPDase 8 contains ten exons and nine introns with arrangement similar to that of mouse E-NTPDase 8 genomic DNA (*19*). The detection of only one improperly spliced form in the human E-NTPDase 8 mRNA suggests a mechanism for transcriptional regulation of the expression of functional human E-NTPDase 8. Proper splicing of the transcript with the two introns into the functional transcript may occur only when the hepatocytes are stimulated under special circumstances.

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