Cloning, purification, and identification of the liver canalicular ecto-ATPase as NTPDase8

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Fausther M, Lecka J, Kukulski F, Lévesque SA, Pelletier J, Zimmermann H, Dranoff JA, Sévigny J. Cloning, purification, and identification of the liver canalicular ecto-ATPase as NTPDase8. Am J Physiol Gastrointest Liver Physiol 292: G785–G795, 2007. First published November 9, 2006; doi:10.1152/ajpgi.00293.2006.—Extracellular nucleotides are critical regulators of biological functions such as regulation of cell volume and proliferation (11, 27, 46, 54). These effects are mediated by specific transmembrane receptors. The hepatic levels of extracellular nucleotides regulate critical liver functions via the activation of specific transmembrane receptors. The hepatic levels of extracellular nucleotides, and therefore the related downstream signaling cascades, are modulated by cell-surface enzymes called ectonucleotidases, including nucleoside triphosphate diphosphohydrolase-1 (NTPDase1/CD39), NTPDase2/CD39L1, and ecto-5′-nucleotidase/CD73. The goal of this study was to determine the molecular identity of the canalicular ecto-ATPase/ATPDase that we hypothesized to correspond to the recently cloned NTPDase8. Human and rat NTPDase8 cDNAs were cloned, and the genes were located on chromosome loci 9q34 and 3p13, respectively. The recombinant proteins, expressed in COS-7 and HEK293T cells, were biochemically characterized. NTPDase8 was also purified from rat liver by Triton X-100 solubilization, followed by DEAE, Affigel Blue, and concanavalin A chromatographies. Importantly, NTPDase8 was responsible for the major ectonucleotidase activity in liver. The ion requirement, apparent Km values, nucleotide hydrolysis profile, and preference as well as the resistance to azide were similar for recombinant NTPDase8s and both purified rat NTPDase8 and porcine canalicular ecto-ATPase/ATPDase. The partial NH2-terminal amino acid sequences of all NTPDase8s share high identity with the purified liver canalicular ecto-ATPase/ATPDase. Histochemical analysis showed high ectonucleotidase activities in bile canaliculi and large blood vessels of rat liver, in agreement with the immunolocalization of NTPDase1, 2, and 8 with antibodies developed for this study. No NTPDase3 expression could be detected in liver. In conclusion, NTPDase8 is the canalicular ecto-ATPase/ATPDase and is responsible for the main hepatic NTPDase activity. The canalicular localization of this enzyme suggests its involvement in the regulation of bile secretion and/or nucleoside salvage.

IN LIVER, EXTRACELLULAR NUCLEOTIDES are critical regulators of biological functions such as regulation of cell volume and proliferation (11, 27, 46, 54). These effects are mediated by specific nucleotide P2 receptors (8) that are subdivided into P2X1–7 (ion-gated channels) and P2Y1, 2, 4, 6, 11–14 (G-protein coupled receptors) (16, 19). The levels of P2 receptor agonists are regulated by various enzymes expressed at the cell surface called ectonucleotidases. This group of enzymes includes members of the ectonucleoside triphosphate diphosphohydrolase (E-NTPase) family, which is composed of eight proteins (NTPDase1–8). NTPDases hydrolyze tri- and diphosphonucleosides into their monophosphate counterparts with different ability and specificity (44, 56).

Among all tissues, the liver has one of the highest ATPase and ADPase activities (37). Histochemical studies showed that most of liver ectonucleotidase activity was associated with the canalicular domain of hepatocytes (18). It was originally believed that this activity, which was different from a classical ATPase activity, was due to a protein structurally similar to the cell adhesion molecule C-CAM 105 (39). However, this protein was later shown to be an embryocarcinogenic antigen that could not explain the high ATPase activity in this tissue (30). Further studies revealed that NTPDase1 is expressed by Kupffer cells and vascular endothelial cells (52) whereas NTPDase2 is produced by portal fibroblasts and activated hepatic stellate cells (14, 17). Yet activity assays indicated that the combined activity of these two NTPDases could not account for the major ectonucleotidase activity observed in the liver (52). Thereafter, the major ectonucleotidase activity in porcine liver was shown to bear the basic properties of an NTPDase (36, 52). Interestingly, this enzyme called hepatic ATPDase was localized in bile canaliculi membrane (52) and was proposed to be responsible for the high ectonucleotidase activity previously detected in liver (18, 40). This ectonucleotidase represents the oldest canalicular marker and has been of great importance to liver cell biologists (18, 41).

We have recently cloned a novel member of the E-NTPDase family in mouse and designated it NTPDase8 (4). In the present work, we report the cloning and biochemical characterization of NTPDase8 in human and rat species and its identification as the hepatic canalicular ecto-ATPase/ATPDase.

MATERIALS AND METHODS

Materials

Culture cell media were obtained from Invitrogen (Burlington, ON, Canada). Agarose, aprotinin, PMSF, nucleotides, tetradecylammonium hydrogen sulfate, EGTA, and sodium deoxycholate were provided by Sigma-Aldrich (Oakville, ON, Canada); sodium azide by Fisher Scientific (Fair Lawn, NJ), Triton X-100 by Roche Diagnostics

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The isolation of rat liver membrane fractions and protein solubilization were performed as previously described (51). Membrane proteins (6 mg/ml) were mixed with an equal volume of 0.6% Triton X-100. Further purification of solubilized NTPDase8 was done by column chromatography using a Pharmacia FPLC system. Unless indicated otherwise, chromatographies were carried out at the flow rate of 1 ml/min and 2-ml fractions were collected. Briefly, Triton-solubilized proteins (500 mg) were applied to a DEAE Sepharose column (DEAE fast flow, 10 cm × 2.5 cm, Amersham Biosciences, Uppsala, Sweden) and resolved in the presence of 0.1% Triton X-100, 7.5% glycerol, and 10 mM Tris, pH 8.0, with a NaCl gradient: no salt for 0–50 min, 0.03 M NaCl for 50–150 min, 0.03–0.13 M NaCl for 150–650 min, and 0.13 M NaCl for 650–750 min. Samples containing NTPDase8, as determined by activity assay and immunoblotting, were pooled, reequilibrated by ultrafiltration in an Amicon stirred cell in 0.1% Triton X-100 and 25 mM Tris/His, pH 5.95 (Millipore PBBT membrane, MWWL 30,000) and applied to an Affigel Blue column (20 cm × 1 cm, Bio-Rad, Hercules, CA). Affigel Blue unbound material was washed out with 20 ml of reequilibrating buffer, and the elution of retained proteins was done by a gradient developed from 0.1% Triton X-100, 7.5% glycerol, and 10 mM Tris, pH 6.8 (buffer A) and 0.1% Triton X-100, 7.5% glycerol, 1 M NaCl, and 10 mM Tris, pH 7.5 (buffer B). The following chromatography program was used: gradient A → B from 0% to 100% for the first 120 min and 100% B for the following 150 min. NTPDase8-enriched fractions were pooled and reequilibrated in concanavalin A (ConA) buffer (1 M NaCl, 1 mM CaCl₂, 1 mM MgCl₂, 1 mM MnCl₂, 0.1% Triton X-100, and 20 mM Pipes/0.01 M CaCl₂, pH 6.8) and loaded on a ConA Sepharose column (Amersham Biosciences, 5 ml) at gravity flow. Unbound proteins were washed out with 6 × bed volume of ConA buffer. NTPDase8 and other remaining glycosylated proteins were desorbed with 0.5 M methyl α-d-mannopyranoside (Sigma-Aldrich, St. Louis, MO) in ConA buffer. For the biochemical characterization, mannopyranoside removal and enzyme concentration (to ~1 mg/ml) were done by ultrafiltration with Centurion [YM-30] filter devices (Amicon).

**Plasmids Cloning.** The plasmid encoding rat NTPDase 1 (GenBank accession no. NM_022587) has been described in a published report (26). The complementary DNA sequences encoding human NTPDase8 (AY430414) or rat NTPDase8 (AY536920) were cloned as follows. Total RNA was isolated from rat liver with Trizol reagent (Invitrogen) or obtained from AMBION (Austin, TX) (kindly donated by Dr. C. Guillemette, Centre de Recherche du Centre Hospitalier de l’Université Laval). The cDNA was synthesized with SuperScriptII (Invitrogen) with oligo(dT)₁₅ as the primer, in accordance with manufacturer’s instructions. For amplification, 10% of the reverse transcription reaction was used as template in a reaction mixture containing 0.6 μM primer, 400 μM dNTP, and 3.5 U Expand High Fidelity PCR System (Roche, Laval, Canada) with Mg²⁺ concentrations of 1.5 mM for rat or 2.5 mM for human. Amplification was done with primers designed from human or rat expressed sequence tags (ESTs; XM_231041 and AI535212, respectively) that revealed highest homology to mouse NTPDase8 (AY364442): one human forward sequence 5′-CCA-GTA-CCA-GCT-CCA-3′ and two reverse sequences 5′-CCA-GAA-AGG-CAC-CTA-GCG3′, 5′-GGG-GTC-CCT-GCT-GTG-TTC-3′; rat sequences, forward 5′-TCA-GCC-CCT-CCC-ACC-ATG-AGA-CTT-3′ and reverse 5′-TG-TAT-CTT-ATC-TGG-GGC-AAC-T-3′. Amplification for human NTPDase8 (or rat) was started by an incubation of 2 min at 94°C, followed by 30 cycles of 30 s (rat: 15 s) denaturation at 94°C, 15 s annealing at 55.5°C (rat: 30 s at 60°C) and 2-min primer extension at 72°C, and ended with 10 min incubation at 72°C. The PCR products of ~1.5 kb were purified on 1% agarose gel using the QIAEX II gel extraction kit (Qiagen, Mississauga, ON, Canada) and ligated into the expression vector pCDNA3.1/V5-His (Invitrogen). Plasmids were purified on 1% agarose gel using the QIAEX II gel extraction kit. Purification of Rat Liver NTPDase8

Characterization of genomic sequences. The human and rat NTPDase8 cDNA sequences were used to blasted the National Center for Biotechnology Information (NCBI) genome database. The sequences identified as NT_024000.16 and NT_024000.16 showed 100% homology with human and rat NTPDase8 cDNAs, respectively. The genomic sequences and exon/intron junctions were analyzed with NCBI BLAST programs.

**PAGE and Immunoblotting Procedures**

Subcellular membrane fractions of rat hepatocytes were prepared following the method of Kipp and Arias (29). Protein concentration was estimated by the Bradford microplate assay using BSA as a standard (6). Protein samples were resuspended in 2% (wt/vol) lithium dodecyl sulfate sample buffer and fractionated by PAGE under nonreducing conditions according to Laemmli (35). Proteins were transferred to Immobilon-P membrane (Millipore, Bedford, MA) by electrophoresis. After incubation with primary antibodies, proteins of interest were visualized with the appropriate horseradish peroxidase-conjugated secondary antibody (anti-rabbit, Amersham Biosciences, Boston, MA; anti-guinea pig, Santa Cruz Biotechnology, Santa Cruz, CA) and the Chemiluminescent Reagent Plus (Perkin-Elmer, Boston, MA), as recommended.
Immunohistochemistry, Immunofluorescence, and Enzyme Histochemistry

For immunohistochemistry, sections of snap-frozen rat liver (6 μm) or cultured cells (10^5 per coverslip) were fixed in cold acetone (Fisher) mixed with 10% phosphate-buffered formalin (Fisher) and were incubated with rabbit polyclonal sera to rat NTPDase1 (rN1-6L), NTPDase2 (BZ3-4F) (14), or a guinea pig polyclonal serum to rat NTPDase8 (rN8-8c), as described previously (53). Immunofluorescence was performed with polyclonal rN8-8c serum and monoclonal anti-multidrug resistance related protein-2 (Mrp-2) antibody (kindly donated by Dr. C. Soroka, Yale Liver Center), as previously described (14). Localization of ectonucleotidase activities was determined using the Wachstein/Meisel lead phosphate method (7).

Fig. 1. Specificity of antibodies to rat NTPDase. The specificity of polyclonal sera to rat NTPDase8 (rN8-8c) and NTPDase1 (rN1-6L) was tested in A by immunocytochemistry and in B by immunoblotting. A: immunocytochemistry on mock or transfected COS-7 cells with plasmids encoding rat NTPDase8 (a) or rat NTPDase1 (b). Transfected cells expressing rat NTPDase8 displayed a high staining with rN8-8c (a, bottom right), whereas untransfected cells (mock) were devoid of immunoreactivity (a, top right) as well as when the preimmune serum was incubated with these cells (a, left). Similar results were obtained with sera to rat NTPDase1, rN1-6L (b). Scale bar = 20 μm. B: immunoblotting of protein extracts from mock (ctl) or transfected COS-7 cells with plasmids encoding rat NTPDase8 (N8) or rat NTPDase1 (N1) and protein fractions from rat and mouse tissues. Protein samples of 6 μg for cell lysates (recombinant proteins) or 10 μg for murine tissues were loaded in each well. Each serum is specific for its antigen, as demonstrated by immunoblotting with transfected cell lysates (left and middle). When tested on protein extracts from murine tissues, rN8-8c detected NTPDase8 in liver (L; highest expression), kidney (K), and jejunum (J) from rat species, but not from mouse (right). Subcellular membrane fractionation of rat liver showed a much higher intensity of the NTPDase8 immunoreactive band in the canicular membrane vesicle fraction (CMVs) than in the total liver membrane fraction (L0). Molecular weights are indicated in kDa.

Table 1. Alignment of amino acid sequences of the purified porcine canalicular ecto-ATPase/ATPase with NTPDase8s and chicken ATPases

<table>
<thead>
<tr>
<th>Enzyme Name</th>
<th>GenBank Accession Number or Reference*</th>
<th>NH2-Terminal Amino Acid Sequence</th>
<th>NH2 Terminal</th>
<th>cDNA-Based Homology</th>
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<tr>
<td>Porcine canalicular ecto-ATPase/ATPase</td>
<td>Sévigny et al. (52)</td>
<td>-GQTRKDORFVPTALLAAA</td>
<td>100</td>
<td>n/a</td>
</tr>
<tr>
<td>Porcine EST</td>
<td>BF192072/BG894445</td>
<td>MGQTRKDORFVPTALLWAA</td>
<td>85.3</td>
<td>100</td>
</tr>
<tr>
<td>Human NTPDase8</td>
<td>AY430414</td>
<td>MGLSRKEQVFLALLGAS</td>
<td>60.0</td>
<td>81.0</td>
</tr>
<tr>
<td>Mouse NTPDase8</td>
<td>AY364442</td>
<td>MGLSRKEQVFLALLGAS</td>
<td>54.9</td>
<td>79.5</td>
</tr>
<tr>
<td>Rat NTPDase8</td>
<td>AY536920</td>
<td>MGLSRKEQVFLALLGAS</td>
<td>42.8</td>
<td>77.7</td>
</tr>
<tr>
<td>Chicken ATPase</td>
<td>AF426405</td>
<td>MEYKGRVAGELITATCV</td>
<td>28.2</td>
<td>61.9</td>
</tr>
<tr>
<td>Chicken stomach ecto-apyrase/ATPase</td>
<td>Lewis-Carl and Kirley (38)</td>
<td>MEYKGRVAGELITATCV</td>
<td>28.2</td>
<td>n/a</td>
</tr>
</tbody>
</table>

Alignment of the NH2-terminal amino acid sequence of the purified porcine canalicular ecto-ATPase/ATPase was done with various NTPDase8s, chicken ATPase, and purified chicken stomach ecto-apyrase/ATPase. The identical amino acids between the different sequences are underlined. The indicated percentage of amino acid identity was calculated by using the sequence of the porcine canalicular ecto-ATPase/ATPase as template and determined by pairwise alignment. Once translated into amino acids the 5’ ends of 2 porcine liver ESTs revealed to be nearly identical to this enzyme. The cDNA-based homology was calculated by comparing the first 260 amino acid sequence deduced from the open reading frame obtained from these overlapping porcine expressed sequence tags (ESTs) with the corresponding sequence of all NTPDase8s and determined by pairwise alignment. All forms of NTPDase8 have high homology with the porcine liver canalicular ecto-ATPase/ATPase, suggesting that all these proteins are orthologs. *References are provided for partial amino acid sequences obtained from purified proteins and accession numbers are given for cDNA sequences used to deduce the amino acid sequence.
RESULTS

Given that the presence of NTPDase1 and NTPDase2 has already been demonstrated in the liver and could not account for the canalicular ectonucleotidase activity, and that there are low levels of NTPDase3 mRNA in this tissue, we hypothesized that NTPDase8 was responsible for this activity. To address this assumption, we first searched for the existence of NTPDase8 in human and rat and cloned the respective cDNAs.

Cloning and Characterization of Human ENTPD8 and Rat Entpd8 cDNAs

The sequence of human NTPDase8 includes an open reading frame of 1,485 nucleotides (rat: 1,482), which is translated into a protein of 495 amino acid residues (rat: 494) with a predicted molecular mass of 53,888 Da (rat: 54,330) and a calculated isoelectric point of 5.19 (rat: 5.83). The deduced amino acid sequence contains seven (rat: eight) potential N-glycosylation sites, five apyrase conserved regions featured by all NTPDases, and various potential phosphorylation sites. Interestingly, there is a conserved potential phosphorylation site for both protein kinase C and casein kinase II on the fourth amino acid of mouse (4), human, and rat NTPDase8s. Hydrophobicity analysis of human and rat NTPDase8s predicts two transmembrane domains, one near the NH2 terminus (amino acids 9 – 30, for both enzymes) and one near the COOH terminus (amino acids 472 – 488 for human and 466 – 488 for rat). Both human and rat cDNAs were deposited in the database (Table 1). Sequence analysis on the website http://www.ncbi.nlm.nih.gov/genome/
seq/ localized the genes to chromosome 9q34 (entry NT_024000.16) for human and chromosome 3p13 (NW_001084810.1) for rat. Alignment of the full cDNA with the human genomic sequence reveals that ENTPD8 covers 3,631 bases and is organized into nine exons (data not shown). The analysis with this recent sequence indicates that the rat gene would be organized similarly to both human ENTPD8 and mouse Entpd8.

Fig. 3. Purification of NTPDase8 from rat liver. The purification procedure is detailed in MATERIALS AND METHODS. A: DEAE column. Liver membrane proteins were solubilized with Triton X-100, loaded on a DEAE column, and eluted by a NaCl gradient from fractions 1 to 281. Three peaks of ATPase activity are distinguishable, and the 3rd and highest peak of activity corresponds to NTPDase8 (N8), as determined by immunoblotting (data not shown). A significant AMPase activity was also associated with the peaks corresponding to NTPDase2 (N2) and NTPDase8. Fractions containing NTPDase8 activity were collected (pooled fractions) and applied to an Affigel Blue column. Note that we took care to avoid NTPDase1 activity in the selected pooled fractions.

B: Affigel Blue column. After washing the column and eluting the retained proteins, 1 peak of ATPase activity corresponding to NTPDase8 as determined by immunoblotting (data not shown) was collected (pooled fractions) and further purified on a concanavalin A (ConA) column. Note that we chose to avoid most AMPase activity in the selected pooled fractions.

C: protein samples from the pooled fractions of each purification step were monitored for NTPDase8 content by SDS-PAGE and immunoblotting. NTPDase8 levels increase with each purification step: N8, NTPDase8 transfected COS-7 cell lysate (4 μg); lane 1, total liver homogenate (5 μg); lane 2, liver particulate fraction (5 μg); lane 3, DEAE chromatography pooled fractions (5 μg = 220 pmol P/min); lane 4, Affigel Blue chromatography pooled fractions (5 μg = 220 pmol P/min); lane 5, ConA chromatography pooled fractions (5 μg = 2,270 pmol P/min). Molecular weights are indicated in kDa.
The NH₂-terminal amino acid sequence deduced from the coding sequence of ENTPD8 shows high homology (60%) with the NH₂-terminal sequence of the cancriotic ecto-ATPase/ATPase purified from porcine liver (Table 1). Two porcine ESTs corresponding to this purified enzyme were obtained from the database. The comparison of the partial sequence of the 260 amino acids derived from these overlapping ESTs revealed over 75% identity with the corresponding sequence of the other mammalian NTPDase8s (Table 1). These data suggest that NTPDase8 and the hepatic cancriotic ecto-ATPase/ATPase are orthologs.

**Table 2. Table summarizing the purification of rat liver NTPDase8**

<table>
<thead>
<tr>
<th>Step</th>
<th>Total Protein, mg</th>
<th>Total Activity, μmol P₃·min⁻¹</th>
<th>Specific Activity, μmol P₃·min⁻¹·mg⁻¹</th>
<th>Purification Fold</th>
<th>Yield, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>19,000</td>
<td>1067</td>
<td>615</td>
<td>461</td>
<td>1</td>
</tr>
<tr>
<td>Particulate fraction</td>
<td>998</td>
<td>187</td>
<td>76</td>
<td>27</td>
<td>1.1</td>
</tr>
<tr>
<td>Particulate fraction + Triton X-100</td>
<td>998</td>
<td>153</td>
<td>112</td>
<td>231</td>
<td>2.1</td>
</tr>
<tr>
<td>Solubilized proteins</td>
<td>918</td>
<td>55</td>
<td>62</td>
<td>108</td>
<td>1.1</td>
</tr>
<tr>
<td>DEAE</td>
<td>160</td>
<td>7</td>
<td>11</td>
<td>20</td>
<td>0.8</td>
</tr>
<tr>
<td>Affigel</td>
<td>9.2</td>
<td>0.80</td>
<td>1.40</td>
<td>0.70</td>
<td>1.5</td>
</tr>
<tr>
<td>ConA</td>
<td>0.86</td>
<td>0.39</td>
<td>0.40</td>
<td>0.10</td>
<td>454</td>
</tr>
</tbody>
</table>

ConA, concanavalin A.

**Purification of NTPDase8 from Rat Liver**

To evaluate the relative contribution of each ectonucleotidase in the liver, we purified ectonucleotidase activities from rat liver and identified the enzymes involved with an emphasis on NTPDase8. DEAE was chosen for the separation of NTPDase1, 2, and 8 because these enzymes are characterized by different predicted isoelectric points of 7.5, 8.8, and 5.83, respectively. This separation resulted in three ATP- and ADP-hydrolyzing fractions (Fig. 3A and data not shown). Immunoblotting revealed that peaks 2 and 3 corresponded to NTPDase1 and NTPDase8, respectively, as expected from their isoelectric points. The first peak of activity could be attributed to NTPDase2 because of its isoelectric point and also by the comparison with the chromatograms from previous work.
purifications (2). A qualitative assessment of surface areas of activity in Fig. 3A suggests that the main nucleotidase activity in the liver is attributable to NTPDase8. Particular attention was taken to exclude fractions containing NTPDase1 in the NTPDase8 pooled fractions that were further separated on an Affigel Blue column. One peak of ATPase and ADPase activities was obtained that did not contain NTPDase1, as verified by immunoblotting (Fig. 3B and data not shown). To exclude contamination with ecto-5'-nucleotidase, fewer fractions with NTPDase8 activity were pooled and applied to a ConA affinity
column, as indicated in the figure. One peak of activity corresponding to NTPDase8 was observed, as confirmed by immunoblotting (data not shown). During the purification process, a clear increase in NTPDase8 contents could be detected (Fig. 3C).

Note that the purification factor of NTPDase8 shown in Table 2 is under evaluated as there are no specific substrates for NTPDase8. Phosphatases, classical ATPases, nucleotide pyrophosphatase/phosphodiesterases (NPPs), and other NTPDases, including intracellular NTPDase 4-7, also hydrolyze the same substrates. Many of these enzymes are present in the liver homogenate and most likely eliminated during the steps of purification. In addition, as highlighted above, to avoid contamination with other ectonucleotidases, some fractions displaying high NTPDase8 activity were discarded during the purification procedure. This resulted in an important loss of NTPDase8 and a much lower purification factor (Table 2). Most importantly, the ConA-purified NTPDase8 fraction was almost completely devoid of any other nucleotidases, allowing its biochemical characterization.

Biochemical Characterization of NTPDase8s

The biochemical properties of human and rat recombinant NTPDase8s were determined with cell lysates and with intact COS-7 and 293T cells transiently transfected with an expression vector (pcDNA3.1) encoding either human or rat NTPDase8. Activity assays were carried out for 12–20 min to ascertain the linearity of the reaction (data not shown). Recombinant human and rat NTPDase8s, as well as purified rat NTPDase8, required Ca\(^{2+}\) or Mg\(^{2+}\) for catalytic activity with a preference for Ca\(^{2+}\) with all nucleotides tested. Therefore the following assays for NTPDase activities were carried out in the presence of Ca\(^{2+}\). Both recombinant NTPDase8s hydrolyzed ATP, UTP, ADP, and UDP and preferred the triphosphonucleosides. In these assays, we observed some variation in the ratio of hydrolysis of ATP/ADP between intact cells and cell lysates as well as between the cell lines transfected (Fig. 4A). Neither recombinant human nor rat NTPDase8 hydrolyzed AMP (data not shown). The purified enzyme from rat liver hydrolyzed tri- and diphosphonucleosides with a ratio ATP/ADP of 0.94 and UTP/UDP of 0.98. The NTPDase8 ConA purified fraction hydrolyzed AMP at the rate of 16% of ATP, which was most likely due to the presence of some remaining ecto-5'-nucleotidase (see Fig. 3B).

Next, we tested whether azide, an inhibitor of NTPDase1 (32), could affect the activity of recombinant and purified NTPDase8s. ATPase and ADPase activities of NTPDase8s were less affected by 10 mM sodium azide than the recombinant NTPDase1s (Fig. 4B and data not shown). Since the purified enzymes were solubilized in Triton X-100, the effect of this detergent was tested. NTPDase1 has previously been reported to be highly sensitive to azide inhibition after solubilization in Triton X-100 (52). Once solubilized in this detergent, recombinant rat NTPDase8 was totally resistant to azide inhibition, as for the purified enzymes.

We also compared the activity of NTPDase1 and NTPDase8 in the presence of a common bile salt, sodium deoxycholate, which has been reported to affect differently bovine spleen NTPDases (dominantly NTPDase1) and porcine liver ATPDase activities (36). ATPase and ADPase activities of these recombinant NTPDases were affected similarly by deoxycholate (Fig. 4C and data not shown), suggesting that the canaliculare microenvironment, rather than the NTPDase, is responsible for the enzyme resistance to deoxycholate.

The apparent \(K_m\) and \(V_{max}\) values of recombinant human NTPDase8 are summarized in Table 3. In agreement with its kinetic constants, NTPDase8 hydrolyzed ATP and UTP first to their respective diphosphonucleoside and then to the monophosphonucleoside with the distinction that ADP was dephosphorylated to AMP much faster than UDP to UMP (Fig. 5). This resulted in an accumulation of UDP. Simultaneous hydrolysis of ATP and UTP confirmed that human NTPDase8 preferred adenine over uracil nucleotides as substrates, as expected from the kinetic parameters. Importantly, purified rat NTPDase8 displayed similar substrate preferences and hydrolysis profiles compared with its recombinant isoform (data not shown).

DISCUSSION

By 1) molecular cloning, 2) biochemical approaches, and 3) immunolocalization, we have demonstrated that the newly cloned ectonucleotidase NTPDase8 accounts for the high ectonucleotidase activity observed in the bile canalculus. Thus the molecular identity of the long-known hepatic canalicular ecto-ATPase is NTPDase8.

1) The existence of NTPDase8 in human and rat liver was established by cDNA cloning based on a sequence homology with the mouse NTPDase8 (4). Importantly, the transfection of COS-7 and HEK293T cells with an expression vector encoding human or rat NTPDase8 led to the appearance of nucleotidase activity at the cell surface, demonstrating that these enzymes are ectonucleotidases. Genomic analysis with these cDNAs revealed that ENTPD8 is located on chromosome 9q34 and rat Entpd8 on chromosome 3p13. The distinct localization of both ENTPD8 and rat Entpd8 confirms that the products of these genes are different from the other previously characterized NTPDases (34). Analysis of the deduced amino acid sequence of each of these NTPDase8s, as well as of chicken ecto-ATPase (33, 38), with the canalicular ecto-ATPase/ATPase purified from porcine liver showed high identity, suggesting that these enzymes are orthologs.

2) Our purification procedure combined with immunoblotting experiments showed that NTPDase8 is responsible for the main ectonucleotidase activity in liver. In rat liver, the combined ecto-ATPase activities of the two other ectonucleotidases NTPDase1 and NTPDase2 were far less than that of NTPDase8 alone. In further support for this notion, ectonucleotidase activity and histochemistry performed in Entpd1\(^{-/-}\) mice

<table>
<thead>
<tr>
<th>Substrate</th>
<th>(K_m) (\mu)M</th>
<th>(V_{max}) (\text{nmol P}	ext{min}^{-1}\text{mg}^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>81 ± 2</td>
<td>790 ± 35</td>
</tr>
<tr>
<td>ADP</td>
<td>137 ± 13</td>
<td>163 ± 9</td>
</tr>
<tr>
<td>UTP</td>
<td>480 ± 32</td>
<td>1100 ± 50</td>
</tr>
<tr>
<td>UDP</td>
<td>241 ± 17</td>
<td>110 ± 4</td>
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</tbody>
</table>

Human NTPDase8 exhibited Michaelis-Menten kinetics for the hydrolysis of ATP, ADP, UTP, and UDP. Apparent \(K_m\) and \(V_{max}\) values were estimated from Eadie-Hofstee plots created with GraphPad Prism software (GraphPad Software, San Diego, CA). Results are expressed as means ± SE of 3 separate experiments, each performed in triplicate.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>(K_m) (\mu)M</th>
<th>(V_{max}) (\text{nmol P}	ext{min}^{-1}\text{mg}^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>81 ± 2</td>
<td>790 ± 35</td>
</tr>
<tr>
<td>ADP</td>
<td>137 ± 13</td>
<td>163 ± 9</td>
</tr>
<tr>
<td>UTP</td>
<td>480 ± 32</td>
<td>1100 ± 50</td>
</tr>
<tr>
<td>UDP</td>
<td>241 ± 17</td>
<td>110 ± 4</td>
</tr>
</tbody>
</table>

Human NTPDase8 exhibited Michaelis-Menten kinetics for the hydrolysis of ATP, ADP, UTP, and UDP. Apparent \(K_m\) and \(V_{max}\) values were estimated from Eadie-Hofstee plots created with GraphPad Prism software (GraphPad Software, San Diego, CA). Results are expressed as means ± SE of 3 separate experiments, each performed in triplicate.
have previously been described for recombinant mouse NTPDase8 (4) and purified porcine hepatic ATPDase (52). We have also observed that the solubilization of rat NTPDase8 with Triton X-100 affected the biochemical properties of the enzyme. For example, the addition of Triton to rat recombinant NTPDase8 made this nucleotidase completely resistant to azide. This effect may be attributable to the transmembrane domains of the enzyme, as shown by Knowles et al. with a mutant of human NTPDase8 lacking both hydrophobic domains (31). In general, the recombinant enzyme cloned by the latter group showed similar biochemical characteristics with the ones presented here with some differences for deoxycholate and azide inhibition that may be due to different buffer compositions.

3) Histochemical data showed that the ectonucleotidase activity in the liver matched the immunolocalization pattern of NTPDase1, 2, and 8. NTPDase8 represented the ectonucleotidase activity of the bile canaliculi. NTPDase1 and NTPDase2 were responsible for the ectonucleotidase activity in blood vessels; NTPDase1 was present on endothelial cells and smooth muscle cells whereas NTPDase2 was on adventitial cells. NTPDase2 was also detected on portal fibroblasts, in the vicinity of bile ducts. These observations for NTPDase1 and NTPDase2 are in agreement with previous reports (14, 52).

Other hepatic ectonucleotidases have been previously reported in the liver and their contribution to nucleotide hydrolysis needs to be considered as well. The expression of NPP1 and NPP3 has been described in rat liver (21, 22, 50, 55). The products of triphosphonucleoside hydrolysis by NPPs are a monophosphonucleoside and a pyrophosphate group, both undetectable by the malachite green method used in this work to estimate ectonucleotidase activity (5, 56). The latter enzymes are meanwhile more active in alkaline pHs than at physiological pH (50). Another class of ectonucleotidases expressed in the liver is the alkaline phosphatase family (56). However, their contribution to the ectonucleotidase activity in the liver may be considered negligible in our experiments, as the hydrolysis of tri-, di-, and monophosphonucleosides was not significantly reduced in the presence of tetramisole, an inhibitor of this enzyme.

Taken together, these data indicate that NTPDase8 is the canalicular ecto-ATPase/ATPase and, moreover, accounts for the major liver ectonucleotidase activity. The highly specific localization and biochemical properties of NTPDase8 suggest potential functions in the liver. NTPDase8, which efficiently hydrolyzes ATP and UTP, may attenuate and/or terminate the activation of receptors for these two nucleotides (P2X1–7 and P2Y2,4,11) but may also favor the activation of ADP and UDP specific receptors (P2Y1,6,12,13), as it produces a transient accumulation of diphosphonucleosides. As a result, NTPDase8 would modulate the activity of P2 receptors present in the hepatocyte membrane bordering the bile canaliculi upstream, but also along the epithelium lining the intrahepatic biliary tree downstream. Interestingly, the expression of multiple P2 receptors (P2X2,4,6 and P2Y1,2,4,6,13) mRNAs has been reported in hepatocytes (12) and cholangiocytes (13, 15, 20). The activation of these nucleotide receptors is involved in the regulation of various functions like cell volume regulation, ATP release, ion secretion, and bile formation (13, 15, 24, 28, 45–48, 57). A potential function of NTPDase2 in the physiology of cholangiocytes has recently been demonstrated in vitro.

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**Fig. 5. Nucleotide hydrolysis and product formation by human recombinant NTPDase8.** The nucleotide hydrolysis pattern was determined by HPLC, as described in MATERIALS AND METHODS. Human NTPDase8 was incubated with ATP and/or UTP (500 μM), and the formation of nucleotide derivatives was followed over a period of 1 h. In A and B, 24 nmol/min of enzymatic activity were used. This amount was doubled for C. A: ATP hydrolysis: ATP (●), ADP (●), AMP (●). Human NTPDase8 dephosphorylates ATP to ADP, which is then hydrolyzed to AMP. No adenosine production was detected. B: UTP hydrolysis. UTP (●), UDP (●), UMP (●). The profile of UTP hydrolysis (UTP → UDP → UMP) is similar to that observed for ATP, with the difference that the conversion of UDP to UMP is slower than that of ADP to AMP. No uridine production was detected. C: concurrent hydrolysis of ATP and UTP: ATP; ADP; AMP; UTP; UDP; UMP. NTPDase8 prefers adenine nucleotides over uracil nucleotides as substrates. Notice that AMP accumulates more rapidly than UMP.

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showed that, despite the absence of NTPDase1, the liver of these mice retained most ATPase and ADPase activities (O. Guckelberger and J. Sévigny, personal observations). Recombinant human and rat NTPDase8 as well as the purified rat NTPDase8 favored hydrolysis of triphosphonucleosides over diphosphonucleosides also with a preference for adenine over uracil nucleotides. All NTPDase8s analyzed preferred Ca2+ over Mg2+ for nucleoside triphosphatase and diphosphatase activities and were resistant to sodium azide. Similar properties
(25). In the latter study, NTPDase2 expressed by portal fibroblasts blocked the mitogenic response of neighboring cholangiocytes, through the modulation of their basolateral P2Y receptors.

The coexpression of NTPDase8 with ecto-5′-nucleotidase/CD73 (data not shown and Ref. 49) in the bile canaliculi suggests that the monophosphonucleosides (e.g., AMP) eventually produced by NTPDase8 are further dephosphorylated to the nucleosides (e.g., adenosine), also responsible for various biological functions in liver. For example, adenosine has been shown to protect the liver against inflammation-induced injury via specific A2A receptor activation (3, 42, 43). By their biological functions in liver. For example, adenosine has been shown to protect the liver against inflammation-induced injury via specific A2A receptor activation (3, 42, 43). By their enzymatic activities, NTPDase8 and ecto-5′-nucleotidase would control the levels of nucleotide and/or nucleosides present in the bile flow and possibly their reuptake and salvage by hepatocytes. Of great importance for such a function, nucleoside transporters are also highly expressed in the canicular domain of hepatocytes (10, 11, 23). There are evidences that nucleotides are released from hepatocytes and bile duct cells in vitro; the concentration of adenosine nucleotides in canicular effluents was estimated in the order of 0.1 to 5 μM (9, 19). Because liver represents the major source of purines for tissues incapable of de novo nucleotide synthesis such as brain, intestinal mucosa, muscles, and bone marrow, adequate nucleotide hydrolysis and nucleoside reuptake in the bile canaliculi may be essential for maintenance of purine balance at the extrahepatic level.

In summary, the canicular ecto-ATPase is NTPDase8. The coexpression of NTPDase8, CD73, and nucleoside transporters in bile canaliculi suggests the importance of extracellular nucleotide signaling in the control of bile secretion and in nucleoside-nucleotide balance and reuptake.

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