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Functional expression of a cDNA encoding a human ecto-ATPase

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1 The metabolism of extracellular nucleotides plays an important role in nucleotide signalling mediated by P2 receptors. The nucleotide sequence encoding a putative human ecto-ATPase named CD39L1 was reported recently. However, the biological activity of this protein has not been established.

2 Based on the sequence of CD39L1 we isolated from mRNA from human ECV-304 cells a sequence encoding a 495 amino acid protein that is identical to CD39L1, with the exception that this sequence contains a 23 amino acid stretch in the putative extracellular loop that is missing in CD39L1. Partial sequence of a genomic DNA clone indicates that the CD39L1 gene corresponds to an alternative spliced form of the human ecto-ATPase.

3 Stable expression of isolated sequence in NIH-3T3 mouse fibroblasts conferred a marked nucleotide hydrolytic activity consistent with the activity of an ecto-ATPase.

4 The human ecto-ATPase hydrolyzed all naturally occurring nucleoside triphosphates in a Ca^{2+}- or Mg^{2+}-dependent manner. Nucleoside diphosphates were hydrolyzed at a rate approximately 5% of that of the corresponding triphosphates. The apparent K_m and V_{max} values were: 394 ± 62 μM and 107 ± 7 nmol Pi min^{-1} 10^6 $cells^{-1}$ for the hydrolysis of ATP, and 102 ± 33 μM and 4 ± 0.4 nmol Pi min^{-1} 10^6 $cells^{-1}$ for the hydrolysis of ADP, respectively.

5 In conclusion, we report here the cloning and functional expression of a human ecto-ATPase. The study of the biochemical properties and the regulatory mechanisms of ecto-ATPases of defined sequence will be valuable in the definition of their role in nucleotide signalling.

Keywords: ecto-ATPase; CD39; CD39L1; nucleotide hydrolysis; ecto-nucleotidases; extracellular nucleotides; NIH3T3 cells; alternative splicing

Abbreviations: ACR, apyrase conserved regions; ADP, adenosine 5′-diphosphate; AMP, adenosine 5′-monophosphate; ATCC, American type culture collection; ATP, adenosine 5′-triphosphate; CTP, cytidine 5′-triphosphate; DSM, German cell culture collection; GTP, guanosine 5′-triphosphate; NDP's, nucleoside 5′-diphosphate; NTP's, nucleoside 5′-triphosphates; PCR, polymerase chain reaction; Pi, inorganic phosphate; UTP, uridine 5′-triphosphate

Introduction

Extracellular ATP and other nucleotides modulate important physiological responses through interaction with G protein-coupled receptors and ligand-gated ion channel receptors known as P2Y and P2X receptors, respectively (Dubyak & El-Moatassim, 1993; Abbracchio & Burnstock, 1994; Harden *et al*., 1995). Nucleotide-promoted signalling is abrogated by the rapid degradation and/or interconversion of extracellular nucleotides by ecto-nucleotidases. For example, ATP is a potent agonist at $P2Y_2$ receptors, but its hydrolysis by ecto-enzymes produces the corresponding nucleotide di- and monophosphate and the nucleoside adenosine; all of these metabolic products are inactive at $P2Y_2$ receptors. On the other hand, ADP is more potent than ATP at $P2Y_1$ receptors and adenosine is the cognate agonist of A1, A2 and A3 adenosine receptors. Although the physiological significance of ecto-nucleotidases is not fully understood, their ubiquitous distribution (Plesner, 1995) and frequent co-localization with P2 receptors indicate that their primary function is associated with nucleotide signalling. Recent reports suggest that ecto-nucleotidases have additional roles including cell adhesion, vesicular transport, and purine recycling (Plesner, 1995; Zimmermann, 1996b; Strobel & Rosenberg, 1993; Kittel & Bacsy, 1994).

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Ecto-nucleotidases comprise a large group of extracellular enzymes expressing different nucleotide selectivities and biochemical properties. Thus, ecto-ATPase hydrolyzes nucleoside 5′-triphosphates (NTP's) into nucleoside 5′-diphosphates (NDP's) and inorganic phosphate (Pi); ecto-ATP diphosphohydrolase, also known as ATPDase, apyrase or CD39, hydrolyzes NTP's and NDP's at approximately the same rate, producing the corresponding nucleoside monophosphates. Other enzymes involved in extracellular nucleotide hydrolysis or interconversion include phosphodiesterases, adenylate kinases, nucleoside diphosphate kinase (NDPK), ecto-5′ nucleotidase, nucleotide pyrophosphatases, dinucleoside polyphosphate hydrolases, etc. (Zimmermann, 1996a).

Ecto-ATPase is an integral membrane protein that requires millimolar concentrations of divalent cations (Ca^{2+} or Mg^{2+}) and hydrolyzes all naturally occurring nucleoside 5′-triphosphates. The activity of ecto-ATPase against nucleoside diphosphates is only 3–5% of the activity observed against nucleoside triphosphates (Kegel *et al*., 1997). Ecto-ATPase activity is not inhibited by the commonly used inhibitors of intracellular ATPases (Plesner, 1995; Zimmermann, 1996a). Genes encoding ecto-ATPases have been cloned from chicken gizzard (Kirley, 1997), rat brain (Kegel *et al*., 1997) and mouse hepatoma cells (Gao *et al*., 1998). The predicted structure of the proteins encoded by these genes suggests that the ecto-ATPase contains two transmembrane domains with very short amino- and carboxy-termini facing the intracellular space, and a large extracellular loop containing several potential N-

glycosylation sites. This structure is remarkably similar to that predicted for ATP diphosphohydrolase (Kirley, 1997; Kegel *et al*., 1997; Gao *et al*., 1998; Zimmermann *et al*., 1998). A human ecto-ATPase sequence named CD39L1 was reported recently by Chadwick & Frischauf (1997). This sequence exhibits a high degree of homology with previously described ecto-ATPases (57–83%) and with the ecto-ATP diphosphohydrolases from different species (40–50%), and sequence comparisons indicated CD39L1 was most prominently different in a 23-amino acid sequence not present in the last third of the putative extracellular loop of the protein. Biological activity of the cloned CD39L1 has not been reported.

In this study we describe the cloning and functional expression of a DNA sequence obtained from human ECV-304 cells. This sequence contains an open reading frame of 1485 base pairs and has homology with the ecto-ATPases previously reported (chicken gizzard, rat brain and mouse hepatoma cells). Moreover, the ECV-304 cell DNA corresponds to the sequence reported by Chadwick & Frischauf (1997), except that the DNA isolated here encodes a 23 amino acid sequence that was not present in the previously reported sequence. Stable expression of this gene in NIH-3T3 mouse fibroblast cells confers a marked nucleotide hydrolytic activity consistent with the activity of an ecto-ATPase.

Methods

Materials

ATP was from Research Biochemicals Inc. (Natick, MA, U.S.A.). UTP, CTP, GTP, XTP, ITP, ADP, UDP, AMP, dATP, dUTP, dCTP, dGTP, adenosine and hygromycin B were from Boehringer Mannheim (Mannheim, Germany). HPLC-grade water and acetonitrile were purchased from Fisher Scientific (Pittsburgh PA, U.S.A.). KH_2PO_4 standard solution was from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All tissue culture reagents were from the Lineberger Comprehensive Cancer Center tissue culture facility at the University of North Carolina. Sources of other products were previously reported (Boyer *et al*., 1997)

Cell culture

The murine packaging cell line PA317 was grown in DMEM containing 4.5 g liter^{-1} of glucose, 10% foetal calf serum, and no antibiotics. ECV304 cells (ATCC CRL-1998) were grown in 199 medium supplemented with 10% foetal calf serum, 50 U ml^{-1} penicillin and 50 μg ml^{-1} streptomycin. NIH-3T3 cells stably expressing the ecto-ATPase and vector control cells were grown in DMEM containing 4.5 g liter^{-1} of glucose, 10% bovine calf serum, and 400 μg ml^{-1} hygromycin B. All cells were maintained at 37°C in a humidified atmosphere of 5% CO_2, 95% air except where indicated.

Human ECV-304 cells were originally introduced to the cell banks as an endothelial cell line. However, recently the origin of this cell line has been questioned, and there is unpublished evidence that ECV-304 cells derived from the human bladder T24 cell line. For updated information on the origin of ECV-304 cells contact the American type culture collection (ATCC) or the German cell culture collection (DSM).

Isolation of the human ecto-ATPase cDNA

The cDNA encoding the human ecto-ATPase was obtained by RT–PCR amplification of mRNA from human ECV304 cells. Messenger RNA was isolated from ECV304 cultures using the Fast Track 2.0 Kit (Invitrogen). First-strand cDNA synthesis was performed using 0.1 μg of mRNA as template and oligo(dT) as primer for the reverse transcriptase (RT–PCR Kit, Stratagene). The PCR reaction (100 μl) was developed in the presence of 5.0 Units of *Pfu* DNA Polymerase (Stratagene) and contained 5 μl of the cDNA obtained in the RT reaction as the template and 0.4 μM of each of the amplification primers. The PCR primers were designed to amplify the complete coding sequence of the putative human ecto-ATPase CD39L1 (GenBank accession number U91510), and corresponded to the 5′- and 3′-untranslated regions. To facilitate the cloning into the expression vector, the primers were designed to contain restriction sites for *Eco*RI and *Xho*I in the 5′ and 3′ ends, respectively. Sense primer, 5′-GAG AGA ATT CCC GCC CAT GGC CGG GAA GGT GCG GTC A-3′, antisense primer, 5′-GAG ACT CGA GAG TAC GGG GTG GGG ATA CAG GGG TTG G-3′. The PCR protocol consisted of 35 cycles of amplification structured in the following steps: 45 s at 94°C, 1 min at 60°C, and 3 min at 72°C, and included an initial 4.5 min denaturation period at 94°C and a final 10 min extension at 72°C in a Perkin Elmer DNA thermal cycler model 480. The amplified PCR product was purified (QIAquick PCR Purification Kit, QIAGEN), digested with *Eco*RI and *Xho*I, and ligated into the retroviral expression vector pLXPIH, previously digested with the same restriction enzymes. The resultant DNA construct was amplified and purified (QIAGEN). DNA was sequenced at the UNC-CH Automated DNA Sequencing Facility on a model 377 DNA sequencer (Perkin Elmer, Applied Biosystems Division) using the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase (Perkin Elmer, Applied Biosystems Division). The PCR amplification product was absolutely dependent on the presence of reverse transcriptase, indicating that the amplified sequence originated from mRNA. The amplified sequence contained an open reading frame encoding a predicted 495 amino acid protein.

Stable expression of the human ecto-ATPase in NIH-3T3 mouse fibroblast cells

Retrovirus harboring the ecto-ATPase was produced as previously described by Comstock *et al*. (1997). Briefly, the pLXPIH vector harbouring the human ecto-ATPase cDNA was transfected into the murine packaging cell line PA317 by the calcium phosphate transfection method. Transfected cells were incubated for 48 h at 32°C in the presence of 5 mM butyrate and the cell supernatant containing packaged retroviruses was collected, filtered, and used to infect wild type NIH-3T3 cells. Infection was carried out for 2 h in the presence of 8 μg ml^{-1} polybrene. After 48 h, infected NIH-3T3 cells were selected for hygromycin B resistance by supplementing the growth medium with 400 μg ml^{-1} hygromycin B. Clonal cell lines that stably express the human ecto-ATPase were obtained. NIH-3T3 cells expressing the empty pLXPIH vector were produced in the same manner and used as controls in further experiments.

Assay of ecto-ATPase activity

NIH-3T3 cells stably expressing the vector control or the ecto-ATPase were seeded in 48-well plates at 5×10^4 cells per well and assayed after cells reached confluence, usually 2–3 days after plating. Briefly, the cells were washed once with 1 ml of phosphate-free saline solution (mM): NaCl 125, KCl 5.2, HEPES 20, (pH 7.4), $CaCl_2$ 2, $MgCl_2$ 1.2 and D-glucose 5, and

incubated at 37°C in 250 μl final volume of the same medium containing the indicated concentrations of nucleotide. The incubation was terminated by transferring the cell-free supernatants of each well to a new plate containing 50 μl per well of 60 mM EDTA at 4°C. The cell supernatants were microwaved for 20 s to inactivate any possible released nucleotidase activity. Nucleotide hydrolysis in the cell supernatants was assayed by HPLC or by the release of inorganic phosphate.

HPLC measurement of nucleotide hydrolysis

Cell supernatants prepared as described above were filtered and nucleotide content determined by reverse phase HPLC on an analytical C18 column (Dinamax C-18, 250 × 4.6 mm, 60 Å, Rainin). Separation of nucleotides and nucleosides was achieved using a paired-ion chromatography protocol with a linear gradient of acetonitrile (5–30%) in 10 mM KH_2PO_4, pH 7.5, and 2 mM tetrabutylammonium hydrogen sulphate, over 20 min at a flow rate of 2 ml min^{-1}. Absorbance at 254 nm was monitored on-line with a Waters UV detector model 490.

Phosphate assay

Inorganic phosphate was determined colorimetrically using a modification of the malachite green-based assay described by Lanzetta *et al*. (1979). Usually, 30 μl of cell supernatants were combined with 100 μl of malachite green reagent, mixed and the absorbance at 595 nm was determined in a plate reader. The inorganic phosphate content in the cell supernatant was determined by comparison against a standard curve constructed with known amounts of phosphate.

Results

Isolation and sequencing of a human ecto-ATPase cDNA

Chadwick & Frischauf (1997) recently reported the cloning and chromosomal localization of a human gene (named CD39L1) that exhibits high homology to chicken muscle ecto-ATPase and to human CD39. Introduction of a gap of 23 amino acids in the CD39L1 sequence was necessary to obtain optimal alignment of the predicted amino acid sequences of these proteins. The reported human gene was not expressed, and therefore, no examination of biological activity was provided. As such, we have isolated a human ecto-ATPase sequence with the goal of studying its biological properties.

Preliminary results indicated that ECV304 cells express a marked nucleotide hydrolytic activity consistent with that of an ecto-ATPase (data not shown). Therefore, this cell line was used as a source for isolation of the ecto-ATPase gene. Using RT–PCR and primers corresponding to the 5′- and 3′-untranslated regions of CD39L1, we isolated a cDNA from ECV304 cells following the procedure detailed in Methods. The isolated sequence contained an open reading frame of 1485 bp encoding a 495 amino acid protein (Figure 1). This sequence corresponded to that reported by Chadwick & Frischauf (1997), with the exception that the open reading frame in the sequence reported here is 69 bp longer resulting in a predicted protein containing an additional 23 amino acids. Importantly, the extra sequence found in our clone corresponded exactly to the gap introduced in CD39L1 to obtain an optimal alignment with related proteins. This observation suggests that the CD39L1 clone reported by Chadwick & Frischauf (1997) likely is an alternatively spliced product. Figure 2 shows the alignment of our sequence with CD39L1, and human CD39.

The analysis of the amino acid sequence of the human ecto-ATPase reported here (see activity data below), predicts two potential transmembrane domains at the extremes of the protein (8 and 12 amino acids from the amino- and carboxy-termini, respectively), suggesting a topology of a large extracellular loop with two short amino and carboxy termini facing the intracellular space. Hydrophobicity and surface probability analysis also predict an hydrophobic region (amino acids 340–375) of the putative extracellular loop that could potentially form a reentry loop, as suggested by Kegel *et al*. (1997) for the rat ecto-ATPase and ecto-ATP diphosphohy-

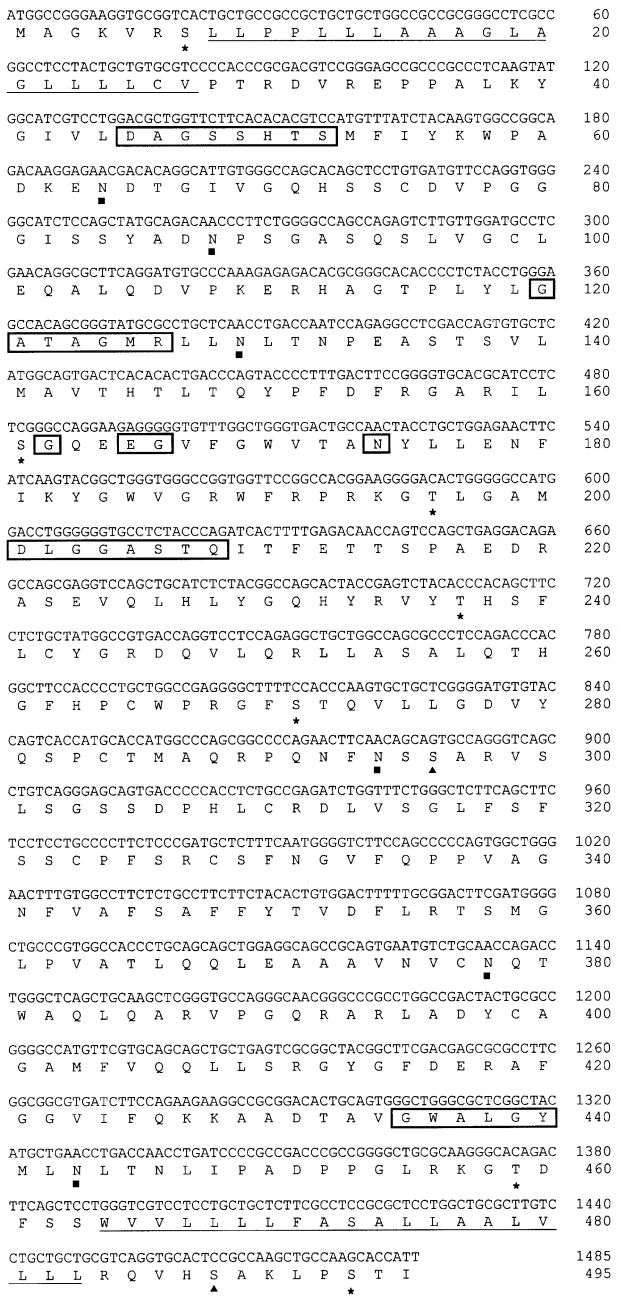

Figure 1 DNA sequence and predicted protein sequence of the human ecto-ATPase. Underlined with solid lines are the two putative transmembrane domains. ■, Potential N-glycosylation sites; *, potential protein kinase A phosphorylation sites; ▲, potential protein kinase C phosphorylation sites. Boxed sequences correspond to apyrase conserved regions. This sequence has been submitted to the GeneBank database with accession number AF144748.

drolase. A consensus site for protein kinase C phosphorylation (Ser 488) and one for protein kinase A (Ser 493) are found in the short intracellular C-terminal domain. Six potential N-glycosylation sites occur in the predicted large extracellular loop. Other potential phosphorylation sites for cyclic AMP dependent protein kinase (residues 161, 196, 237, 271 and 459), and a second protein kinase C phosphorylation site (Ser 296) are found in the predicted extracellular loop. The human ecto-ATPase (Figure 1) and the alternatively spliced form, CD39L1, contain the five 'apyrase conserved regions' (ACR) present in other ecto-ATPases and in ATP diphosphohydrolases (Handa & Guidotti, 1996; Vasconcelos *et al*. 1996). The amino acid identity of the human ecto-ATPase with ecto-ATPases from other species ranges from 57% identity to chicken to 83% identity to rat. The identity to human ecto-ATP diphosphohydrolase is 41% (Figure 2).

Expression of human ecto-ATPase in mammalian cells

Experiments were conducted to identify cell lines suitable for the expression of the ecto-ATPase, and several cells that express no or low levels of endogenous ecto-nucleotidase

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h-eATPase           MAGKVRSLLPPLLLAAAGLAGLLLLCVPTRDVRE-PPALKYGIVLDAGSSHTSMFIYKWPADKENDTG
h-CD39L1            MAGKVRSLLPPLLLAAAGLAGLLLLCVPTRDVRE-PPALKYGIVLDAGSSHTSMFIYKWPADKENDTG
h-CD39      MEDTKESNVKTFCSKNILAILGFSSII-AVIALLAVGLTQNKALPENVKYGIVLDAGSSHTSLYIYKWPAEKENDTG

h-eATPase   IVGQHSSCDVPGGGISSYADNPSGASQSLVGCLEQALQDVPKERHAGTPLYLGATAGMRLLNLTNPEASTSVLMAVT
h-CD39L1    IVGQHSSCDVPGGGISSYADNPSGASQSLVGCLEQALQDVPKERHAGTPLYLGATAGMRLLNLTNPEASTSVLMAVT
h-CD39      VVHQVEECRVKGPGISKFVQKVNEIGIYLTDCMERAREVIPRSQHQETPVYLGATAGMRLLRMESEELADRVLDVVE

h-eATPase   HTLTQYPFDFRGARILSGQEEGVFGWVTANYLLENFI-KYGW--VGRWFRPRKGTLGAMDLGGASTQITFETTSPA-
h-CD39L1    HTLTQYPFDFRGARILSGQEEGVFGWVTANYLLENFI-KYGW--VGRWFRPRKGTLGAMDLGGASTQITFETTSPA-
h-CD39      RSLSNYPFDFQGARIITGQEEGAYGWITINYLLGKFSQKTRWFSIVPYETNNQETFGALDLGGASTQVTFVPQNQTI

h-eATPase   EDRASEVQLHLYGQHYRVYTHSFLCYGRDQVLQRLLASALQTHGFH----PCWPRGFSTQVLLGDVYQSPCTMAQRP
h-CD39L1    EDRASEVQLHLYGQHYRVYTHSFLCYGRDQVLQRLLASALQTHGFH----PCWPRGFSTQVLLGDVYQSPCTMAQRP
h-CD39      ESPDNALQFRLYGKDYNVYTHSFLCYGKDQALWQKLAKDIQVASNEILRDPCFHPGYKKVVNVSDLYKTPCT--KRF

h-eATPase   QNFNSSARVSLSGSSDPHLCRDLVSGLFSFSSCPFSRCSFNGVFQPPVAGNFVAFSAFFYTVDFLRTSMGLPVATLQ
h-CD39L1    QNFNSSARVSLSGSSDPHLCRDLVSGLFSFSSCPFSRCSFNGVFQPPVAGNFVAFSAFFYTVDFLRTSMGLPVATLQ
h-CD39      EMTLPFQQFEIQGIGNYQQCHQSILELFNTSYCPYSQCAFNGIFLPPLQGDFGAFSAFYFVMKFLNLTS-EKVSQEK

h-eATPase   QLEAAAVNVCNQTWAQLQARVPGQRAR-LADYCAGAMFVQQLLSRGYGFDERAFGGVIFQKKAADTAVGWALGYMLN
h-CD39L1    QLEAAAVNVCNQTWAQ-----------------------QLLSRGYGFDERAFGGVIFQKKAADTAVGWALGYMLN
h-CD39      VTE-MMKKFCAQPWEEIKTSYAGVKEKYLSEYCFSGTYILSLLLQGYHFTADSWEHIHFIGKIQGSDAGWTLGYMLN

h-eATPase   LTNLIPADPPGLRKGTDFSSWVVLLLLFASALLAALVLLLRQVHSAKLPSTI*
h-CD39L1    LTNLIPADPPGLRKGTDFSSWVVLLLLFASALLAALVLLLRQVHSAKLPSTI*
h-CD39      LTNMIPAEQPL-STPLSHSTYWFLMVLFSLVLFTVAIIGLLIFH--K-PSYFWKDMV*
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Figure 2 Alignment of deduced amino acid sequences of the human ecto-ATPase, CD39L1, and human CD39. Shaded areas indicate amino acid identity to the human ecto-ATPase described here. Gaps (−) were introduced to improve alignment. GeneBank accession numbers are: U91510 for CD39L1 and S73813 or U87967 for CD39.

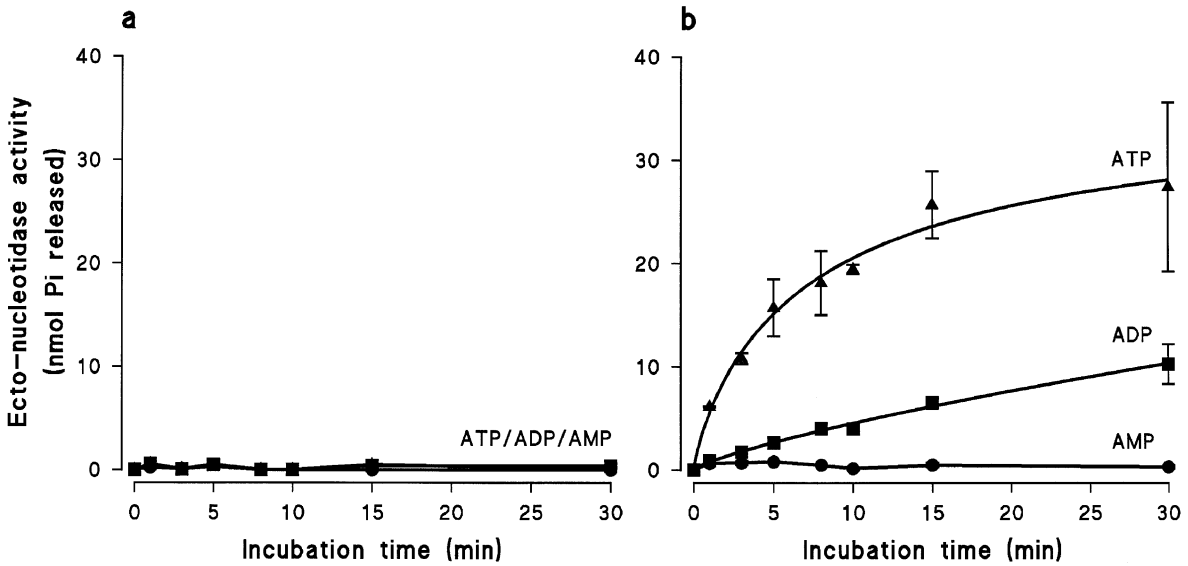

Figure 3 Rate of hydrolysis of ATP, ADP and AMP by NIH-3T3 cells expressing the human ecto-ATPase. NIH-3T3 cells expressing the empty pLXPIH vector (a) or the vector containing the ecto-ATPase gene (b) were incubated at 37°C with 100 μM ATP, ADP or AMP as described in Methods. At indicated times the reaction was stopped and the cell-free supernatant was removed from the cells. Nucleotide hydrolysis was estimated as the release of inorganic phosphate. Data shown are the mean ± s.e.mean of triplicate assays from a representative experiment repeated at least five times using confluent monolayers of cells seeded in 48-well culture plates.

activity were identified. For example, essentially no nucleotide hydrolytic activity was found in NIH-3T3 mouse fibroblasts during incubation with 100 μM ATP, ADP and AMP (data not shown). This cell line was chosen for expression of the human ecto-ATPase.

The retroviral vector pLXPIH alone or vector engineered to contain the human ecto-ATPase cDNA were transfected into the viral packaging cell line PA317, and retroviruses from these constructs were used to infect NIH-3T3 murine fibroblast cells. Clonal cell lines derived from populations of infected cells were isolated and tested for expression of ecto-nucleotidase activity.

Stable expression of the human ecto-ATPase in NIH-3T3 cells resulted in a high hydrolytic activity against extracellular ATP (Figure 3), whereas under the same experimental conditions, ADP was hydrolyzed at much lower rate (Figure 3b). In contrast, no nucleotide hydrolytic activity was observed in cells infected with retrovirus containing empty pLXPIH vector (Figure 3a). AMP was not hydrolyzed by the ecto-ATPase (Figure 3b). These results indicate that the cDNA sequence isolated and expressed in this study corresponds to a functional human ecto-ATPase, and that CD39L1 originally reported by Chadwick & Frischauf (1997) corresponded to an alternative spliced form of the human ecto-ATPase.

To further investigate the biochemical characteristics of the ecto-ATPase, we determined the substrate specificity of the enzyme for all the naturally occurring nucleoside triphosphates UTP, ATP, CTP, GTP, dATP, dUTP, dCTP, dGTP, ITP and XTP. NIH-3T3 cells stably expressing the ecto-ATPase hydrolyzed in a time- and Ca^{2+} or Mg^{2+} concentration-dependent manner all the natural nucleoside triphosphates with approximately the same rate. The nucleoside diphosphate forms (ADP and UDP) were hydrolyzed at a rate of approximately 5–10% of that of the corresponding triphosphates (Table 1).

To obtain the apparent kinetic parameters for the hydrolysis of nucleoside triphosphates and nucleoside diphosphates by the human ecto-ATPase, we measured the initial rates of hydrolysis of ATP and ADP at concentrations of nucleotide ranging from 1 μM to 1 mM (Figure 4). K_m and V_{max} values for the human ecto-ATPase were obtained from the substrate concentration-activity data, according to the Michaelis-Menten equation (Segel, 1993). These values were of 394 ± 62 μM and 107 ± 7 nmol Pi min^{-1} 10^6 cells^{-1} for ATP, and 102 ± 33 μM and 4 ± 0.4 nmol Pi min^{-1} 10^6 cells^{-1} for ADP, respectively ($n = 5$ experiments).

Table 1 Substrate specificity of the human ecto-ATPase

Substrate (100 μM)	*Nucleotide-hydrolysis* (% of ATP)
ATP	100
ADP	10.6±2.4
UTP	64.1±2.6
UDP	5.8±2.9
CTP	69.0±6.2
GTP	92.9±11.2
ITP	68.2±2.9
XTP	121.4±8.8

Rates of phosphate release were expressed as the per cent relative to the rate of ATP hydrolysis, that was of 22.2±6.3 nmol Pi min^{-1} 10^6 cells^{-1} at 37°C. Data are mean±s.e.mean of three experiments each in quintuplicate.

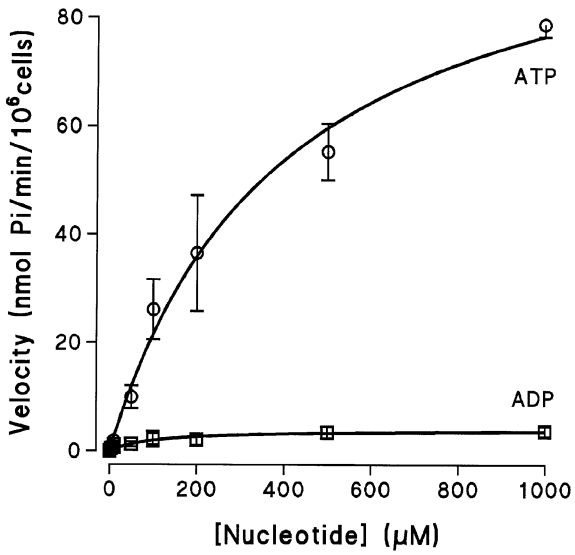

Figure 4 Substrate dependence for the hydrolysis of ATP and ADP by the human ecto-ATPase. NIH-3T3 cells stably expressing the ecto-ATPase were incubated in the presence of the indicated concentrations of ATP and ADP for 3 min. The rates of hydrolysis were measured as the release of inorganic phosphate and expressed as nmol Pi min^{-1} 10^6 cells^{-1} and were obtained from incubations in which less than 10% of the substrate was hydrolyzed. The data shown are the mean±s.d.mean from five experiments performed with triplicate samples.

Discussion

A cDNA encoding an ecto-ATPase was isolated from human ECV-304 cells and its predicted 495 amino acid sequence was found identical in length, and highly homologous to ecto-ATPases previously reported from rat brain (Kegel *et al*., 1997), mouse hepatoma cells (Gao *et al*., 1998), and chicken muscle (Kirley *et al*., 1997). The human cDNA sequence reported recently by Chadwick & Frischauf (1997), known as CD39L1, corresponds to the same gene reported here, except that our sequence is 23 amino acids longer. The alignment of the amino acid sequences, the predicted protein sizes, and the structures of ecto-ATPases from other species suggested that the sequence reported by Chadwick & Frischauf (1997) corresponded to an alternative splice of the ecto-ATPase gene. Indeed, we have partially sequenced a human genomic DNA clone and localized the sequence encoding the 23 amino acids missing in CD39L1 as the 5′ end of an exonic sequence (data not shown). The intron-exon boundaries for this 69 bp fragment conform to the splice donor and acceptor consensus sequences (data not shown). Further evidence that this 23 amino acid sequence is an integral part of the coding sequence of the human ecto-ATPase comes from the identity of the predicted sequence with corresponding regions in ecto-ATPases from other species (83% identity to rat and mouse ecto-ATPases, and 57% to chicken ecto-ATPase). Addition of the 69-bp fragment to the coding sequence of CD39L1 results in a predicted 495 amino acid protein identical in size to the human ecto-ATPase reported here and ecto-ATPases from other species.

The predicted structure of the human ecto-ATPase is similar to that suggested for the ecto-ATP diphosphohydrolase (CD39), and for the rat, mouse, and chicken ecto-ATPases. That is, the existence of two transmembrane domains at the N- and C-terminus result in a large extracellular loop and in two short 8- and 12-amino acid intracellular domains at the N- and C-terminus, respectively. A high degree of structural conserva-

tion is observed among ecto-ATPases and ecto-ATP diphosphohydrolases. For instance, Handa & Guidotti (1996) and Vasconcelos *et al*. (1996) identified five well-conserved regions ('apyrase conserved regions') in the sequence of potato apyrase, human and mouse CD39, and other NTPases from plants, yeast, and protozoans. The core of these non-contiguous conserved domains also are present in the human ecto-ATPase, where as previously predicted, they likely play an important role in the catalytic site of these proteins. All ten cysteine residues present in the predicted extracellular loop of the human ecto-ATPase are conserved in chick, mouse, and rat ecto-ATPases and in mouse and human ecto-ATP diphosphohydrolases. These residues might be involved in the formation of intra-loop disulphide bonds, and in the formation of multi-subunit complexes as has been suggested by Stout & Kirley (1996). All members of the ecto-ATPase and ecto-ATP diphosphohydrolase families are predicted to be highly glycosylated. The human ecto-ATPase contains six potential N-glycosylation sites in the predicted extracellular loop, two of them (Asn 64 and Asn 443) are conserved among human, rat, mouse, and chicken ecto-ATPases and in the human ecto-ATP diphosphohydrolase.

Essentially nothing is known about the regulatory mechanisms of ecto-nucleotidases. Interestingly, human, rat, and mouse ecto-ATPases contain potential protein kinase A phosphorylation sites in the N-terminal (Ser 7) and C-terminal (Ser 493) intracellular domains. Additionally, the human and chick ecto-ATPases express another potential protein kinase C phosphorylation site (Ser 488) in the C-terminus (Figure 1). The presence of these sites suggest the possibility for the existence of a regulatory mechanism of enzyme activity by phosphorylation by intracellular protein kinases A and C, and for the cross talk between extracellular nucleotide metabolism and other intracellular signaling mechanisms. Since only approximately 4% of the ecto-ATPase is in the intracellular space (8 and 12 amino acids in the N- and C-terminus, respectively), it is possible that complex regulatory mechanisms could occur in the large extracellular loop of the protein. The existence of ecto-protein kinases, such as protein kinase C, casein kinase II, and cyclic AMP -dependent protein kinase (Chen *et al*., 1996; Walter *et al*., 1996) and the extracellular location of conserved potential phosphorylation sites for these kinases in ecto-ATPases, open the possibility of extracellular regulation of the catalytic activity of these proteins. However, no evidence for such regulation has been reported.

Stable expression of the human ECV-304 cell clone in NIH-3T3 cells conferred a marked Ca^{2+}- or Mg^{2+}-dependent nucleoside triphosphate-selective hydrolytic activity associated with the extracellular face of the plasma membrane of these cells. Therefore, the data presented here indicate that the cDNA isolated from ECV-304 cells corresponds to a functional ecto-ATPase (Figures 3 and 4). It will be important to determine if CD39L1, the alternatively spliced variant reported by Chadwick & Frischauf (1997), encodes a functional protein, and in that case, to compare its biochemical properties with those of the protein reported here.

ATP levels in resting cells are the result of multiple complex processes including nucleotide release, breakdown, interconversion, and uptake. Extracellular levels of ATP in resting cultured cells are in the low nanomolar range, and these levels can increase significantly in response to a variety of factors, such as mechanical stimulation, release of nucleotide containing granules, or by cell lysis (Lazarowski *et al*., 1997). The apparent K_m value of the human ecto-ATPase for ATP (394 ± 62 μM) was significantly higher than the values reported for purified preparations of other ecto-nucleotidases. However, under some physiological circumstances, e.g. platelet activation, vascular endothelial cells are locally exposed to millimolar levels of nucleotides, and in this context the apparent K_m value for ATP as substrate is in a physiologically relevant range. Two other issues should be considered. The nucleotide concentrations reported here are those of the bulk phase, and these concentrations could be significantly different than concentrations at the membrane surface. Similarly, the concentrations of ATP typically measured upon release from cells also are determined in the bulk phase, and functional data indicate that much higher concentrations occur at the level of the plasma membrane (Slakey *et al*., 1990; Beigi *et al*., 1999). Thus, the assessment of relevant concentration of substrate is very different when the activity of the enzyme is measured *in situ* as an ecto-enzyme than with purified enzyme or membrane preparations in solution. More controlled approaches to address these issues will be necessary for accurate kinetic analysis of ecto-ATPases in intact cell preparations.

In conclusion, we report here the isolation of the cDNA and for the first time the functional expression of a human ecto-ATPase. The study of the biochemical properties and the regulatory mechanisms of ecto-ATPases will be valuable in the definition of their role in nucleotide signaling and in other physiological functions.

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