CD39-L4 Is a Secreted Human Apyrase, Specific for the Hydrolysis of Nucleoside Diphosphates*

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The human ecto-apyrase gene family consists of five reported members (CD39, CD39-L1, CD39-L2, CD39-L3, and CD39-L4). The family can be subdivided into two groups by conservation of proposed structural domains. The CD39, CD39-L1, and CD39-L3 genes all encode hydrophobic portions in their carboxy and amino termini, serving as transmembrane domains for CD39 and potentially for the other two members. CD39-L2 and CD39-L4 genes encode hydrophobic portions in their amino termini, suggesting that they might encode secreted apyrases. We demonstrate that the CD39-L4 gene encodes the first reported human secreted ecto-apyrase. COS-7 cells transfected with a CD39-L4 expression construct utilizing the naturally occurring leader peptide express recombinant protein outside of the cells. This expression can be blocked by brefeldin A, a chemical that inhibits a step in mammalian secretory pathways. We also demonstrate expression of CD39-L4 message in macrophages, suggesting that the protein is present in the circulation. Furthermore, we show that CD39-L4 is an E-type apyrase, is dependent on calcium and magnesium cations, and has high degree of specificity for NDPs over NTPs as enzymatic substrates. A potential physiological role in hemostasis and platelet aggregation is presented.

CD39 was originally identified as a lymphoid activation marker (1, 2). Molecular cloning revealed that the sequence encoded four regions (termed apyrase conserved regions or ACRs) with significant homologies to apyrases. Human apyrases are enzymes that hydrolyze adenosine tri- and diphosphates as substrates (3). Hydrophobic domains anchor CD39 to the surface of lymphocytes and endothelial cells, positioning their enzymatic domains outside of the cell. The ecto-enzymatic activity of CD39 has been proposed to regulate a variety of physiological states including cardiac function, hormone secretion, immune responses, neurotransmission, and platelet aggregation (4–7), all by modulating circulating levels of nucleotides in the blood.

CD39 has been proposed to play a role in platelet aggregation because of its ATP diphosphohydrolase activity, which could modulate levels of circulating ADP in the microenvironment of the vascular endothelium (6). Platelets adhere to sites of vascular injury, are activated, and release ADP, serotonin, thromboxane A2, and other signaling molecules. As a result, ADP from the releasate promotes activation, recruitment, and aggregation of platelets in the injury microenvironment (8). Interactions between activated platelet surfaces and coagulation proteins result in thrombin generation, further platelet activation, and formation of an insoluble fibrin plug. Therefore, enzymes able to modulate the levels of ADP at such sites could represent key mediators of hemostasis and clot formation.

We show that CD39-L4 is not only naturally secreted from mammalian cells and soluble once secreted but that it has a specificity for NDPs over NTPs as substrates. Specifically, the hydrolysis of ADP potentially classes CD39-L4 as a mediator of hemostasis. Expression of CD39-L4 message in macrophages indicates that the protein might be present in blood and have a role in modulating levels of circulating ADP. We propose that platelet activation and aggregation can be attenuated through CD39-L4 expression at sites of vascular injury through the hydrolysis of ADP.

MATERIALS AND METHODS

Reagents—All reagents were of the highest purity grade available. All nucleotides, N-ethylmaleimide, ouabain, sodium azide, and sodium fluoride were purchased from Sigma. The monoclonal antibody used against the Arg-Gly-Ser-His$_6$ epitope and the nickel resin (Ni-NTA)$^{1}$ were purchased from Qiagen. Ap$_5$A and the Fugene-6 transfection reagent were purchased from Roche Molecular Biochemicals. Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), and penicillin/streptomycin were purchased from Life Technologies, Inc. The inorganic phosphorus diagnostic kit (phosphor reagent) was purchased from Sigma. The QuickChange™ site-directed mutagenesis kit was purchased from Stratagene. The following human cDNA libraries were purchased from Life Technologies, Inc. The inorganic phosphorus diagnostic kit (phosphor reagent) was purchased from Sigma. The QuickChange™ site-directed mutagenesis kit was purchased from Stratagene. The following human cDNA libraries were purchased from Life Technologies: adult brain, adult heart, adult kidney, adult lung, adult liver, adult spleen, adult testis, fetal brain, and leukocyte. Adult lung, placenta, bone marrow, and fetal kidney libraries were purchased from CLONTECH. Adult ovaries, fetal liver, and macrophage were purchased from Invitrogen. Fetal skin was purchased as mRNA from Invitrogen and made in-house into a cDNA library. Fetal liver/ spleen was obtained from Soares (10).

DNA Methods—The CD39-L4 cDNA sequence was initially isolated from a macrophage cDNA library (Invitrogen). The sense primer (5'-TAAAGCTTGGGAAAA GAATGGCCACTTC-3') with a HindIII site and the antisense primer (5'-AGACTCGAG GTGGCCTCAATGGGAGATG-CC-3') with a XhoI site were used to subclone the coding sequences into the mammalian expression vector pcDNA3.1 (Invitrogen). The nucleotide sequence of the insert was found to be identical to that reported for the CD39-L4 cDNA (4). The coding region was further modified so that it would include a Gly-Ser-His$_6$ epitope tag immediately following Arg$_5$. Briefly, two partially overlapping complementary oligonucleotides (5'-GGCGCTGTCTCCCACAGAGGATCGCATCACCATCACCATC-3' and 5'-AACCAAGTCTGCTGGTTGT-9') were used on the CD39-L4 pcDNA3.1 template. The primers were extended in opposite directions around the plasmid using a 12-cycle PCR program (95 °C, 1 min; 60 °C, 1 min; 72 °C, 15 min) (Stratagene). The reaction was treated with DpnI to digest the methylated parental DNA and then transformed into Escherichia coli. Colonies were screened for the insertion. Expression of CD39-L4 in COS-7 Cells—COS-7 cells obtained from the American Type Culture Collection were grown in DMEM supplemented with 10% FBS and 100 units/ml penicillin G and 100 µg/ml

*$^{2}$ The abbreviations used are: NTa, nitrotriacetic acid; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis.

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streptomycin sulfate at 37 °C in 10% CO₂. Transfections were performed at 75% confluence in 10-cm plates with Fugene-6 according to the instructions of the manufacturer. In summary, the cells in 7 ml of medium were incubated with 16 μl of Fugene-6 and 8 μg of DNA for 14–18 h. At the end of the transfection, the medium was replaced with DMEM containing low serum (1% FBS). The cells were then incubated for 24–48 h prior to harvesting. For assays utilizing brefeldin A–treated cells, brefeldin A was dissolved in ethanol and added to the transfected cells 48 h after transfection. Both, control and brefeldin A-treated cells were washed once with phosphate-buffered saline (PBS) and incubated for 8 h in medium with none or with varying dosages of brefeldin A.

**Protein Preparation**—The protein was harvested in some experiments from both cells and medium. Cells were washed twice with PBS and then scraped from plates. Upon centrifugation, the cells were resuspended in PBS containing 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin, and 0.2 μg/ml aprotinin. After a brief sonication and centrifugation step to clear the lysate, the samples were then incubated with Ni-NTA resin at 4 °C for 2–3 h. The histidine-tagged protein complexed to the resin was washed three times with PBS before loading onto a 10% SDS-PAGE gel for Western blot analysis. The media was centrifuged initially to clear any cell debris, adjusted to pH 8.0 with 20 mM Tris, and incubated at 4 °C for 2–3 h with 100 μl of Ni-NTA resin/10 ml of medium. The Ni-NTA resin was washed three times with assay buffer A (15 mM Tris, pH 7.5, 134 mM NaCl, and 5 mM glucose) and resuspended in a 30% suspension in the same buffer. In some experiments, the assay buffer included one or more of the following reagents: 1 mM EGTA, 2 mM CaCl₂, and 2 mM MgCl₂.

**Assay for Nucleotidase Activity**—Nucleotidase activity was determined by measuring the amount of inorganic phosphate released from nucleotide substrates using the technique of Daly and Ertingshausen (9). In this reaction, the complex of inorganic phosphorus with phosphor reagent (ammonium molybdate in the presence of sulfuric acid) produces an unreduced phosphomolybdate compound. The absorbance of this complex at 340 nm is directly proportional to the inorganic phosphorus concentration. The protein still tethered to the resin as a 30% suspension in the same buffer. In some experiments, the assay buffer included one or more of the following reagents: 1 mM EGTA, 2 mM CaCl₂, and 2 mM MgCl₂.

**Western Blots**—The protein samples were treated with 4-fold loading buffer (250 mM Tris/HCl, pH 6.8, 8% SDS, 40% glycerol, 4% 2-mercaptoethanol, 0.05% bromophenol blue dye) and boiled for 5 min prior to loading onto a 10% Ready-gel (Bio-Rad). The gels were transferred to Immobilon-P membranes (Millipore) using a Trans-blot semi-dry transfer cell (Bio-Rad) in transfer buffer (39 mM glycine, 14 mM NaCl, and 5 mM glucose) and incubated at 4 °C for 2–3 h with 100 μl of Ni-NTA resin/10 ml of medium. The Ni-NTA resin was washed three times with assay buffer A (15 mM Tris, pH 7.5, 134 mM NaCl, and 5 mM glucose) and resuspended in a 30% suspension in the same buffer. In some experiments, the assay buffer included one or more of the following reagents: 1 mM EGTA, 2 mM CaCl₂, and 2 mM MgCl₂.

**Expression and Secretion of CD39-L4 by COS-7 Cells transfected with a CD39-L4 expression vector.** A, immunoblot analysis of COS-7 cells transfected with vector alone (pcDNA3.1) or with the CD39-L4-His₆ expression vector (CD39-L4-His₆ pcDNA3.1). The cell and the medium were incubated with Ni-NTA resin to concentrate the available protein. The amount of protein loaded onto the gel corresponds to one-tenth the amount of protein recovered from the fractions. Lane 1, cell extract from CD39-L4-His₆-transfected cells; lane 2, cell extract from pcDNA3.1; lane 3, media from CD39-L4-His₆-transfected cells; lane 4, media from pcDNA3.1-transfected cells. B, cells transfected with the CD39-L4-His₆ pcDNA3.1 were washed with PBS and incubated for 8 h in medium containing brefeldin A at 0, 0.1, 0.3, or 1.0 μg/ml. Cell lysates and medium were processed and subjected to immunoblot analysis as in panel A.

**RESULTS**

**Expression and Secretion of CD39-L4**—The deduced amino acid sequence of CD39-L4 encodes a potential amino-terminal leader sequence (4). The computer server SignalP² (10) predicted the location of a signal peptide cleavage site immediately after Ala⁹⁰. To immunologically detect the protein, we inserted a Gly-Ser-His₆ epitope immediately after Arg⁶⁰. To ascertain whether CD39-L4-His₆ is secreted, the coding region of the CD39-L4-His₆ protein was inserted into the pcDNA3.1 expression vector and transiently transfected into COS-7 cells. After a 24-h incubation in 10% serum-containing medium, the cell monolayer was shifted to 1% serum-containing media for 24 h. The CD39-L4-His₆ protein was concentrated by treating the cell lysates and medium with Ni-NTA-agarose (Qiagen) followed by SDS-PAGE and immunoblot analysis with an antibody against the Arg-Gly-Ser-His₆ epitope (Fig. 1A). CD39-L4 was detected in both the cell lysate and the medium from cells transfected with the CD39-L4-His₆ expression vector, but not from control cells. Although the predicted molecular mass of CD39-L4-His₆ is 46 kDa, the immunoreactive protein exhibited a mobility by SDS-PAGE corresponding to a molecular mass of around 51 kDa in the media and around 48 kDa in the cell lysate. This difference may be because of posttranslational modifications in the protein. There are three potential N-glycosylation sites (Asn-X-(Ser/Thr)(Thr)) in the CD39-L4 predicted amino acid sequence (4).

Secretion of CD39-L4 was also examined by treatment of the transfected cells with brefeldin A, an inhibitor of translocation of secretory proteins from the endoplasmic reticulum to the Golgi apparatus (11). Increasing dosages of brefeldin A blocked secretion of CD39-L4-His₆ and led to massive intracellular accumulation (Fig. 1B). Together, these results show that CD39-L4 has the characteristics of a secretory protein.

**Tissue-specific expression of CD39-L4** was assayed using a PCR-based approach to test for the presence or absence of a product from a series of commercially available cDNA.
CD39-L4 is a Soluble Human NDPase

Control ecto-apyrase activity was determined with protein tethered to the Ni-NTA resin as described under "Materials and Methods." The protein was in buffer A containing 2 mM CaCl₂ and 2 mM MgCl₂. The assay was started by adding ADP to 1 mM followed by a 30-min incubation at 37 °C. Each inhibitor was assayed in triplicate.

**Table I**

Characterization of CD39-L4 activity

| Inhibitors  | % Control |
|------------|-----------|
| Control    | 100 ± 7   |
| Ouabain (1 mM) | 96 ± 6   |
| NEM (10 mM) | 106 ± 5   |
| Na₃ (1 mM)  | 100 ± 12  |
| NaF (10 mM) | 113 ± 5   |
| Ap5A (10 μM)| 121 ± 9   |
| EGTA (2 mM) | 35 ± 5    |
| EDTA (2 mM) | 52 ± 3    |

**Table II**

Substrate specificity of CD39-L4

| Nucleotide | % Control |
|------------|-----------|
| ADP        | 100 ± 15  |
| ATP        | 5 ± 1     |
| AMP        | 0 ± 0     |
| CTP        | 26 ± 2    |
| GTP        | 34 ± 1    |
| UTP        | 12 ± 4    |
| CDP        | 268 ± 11  |
| GDP        | 334 ± 38  |
| UDP        | 408 ± 14  |

**Fig. 2.** CD39-L4 is an ecto-apyrase stimulated by divalent cations. Media from cells transfected with vector alone or with the CD39-L4-His6 pcDNA3.1 construct were incubated with Ni-NTA resin to concentrate the available protein. The concentration of cDNA used (20 ng) (data not shown), suggesting that the protein while present in the circulation may be found at low levels.

**CD39-L4 Is a Nucleotidase Stimulated by Divalent Cations—**

The high degree of conservation in the apyrase-conserved regions of CD39-L4 suggests similar function to other apyrases. To test this hypothesis, COS-7 cells were transfected with the CD39-L4-His₆ construct. The medium from transfected cells was incubated with Ni-NTA resin (Qiagen) to capture the His₆-tagged protein, the resin was washed with assay buffer, and the protein still tethered to the resin in a suspension was assayed for ADPase activity. CD39-L4 protein from transfected cells displayed a 2.3-fold increase in activity over the cells transfected with the vector alone (Fig. 2). When Ca²⁺ and Mg²⁺ were added, the activity increased 3.6- and 6-fold, respectively.

**Characterization of CD39-L4 Activity—**

CD39-L4 protein was assayed for ADPase activity in the presence of different kinds of inhibitors of ATPases. Table I shows that the inhibitors of vascular ATPases (N-ethylmaleimide), mitochondrial ATPases (N₃⁻ and Na⁺/K⁺-ATPase (ouabain) did not significantly inhibit the Ca²⁺/Mg²⁺-stimulated activity. Inhibitors of phosphatases (F⁻) and adenylate kinase (Ap5A) did not inhibit activity. However, metal chelators (EGTA and EDTA) significantly inhibited activity. These results show that the overwhelming majority of the activity in the assays originates from a protein bound to the resin with characteristics of an E-type apyrase (14).

The nucleotide specificity of the CD39-L4 protein is shown in Table II. The relative activity of the nucleotide triphosphates varies almost 7-fold, with ATP being the poorest substrate. No released phosphate was detected with AMP. ADP was hydrolyzed at a rate approximately 20-fold higher than that of ATP. Interestingly, the other NDPs were also very efficiently hydrolyzed by CD39-L4. Taken together, these results indicate that CD39-L4 defines a new class of E-type apyrases (12) in humans with a specificity for NDPs as enzymatic substrates.

**DISCUSSION**

Circulating nucleotides are known to be important signaling molecules, potentiating a variety of physiological responses (13). Therefore, membrane-bound and circulating ecto-enzymes that reduce excess levels of these molecules have important roles in maintaining normal physiology and health. The first human gene to encoding a protein with ecto-ATP diphosphohydrolase activity was CD39 (14). Expressed on the vascular epithelium, this molecule has been proposed to regulate hemostasis by modulating levels of ADP proximal to the walls of the vasculature (6). However, because CD39 is membrane-bound, it is difficult to estimate the effects it has on total circulating ADP levels in the blood under normal physiological conditions. CD39 also hydrolyzes ATP as a substrate (15), which is not a signal for platelet aggregation.

We have demonstrated that CD39-L4 is secreted from mammalian cells through two lines of evidence. First, crude cell fractionation studies found the protein accumulating in the media (Fig. 1A). Second, we used brefeldin A, a chemical that blocks Golgi functions by inducing a resorption of the Golgi apparatus into the endoplasmic reticulum, and fusion of the trans-Golgi network with the endosomal system (20) to show that the protein is secreted through the traditional mammalian secretory pathway (Fig. 1B). These two experiments strongly suggest that the amino-terminus hydrophobic domain of CD39-L4 encodes a signal peptide sequence. We also observed that the intracellular pool of CD39-L4 protein has a smaller molecular mass, maybe as a result of underglycosylation.

Our expression data showed a very restricted pattern of expression to a macrophage library, suggesting that the protein is present in blood. CD39-L4 clearly hydrolyzes ADP preferentially over ATP, indicating that one of its physiological roles is to reduce levels of circulating ADP and not ATP. We propose a model where macrophages at sites of vascular injury secrete CD39-L4, which degrades excess levels of ADP, attenuating platelet aggregation. In this model, decreased levels of CD39-L4 or its activity in the blood could lead to vascular occlusions, stroke, or other forms of cardiovascular disease. We are currently investigating the validity of this model with a number of different experimental approaches.

The ability that CD39-L4 has to hydrolyze NDPs other than ADP has implications outside of the circulatory system. For instance, it has been reported that UDP is the most potent
agonist for the human P2Y<sub>6</sub> receptor (21). This receptor is expressed in several tissues including infiltrating T cells present in inflammatory bowel disease (22). In this microenvironment, a molecule with the enzymatic properties of CD39-L4 could influence T cell responses by modifying the extracellular half-life of UDP. Another role for CD39-L4 has been suggested by the report that mouse cd39-l4 maps closely to a locus associated with audiogenic brain seizures in mice (4, 17). This locus known as Asp-1 is thought to be linked or to correspond to a factor that influences Ca<sup>2+</sup>-ATPase activity (18).

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