CD39L2, a Gene Encoding a Human Nucleoside Diphosphatase, Predominantly Expressed in the Heart

George Yeung, Julio J. Mulero, Daniel W. McGowan, Sanjeev S. Bajwa, and John E. Ford*

Functional Genomics Department, Immunology Group, Hyseq Inc., 670 Almanor Avenue, Sunnyvale, California 94086

Received April 26, 2000; Revised Manuscript Received July 3, 2000

ABSTRACT: E-NTPDases are extracellular enzymes that hydrolyze nucleotides. The human E-NTPDase gene family currently consists of five reported members (CD39, CD39L1, CD39L2, CD39L3, and CD39L4). Both membrane-bound and secreted family members have been predicted by encoded transmembrane and leader peptide motifs. In this report, we demonstrate that the human *CD39L2* gene is expressed predominantly in the heart. In situ hybridization results from heart indicate that the *CD39L2* message is expressed in muscle and capillary endothelial cells. We also show that the *CD39L2* gene encodes an extracellular E-NTPDase. Flow cytometric experiments show that transiently expressed CD39L2 is present on the surface of COS-7 cells. Transfected cells also produce recombinant glycosylated protein in the medium, and this process can be blocked by brefeldin A, an inhibitor of the mammalian secretory pathway. The enzymology of CD39L2 shows characteristic features of a typical E-NTPDase, but with a much higher degree of specificity for NDPs over NTPs as enzymatic substrates. The kinetics of the ADPase activity exhibit positive cooperativity. The predominance of *CD39L2* expression in the heart supports a functional role in regulating platelet activation and recruitment in this organ.

CD39 was originally identified as a leukocyte marker, expressed on the surface of activated lymphocytes (*1*, *2*). Sequence motifs (*3*, *4*) and enzymatic assays (*4*−*6*) defined CD39 as an E-NTPDase.[1] NTPDases are enzymes that hydrolyze adenosine tri- and diphosphates as substrates (*7*). Hydrophobic domains at the amino and carboxy termini anchor CD39 to the surface of lymphocytes and endothelial cells, positioning its enzymatic activity outside of the cell. The ecto-enzymatic activity of CD39 has been proposed to modulate circulating levels of nucleotides in the blood, regulating a variety of physiological states, including cardiac function, hormone secretion, immune responses, neurotransmission, and platelet aggregation (*4*, *5*, *8*−*11*). Interestingly, CD39 null mice are deficient in their ability to modulate hemostasis (*12*), and this defect has been attributed to purinergic receptor desensitization.

CD39 modulates platelet aggregation for metabolizing the ADP released from activated platelets (*4*, *10*, *11*). Platelets adhere to sites of vascular injury and release ADP, promoting further aggregation of platelets. Therefore, enzymes that are able to modulate the levels of ADP at such sites could represent key mediators of hemostasis. To date, four members of the CD39 family [CD39, CD39L1 (*13*), CD39L3 (*14*), and CD39L4 (*15*)] have been characterized enzymatically. A human Golgi uridine diphosphatase with related sequence (*16*) has also been characterized. The enzymatic and biochemical characteristics of CD39L4 define a novel subclass of human E-NTPDases, a secreted and soluble type, possessing a substrate specificity for NDPs. Specificity for the hydrolysis of ADP over ATP implicates this novel type of human E-NTPDase as a regulator for excessive platelet aggregation which could possibly lead to thrombosis.

In this report, we demonstrate that CD39L2 is expressed as both a cell membrane-bound and soluble glycoprotein, and its processed message is predominantly expressed in the heart. Furthermore, CD39L2 specifically hydrolyzes NDPs, placing it into the CD39L4 subfamily of E-NTPDases. The specific hydrolysis of ADP suggests that CD39L2 may participate in regulating hemostasis. Expression of the *CD39L2* message in heart tissue implicates CD39L2 as a mediator of platelet aggregation in this organ. Because of this tissue specific expression, we have characterized the enzymatic activity of CD39L2 with respect to adenosine diphosphate hydrolysis.

MATERIALS AND METHODS

Reagents. All reagents were of the highest purity grade available. All nucleotides, NEM, ouabain, sodium azide (N_3^-), and sodium fluoride (F^-) were purchased from Sigma (St. Louis, MO). The monoclonal antibody used against the *myc* epitope was purchased from Invitrogen (Carlsbad, CA), and the nickel resin (Ni−NTA) was purchased from Qiagen (Valencia, CA). Ap5A and the Fugene-6 transfection reagent were purchased from Roche Molecular Biochemicals (Indianapolis, IN). DMEM, FBS, and penicillin/streptomycin were purchased from Gibco BRL (Gaithersburg, MD). The

* To whom correspondence should be addressed: Hyseq Inc., 670 Almanor Ave., Sunnyvale, CA 94086. Telephone: (408) 524-8100. Fax: (408) 524-8141. E-mail: ford@sbh.com.

[1] Abbreviations: Ap5A, P^1,P^5-di(adenosine)-5′-pentaphosphate; BCIP/NTP, 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium; cDNA, complementary DNA; DIG, digoxigenin; DMEM, Dulbecco's modified Eagle's medium; E-NTPDase, E-nucleoside triphosphate dephosphohydrolase; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; FBS, fetal bovine serum; NEM, *N*-ethylmaleimide; PCR, polymerase chain reaction; PBS, phosphate-buffered saline.

10.1021/bi000959z CCC: $19.00
Published on Web 09/27/2000

inorganic phosphorus diagnostic kit (phosphorus reagent) was purchased from Sigma. Human brain, heart, kidney, lung, spleen, testis, and fetal brain SuperScript cDNA libraries were purchased from Gibco BRL. Human lymph node, placenta, bone marrow, leukocyte, and stimulated leukocyte MATCHMAKER cDNA libraries were purchased from CLONTECH Laboratories, Inc. (Palo Alto, CA). Human ovary and fetal liver cDNA libraries were purchased from Invitrogen.

RNA Expression Analysis. The expression of *CD39L2* in various tissues was analyzed using both a semiquantitative PCR (*15*) and a Northern blot analysis. For the PCR-based analysis, human cDNA libraries were used as sources of expressed genes from tissues of interest. Plasmid DNA (20 ng) from each library was used as a template for PCR amplification. *CD39L2* gene specific primers (5′-CGTATCCCGCGGGTGGAGGCCGGGGTG-3′ and 5′-CTTCTGCAAGTCCCAGAGCCAGTGTGC-3′) were used to amplify a 1736-nucleotide portion of the *CD39L2* mRNA sequence. Primers specific to human β-actin gene (5′-CGGGATCCCTGTGCTACGTCGCCCTGGAC-3′ and 5′-CGGAATTCACTGGCGCAGGCGGTGATCTCCTT-3′) were used to amplify a 315-nucleotide portion to serve as a positive control for the cDNA libraries. The PCR conditions were as follows: 96 °C for 2.5 min (1 cycle); 96 °C for 45 s, 60 °C for 45 s, and 72 °C for 2.5 min (3 cycles); 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 2.5 min (30 cycles); and 72 °C for 6 min (1 cycle). Amplified products were separated on a 1.2% agarose gel. For the Northern blot analysis, a human Multiple Tissue Northern (MTN) blot was purchased from CLONTECH. A 1008-nucleotide portion of *CD39L2* gene, corresponding to the 3′-untranslated region (3′-UTR), was amplified by PCR using gene specific primers (5′-CATCCTGAGGAGCCACAGCAC-3′ and 5′-AGGTTCAGCTCGTGCCGGGCA-3′). A 315-nucleotide portion of the human β-actin gene was amplified with the same gene specific primers as described above. The probes used in the hybridization were generated by labeling the PCR products with the Prime-It II Random Primer Labeling Kit from Stratagene (La Jolla, CA) in the presence of [α-^{33}P]dCTP. The hybridization was performed using the ExpressHyb hybridization solution from CLONTECH according to the instructions of the manufacturer.

In Situ Hybridization. Tissues were hybridized with DIG-labeled riboprobes derived from *CD39L2* coding sequence nucleotides 944−1134. Riboprobes were prepared using the DIG RNA Labeling Kit (Roche Molecular Biochemicals) as described by the instructions of the manufacturer. Automated in situ hybridization was performed by QualTek Molecular labs (Santa Barbara, CA) using a modified version of a previously published procedure (*17*). The Ventana Medical Systems, Inc. (Tucson, AZ), TechMate Automated Staining System was used for this procedure. All tissues were fixed in 10% neutral buffered formalin, paraffin-embedded, and cut into 4 μm thick sections. Sections were placed onto Ventana's ChemMate Capillary Gap Slides (POP075).

DNA Methods. The *CD39L2* coding sequence was subcloned into pcDNA3.1/*myc*-His(+)A (Invitrogen) via the *Eco*RI and *Xba*I sites. Briefly, a human adult heart cDNA library (Gibco BRL) was subjected to PCR using gene specific primers (5′-CGTATCCCGCGGGTGGAGGCCGGGGTG-3′ and 5′-CTTCTGCAAGTCCCAGAGCCAGTGTGC-3′). The resulted products were diluted 100-fold and subjected to a second round of PCR with primers (5′-GGAGCCCAAAAGACCGGCTGC-3′ and 5′-TGAAGTCACGTCCAGGACAGG-3′). The product represented a single band by agarose gel and was purified and sequenced to confirm its identity. Primers corresponding to the translational start region and the carboxy-terminal region, excluding the stop codon, of the *CD39L2* coding sequence, (5′-CGGAATTCAACATGAAAAAAGGTAATCCGTTATGAA-3′ and 5′-TGTCTAGATGAGGCTGGACTCTTCTG-3′) were used on the purified DNA to produce a fragment of DNA corresponding to the entire coding region of the *CD39L2* gene, flanked by *Eco*RI and *Xba*I sites. This PCR product was digested accordingly to generate overhang ends that were ligated into the *Eco*RI and *Xba*I sites of pcDNA3.1/*myc*-His(+)A. The resultant mammalian expression plasmid (pCD39L2mychis) allows for expression of the *CD39L2* coding sequence fused in-frame with the *myc*-6His epitope at the carboxy terminus.

Expression of CD39L2 in COS-7 Cells. Transfection of COS-7 cells was performed as described previously (*15*). Briefly, the cells in 10 mL of medium were incubated with 16 μL of Fugene-6 and 4 μg of DNA for 48 h. The medium was then replaced with serum-free DMEM and incubated for an additional 48 h prior to harvesting. For studies utilizing brefeldin A-treated cells, brefeldin A was dissolved in ethanol and the mixture added to the transfected cells 48 h after transfection. Both control and brefeldin A-treated cells were washed once with PBS and incubated for 8 h in serum-free DMEM with none or varying doses of brefeldin A.

Protein Preparation. After the conditioned medium was collected from the transfected COS cells, 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, the cytosolic fraction was separated from the insoluble membrane fraction by centrifugation. Purification of proteins from the cytosolic fractions has been described previously (*15*). For purification of proteins from the conditioned medium, the medium was centrifuged initially to clear any cell debris, adjusted to contain 6 mM sodium phosphate (pH 7.6), 0.5 μg/mL leupeptin, 0.7 μg/mL pepstatin, and 0.2 μg/mL aprotinin, 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 twice with Tris wash buffer [50 mM Tris-HCl (pH 7.5), 300 mM NaCl, and 5 mM imidazole], followed by three washes with apyrase assay buffer [15 mM Tris-HCl (pH 7.5), 134 mM NaCl, and 5 mM glucose] and resuspended in a 50% suspension in the same buffer.

Western Blots. Western blot transfer has been described previously (*15*). To detect *myc* epitope-tagged recombinant proteins, the blot was incubated with a 2000-fold dilution of the anti-c-*myc* monoclonal antibody (Invitrogen) at room temperature for 2 h. The secondary antibody (anti-mouse IgG-AP) (Santa Cruz Biotechnology, Santa Cruz, CA) was diluted 1000-fold and incubated for 1 h at room temperature. Bound antibody was detected by using Sigma Fast BCIP/NTP as the alkaline phosphatase substrate according to the instructions of the manufacturer.

Flow Cytometric Analysis. COS-7 cells were transfected as described above with either pcDNA3.1/*myc*-His(+)A or

pCD39L2mychis. After transfection for 72 h, the cells were washed twice with PBS and dislodged with 10 mM EDTA in PBS. Cells were pelleted by centrifugation at 300*g* for 5 min, washed with PBS, and resuspended in binding buffer (PBS containing 3% FBS and 0.02% sodium azide) at a concentration of 1×10^6 cells per 100 μL. The cells were first stained with 20 μg/mL monoclonal anti-*myc* antibody for 30 min at 4 °C. The cells were then washed with binding buffer and stained with 20 μg/mL R-phycoerythrin-conjugated goat anti-mouse IgG antibody (Molecular Probes, Eugene, OR). After being washed with binding buffer, the cells were resuspended in 1 mL of binding buffer and analyzed on the FACScalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA).

Deglycosylation Reaction. Deglycosylation reactions were performed using peptide *N*-glycosidase F (New England Biolabs, Beverly, MA) according to the instructions of the manufacturer. Briefly, the CD39L2 protein sample was denatured with denaturing buffer (0.5% SDS and 1% β-mercaptoethanol) at 100 °C for 10 min. It was then incubated with peptide *N*-glycosidase F in G7 buffer [50 mM sodium phosphate (pH 7)], supplemented with 1% NP-40, at 37 °C for 2 h.

Assay for Nucleotidase Activity. Nucleotidase activity assays were performed as described previously (*15*). Briefly, the protein still tethered to the nickel resin as a 50% suspension in apyrase assay buffer was assayed by the addition of the nucleotide to a final concentration of 15 mM and incubated at 37 °C for 20 min. The reaction was stopped by adding 100 volumes of phosphorus reagent. The amount of phosphate released from the reaction was quantitated by comparing it to a calcium/phosphorus combined standard (Sigma).

Determination of Kinetic Parameters for ADP Hydrolysis. Recombinant CD39L2 was eluted off the Ni−NTA resin with acidic elution buffer [0.1 M sodium acetate (pH 4.5), 0.3 M NaCl, and 0.2 mg/mL inactivated peroxidase (Sigma) as a carrier protein]. Tris-HCl (pH 9) was then added to a final concentration of 70 mM to neutralize the eluted protein sample. Proteins were concentrated 2-fold, while small solute concentrations were reduced by 80% using Microcon concentrators. Reactions were carried out in the presence of the apyrase assay buffer with 15 mM $CaCl_2$, 1 mM ouabain, 10 mM NEM, 10 μM Ap5A, and varying concentrations of ADP, incubated for 15 min at 37 °C. The amount of phosphate produced by the reaction was assayed as described above. The amount of the eluted protein used in the reaction was determined by visualizing with Gelcode Blue (Pierce) staining following SDS−PAGE, and comparing to a series of bovine serum albumin standards with known concentrations. Because the CD39L2 protein is not purified to homogeneity, the location of the CD39L2 band was identified by immunodetection on a duplicated lane from the same gel.

RESULTS

Tissue Specific Expression of CD39L2. *CD39L2* specific primers were designed to perform expression analysis using a semiquantitative PCR approach (*15*). Briefly, human cDNA libraries were used as a template for PCR analysis, and expression levels were normalized against actin levels. Out of 15 libraries that were tested, only adult heart and fetal brain yielded products (Figure 1A). The PCR products were sequenced to confirm primer specificity. Only the PCR products derived from heart corresponded to the reported *CD39L2* sequence (*8*). This heart-derived sequence was used in all experiments described in this report. The adult heart expression of *CD39L2* is also confirmed by Northern blot analysis (Figure 1B). The heart displayed the highest level of expression among all the tissues that were sampled, being at least 2-fold higher than that of brain and up to 7-fold higher than those of the other tissues that were tested. The fetal brain-derived product represents a variant possessing 43 bp of additional sequence inserted into exon 14, which shifts the reading frame of the last 260 bp of coding sequence (data not shown). The enzymatic activity of this brain specific variant remains to be analyzed.

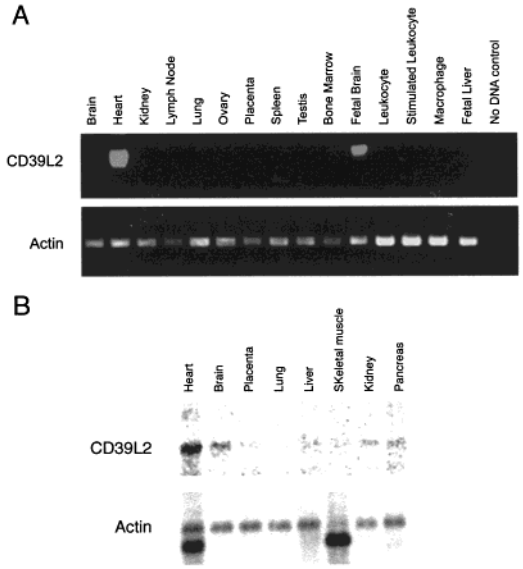

FIGURE 1: Tissue specific expression of *CD39L2*. (A) PCR-based expression analysis. Primers specific to *CD39L2* (top panel) and actin (bottom panel) are described in Materials and Methods. DNA (20 ng) from each cDNA library was used as a template. PCRs were performed as described in Materials and Methods. (B) Northern blot analysis. Probe preparation and hybridization are described in Materials and Methods. The top panel shows the *CD39L2* gene specific expression on the human MTN blot (CLONTECH), and the bottom panel shows the corresponding human β-actin expression on the same blot.

In Situ Hybridization Analysis of CD39L2 mRNA. To determine the cell types of heart-derived *CD39L2*, in situ hybridization was performed with adult heart tissue sections using DIG-labeled antisense riboprobe derived from the coding sequence of *CD39L2*. Binding of the antisense probe can be detected distinctively in cardiac muscles cells (Figure 2A) and capillary endothelial cells (Figure 2C, as indicated by arrows), while hybridization with the sense probe produced no signal (Figure 2B,D).

Cellular Localization of CD39L2. To determine the cellular localization of the CD39L2 protein, the coding sequence of *CD39L2* was inserted into the mammalian expression vector pcDNA3.1/*myc*-His(+)A by placing the carboxy terminus of the CD39L2 protein in-frame with the six-histidine tag for purification and a *myc* epitope for detection by immunoblotting. This plasmid, pCD39L2mychis, was transiently transfected into COS-7 cells, and Western blot analysis was performed on the protein isolated from cytosolic, membrane, and medium fractions. Recombinant protein was detected

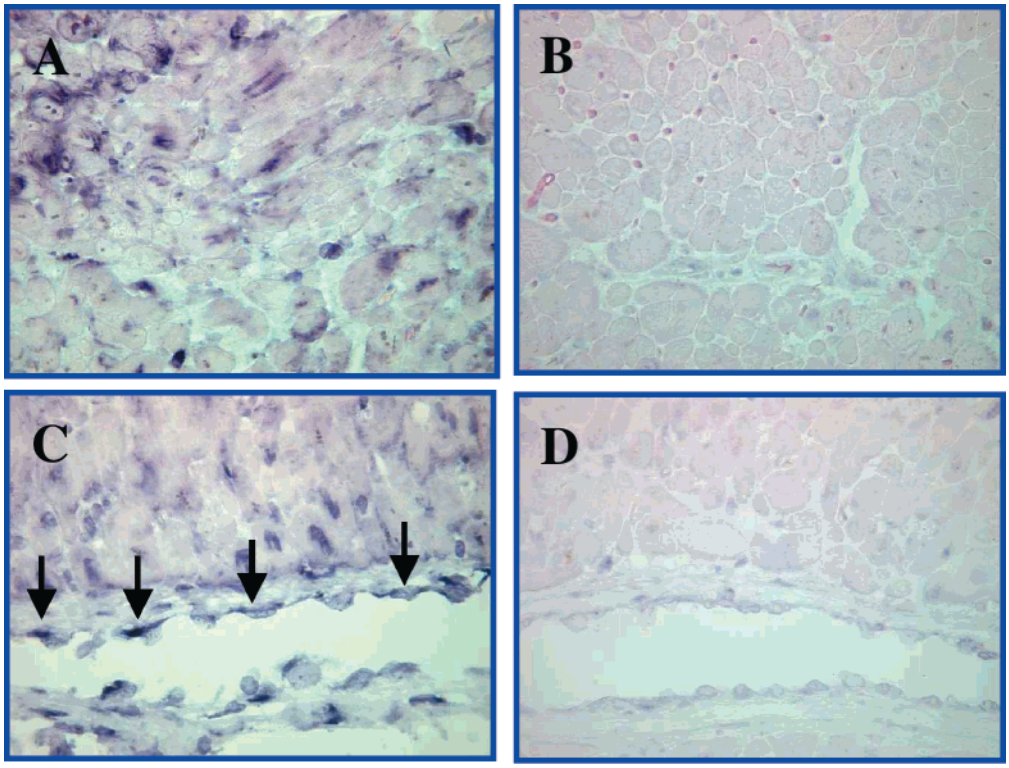

FIGURE 2: In situ hybridization of DIG-labeled *CD39L2* riboprobes to heart tissue sections. Hybridization conditions include proteinase K treatment, with stringency rinses at 2× and 0.1× SSC at 54 °C. Sheep anti-DIG antibodies with alkaline phosphatase conjugation were used as second antibodies. BCIP/NBT was used as the chromogen (blue-black). The slides were subsequently counterstained with Eosin (pink). (A) Hybridized with antisense probe. High-power view showing differential staining of the cardiac muscle cells. (B) Negative control for panel A, hybridized with the sense probe. (C) Hybridized with the antisense probe. High-power view showing differential staining of the capillary endothelial cells (indicated by arrows) and the cardiac muscle cells. (D) Negative control for panel C, hybridized with the sense probe.

in medium and membrane fractions of the *CD39L2*-transfected cells, but not in cytosolic fraction or control transfection samples (Figure 3A). The relative band intensities suggest that the majority of the recombinant CD39L2 protein is released into the medium with a small portion associated with the membrane (Figure 3A, lanes 5 and 6). The predicted molecular mass of unprocessed CD39L2 is 53 kDa. However, membrane and secreted fractions displayed slower electrophoretic mobility than the predicted encoded protein, suggesting that CD39L2 contains post-translational modifications. Both membrane and medium fractions also run as multiple bands (the single band observed in Figure 3A, lane 6, was resolved into at least a triplet upon extended electrophoresis), suggesting that post-translational modifications are heterogeneous.

To investigate if the recombinant CD39L2 is released into the medium through the conventional secretory pathway, a cellular localization assay was performed using various concentrations of brefeldin A, an inhibitor of the mammalian secretory pathway (*18*). The level of extracellular recombinant CD39L2 decreased with the addition of brefeldin A. Correspondingly, recombinant CD39L2 also accumulated in the intracellular fraction with the addition of brefeldin A (Figure 3B). A control sample with ethanol alone without brefeldin A did not inhibit the accumulation of CD39L2 in the medium by any significant extent (data not shown).

Flow cytometric analysis was used to determine if recombinant CD39L2 is expressed on the extracellular membrane. Recombinant CD39L2 was detected only on cells transfected with pCD39L2mychis, while cells from the control transfection showed no monoclonal anti-*myc* antibody binding (Figure 3C). High-stringency washing condition using 0.15 M Na_2CO_3 failed to reduce the magnitude of the signal detected by the anti-myc antibodies, indicating that the association of recombinant CD39L2 is not merely electrostatic (data not shown).

Post-Translational Modifications of CD39L2. To determine whether the CD39L2 protein is glycosylated, the electrophoretic mobility of the protein was compared before and after treatment with peptide *N*-glycosidase F. CD39L2 treated with peptide *N*-glycosidase F migrated as a triplet and at a lower molecular weight on SDS−PAGE than the untreated protein (Figure 4). Therefore, CD39L2 contains N-linked glycosylations that can be removed by peptide *N*-glycosidase F. The multiple-band pattern is consistent with additional modifications.

Biochemical Properties of CD39L2. To demonstrate that CD39L2 encodes an E-NTPDase, the recombinant CD39L2 protein expressed from transiently transfected COS cells was tested for its NDPase and NTPase activities. As shown in Figure 5A, ADPase activity of CD39L2 from transfected medium is significantly higher than that from control transfection. Furthermore, CD39L2 ADPase activity was stimulated upon addition of divalent cations. Addition of Mg^{2+} stimulates activity 8-fold, and Ca^{2+} stimulates activity 17-fold. Adenosine diphosphatase (ADPase) activity of CD39L2 is not affected by inhibitors of vacuolar adenosine triphosphatase (ATPases) (NEM), mitochondrial ATPase (N_3^-), and Na^+,K^+-ATPase (ouabain) (Figure 5B). Ap5A, an inhibitor of adenylate kinase, and F^-, an inhibitor of

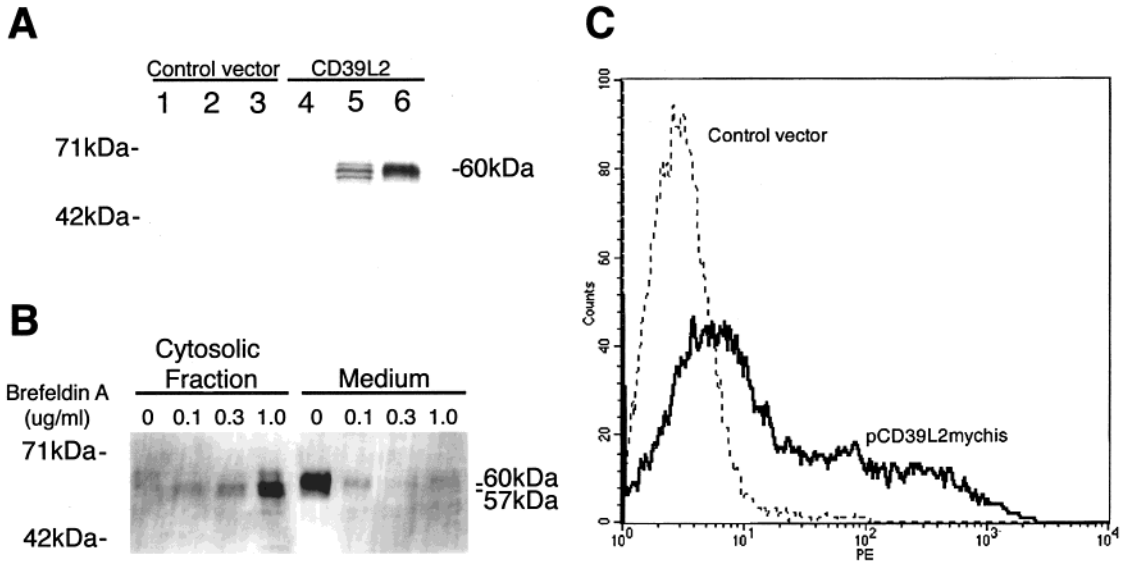

FIGURE 3: Expression and secretion of CD39L2 by COS-7 cells transfected with a CD39L2 expression vector. (A) Immunoblot analysis of COS-7 cells transfected with the control vector [pcDNA3.1/*myc*-His(+)A] or with the CD39L2 expression vector (pCD39L2mychis). The cytosolic fractions and the medium were incubated with Ni−NTA resin to concentrate the available protein: lanes 1 and 4, cytosolic fractions from control vector and CD39L2-transfected cells, respectively; lanes 2 and 5, membrane fractions from control vector and CD39L2-transfected cells, respectively; and lanes 3 and 6, medium from control vector and CD39L2-transfected cells, respectively. The amount of protein loaded corresponds to 10% of the amount of protein recovered per 100 mm plate of cells. (B) Cells transfected with the pCD39L2mychis plasmid 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. Cytosolic fractions and medium were processed and subjected to immunoblot analysis as described for panel A. (C) FACS analysis. Cells transfected with the control vector (dashed line) or pCD39L2mychis (solid line) were collected 72 h after transfection. Flow cytometry was performed as described in Materials and Methods.

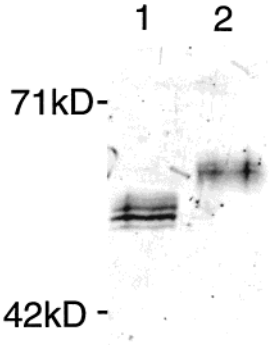

FIGURE 4: Post-translational modifications of CD39L2. Deglycosylation of CD39L2 by peptide *N*-glycosidase F. CD39L2 protein was purified from transfected COS cell medium as described in Materials and Methods, treated with or without peptide *N*-glycosidase F, and subjected to immunoblot analysis: lane 1, CD39L2 treated with peptide *N*-glycosidase F; and lane 2, CD39L2 treated under identical conditions but without peptide *N*-glycosidase F.

phosphatases, also did not inhibit activity. The inhibition of activity by EDTA and EGTA confirms the dependency of CD39L2 activity on divalent cations. These results indicate that CD39L2 exhibits characteristic features of E-NTPDases (*19*). As shown in Figure 5C, CD39L2 shows a substrate specificity for ADP over ATP and AMP. Enzymatic activities with CDP, GDP, and UDP as substrates are 73, 280, and 228%, respectively, relative to ADP hydrolysis, and with CTP, GTP, and UTP as substrates are 7.56, 15.5, and 9.97%, respectively. This specificity for nucleotide diphosphate hydrolysis resembles that of CD39L4 (*15*) and places CD39L2 into the same subclass of E-NTPDases.

Effect of Excess Free Ca^{2+} Ions on ADP Hydrolysis. It is known that excess free Ca^{2+} ions can inhibit activities of CD39 and other apyrases (*20*, *21*). To investigate if CD39L2 is also inhibited in a similar manner, titration analyses of Ca^{2+} concentration on ADPase activity were performed at various ADP concentrations. As shown in Figure 6, a high Ca^{2+} concentration has no inhibitory effect on CD39L2 ADPase activity at low substrate concentrations. For high substrate concentrations, a correspondingly high Ca^{2+} concentration is required for full activation.

Kinetic Analysis of ADP Hydrolysis by CD39L2. To determine the kinetic characteristics of ADP hydrolysis by CD39L2, recombinant soluble CD39L2 obtained by low-pH elution of CD39L2-bound Ni−NTA resin was used. The rate of product release was found to be linear within the first 20 min of reaction (data not shown); therefore, the initial velocity V_0 is taken to be the rate of reaction over the first 15 min. V_0 is determined over a range of substrate concentrations, and is found to respond in a sigmoidal fashion, indicating positive cooperativity (Figure 7). By curve fitting to the Hill's equation, we found $[S]_{0.5}$ to be 10.6 mM and V_{max} 1028 pmol/min. The amount of partially purified CD39L2 protein used in each reaction was estimated to be 3.5 ng, and the specific activity of V_{max} is calculated to be 290 μmol min^{-1} mg^{-1}.

DISCUSSION

The CD39 gene family can be divided into two subfamilies and currently consists of five members. CD39, CD39L1, and CD39L3 form one subfamily defined by two hydrophobic transmembrane domains (*8*). CD39L3 was recently shown experimentally to be expressed as a membrane-bound protein (*14*), confirming this prediction. CD39L2 and CD39L4 possess a single hydrophobic region at the amino terminus. We recently characterized CD39L4, which encodes a signal peptide-like sequence, and demonstrated that it is in fact

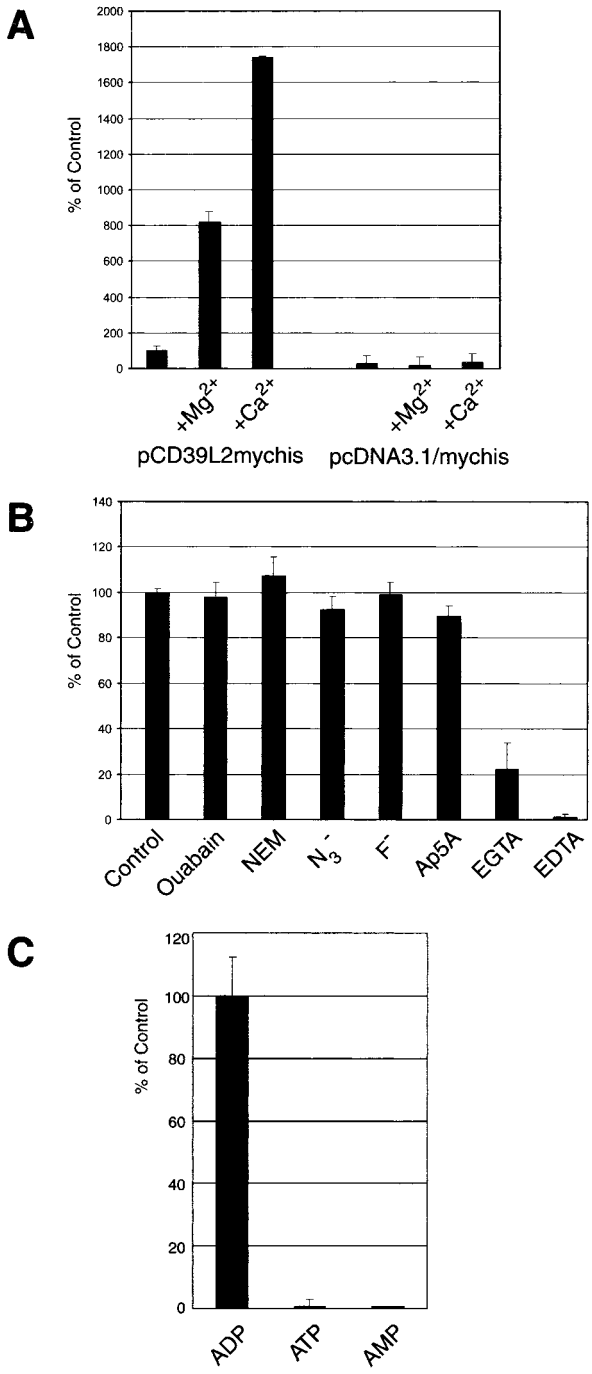

FIGURE 5: Biochemical characterization of CD39L2. CD39L2 activity was determined with protein tethered to the Ni−NTA resin as described in Materials and Methods. Each sample was assayed in triplicate at 37 °C for 20 min. (A) Medium from cells transfected with pcDNA3.1/*myc*-His(+)A or with pCD39L2mychis was incubated with Ni−NTA resin to concentrate the available protein. The concentrate was resuspended in assay buffer A. $CaCl_2$ and $MgCl_2$ were added to final concentrations of 15 mM when needed. The values are expressed relative to the transfected sample with no divalent cation. (B) The protein was in assay buffer A containing 15 mM $CaCl_2$. The assay was started by adding ADP to a final concentration of 15 mM. The concentrations of the inhibitors that were used were as follows: 1 mM ouabain, 10 mM NEM, 1 mM N_3^-, 10 mM F^-, 10 μM Ap5A, 2 mM EGTA, and 2 mM EDTA. The values are expressed relative to the sample with no inhibitor. (C) The protein was in assay buffer A containing 15 mM $CaCl_2$. The assay was started by adding the nucleotides to a final concentration of 15 mM. The values are expressed relative to ADP.

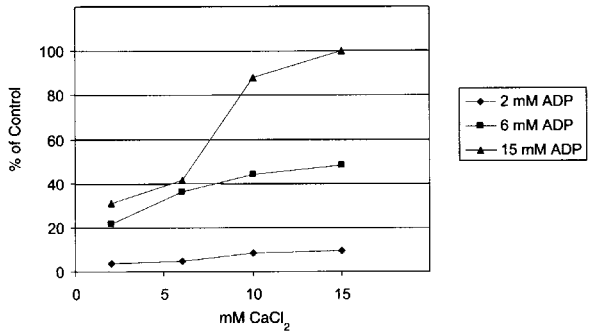

FIGURE 6: Effect of Ca^{2+} on ADPase activity of CD39L2. CD39L2 activity was determined with protein tethered to the Ni−NTA resin as described in Materials and Methods. The protein was in assay buffer A containing various concentrations of $CaCl_2$. The assay was started by adding the required amount of ADP. Each sample was assayed in triplicate at 37 °C for 20 min. The values are expressed relative to the reaction mixtures containing 15 mM $CaCl_2$ and 15 mM ADP.

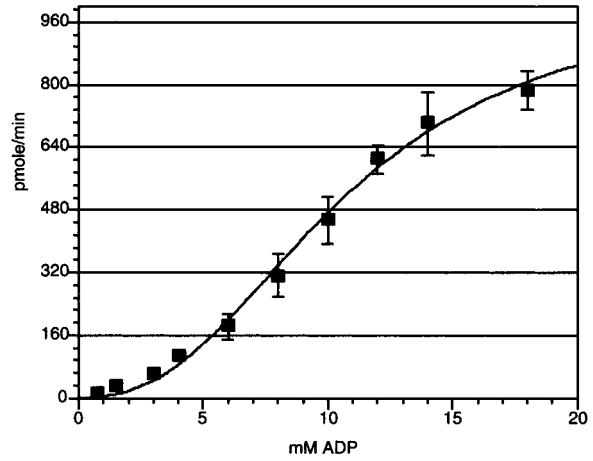

FIGURE 7: Determination of $[S]_{0.5}$ and V_{max} of CD39L2 ADPase activity. CD39L2 protein was eluted as described in Materials and Methods. Each data point represents an average of three separate experiments. Curve fitting of Hill's equation $[V_0 = (V_{max}[S]^n)/(K + [S]^n)]$ to the data points was performed by using DeltaGraph 4.0 software (SPSS Inc., Chicago, IL), and the resulting R^2 value is 0.995. The cooperativity, n, is determined to be 2.48, where V_{max} = 1028 pmol/min and T = 364.5. The substrate at half-maximal velocity $[S]_{0.5} = K^{1/n}$ = 10.6 mM.

secreted (*15*), defining a novel subtype of human E-NTPDase.

CD39L2 has features which indicate it encodes a cell surface protein. A hydrophobic region 38 amino acids from the start methionine encodes a predicted transmembrane domain by the TMHMM server (http://genome.cbs.dtu.dk/services/TMHMM-1.0) (*22*). We demonstrate that recombinant CD39L2 is present in the membrane fraction and is expressed on the cell surface as shown by flow cytometry. These data support the notion that CD39L2 is a cell surface protein. However, membranes from the Golgi and endoplasmic reticulum are likely also to be present in the membrane fraction (*16*). We have also shown that recombinant CD39L2 is released into the culture medium. One possible explanation is that the released form is derived from the transmembrane form via a proteolytic event, as in the cases of tumor necrosis factor and other membrane-derived soluble proteins (*23*, *24*).

CD39L2 is post-translationally modified during its biosynthesis as the recombinant proteins from both membrane

and medium fractions display slower electrophoretic mobility than the predicted molecular mass of 53 kDa. The multiple-band pattern displayed by all forms of recombinant CD39L2 suggests heterogeneous modifications. By using brefeldin A to block endoplasmic reticulum to Golgi protein transport, the CD39L2 protein appears to be smaller than the secreted form (Figure 3B). Apparently, the proteins trapped in the endoplasmic reticulum either are partially processed or undergo degradation of their oligosaccharide chains. Two potential N-glycosylation sites (Asn-X-Ser/Thr) in the predicted protein sequence suggest that CD39L2 might be glycosylated, and this prediction has been confirmed by peptide *N*-glycosidase F treatment. Multiple bands are detected by Western analysis of CD39L2 after glycosidase treatment which indicates other types of modifications occur to CD39L2. Palmitoylation, another type of post-translational modification, has been demonstrated on CD39 (*25*).

Structural and biochemical features of CD39L2 and CD39L4 place these enzymes into a separate subclass. Both proteins possess a single hydrophobic region at the amino terminus, and are more homologous to one another in the apyrase conserved regions than to other family members (*8*). Soluble CD39L2 demonstrates typical E-NTPDase activities, such as being stimulated by divalent cations and being insensitive to a wide range of phosphatase inhibitors. However, CD39L2 has a substrate specificity for NDPs, making it more similar to CD39L4 than the other characterized E-NTPDases (*5*, *10*). Therefore, sequence homologies and enzymatic properties allow CD39L2 and CD39L4 to be grouped into a novel subfamily of E-NTPDases.

To date, CD39L2 is the only E-NTPDase that demonstrates positive cooperativity (*10*, *19*, *26*, *27*). We have recently shown that CD39L4 ADPase activity does not possess cooperativity (*28*). Whether CD39L2 is unique among the remaining CD39 family members in exhibiting cooperativity remains to be determined.

CD39L2 differs from *CD39L4* in its tissue specific expression. Expression of the *CD39L4* message has been reported in macrophages (*15*) and in liver, kidney, prostate, colon, and testis (*8*), while the *CD39L2* message appears to be expressed predominantly in heart. However, variant forms have been reported to be expressed in a variety of other human tissues (*8*), including brain. Sequence analysis of cDNAs isolated from the brain and fetal brain libraries indicates that *CD39L2* messages in those tissues are significantly different due to alternative splicing (data not shown). The functional significance of these variants is yet to be determined.

It is critical to maintain circulation throughout the heart. Coronary occlusion results in mortality by myocardial infarction and other acute events (*29*−*31*). In this report, we demonstrate the expression of the CD39L2 message in capillary endothelial cells in heart. If CD39L2 is expressed in a functional manner in the capillary endothelium, it would then be available to metabolize ADP in platelet releasate and therefore to block further platelet activation and recruitment to this microenvironment.

Optimal ADP concentrations for CD39L2 are estimated in this report to be in the millimolar range. Therefore, we postulate that CD39L2 could be responsible for preventing large spikes of ADP released from platelets. The specific activity of 290 μmol min^{-1} mg^{-1} reported here for CD39L2 is significantly higher than that reported for the truncated soluble CD39, which is 11 μmol min^{-1} mg^{-1} at saturation (*10*). Therefore, CD39L2 exhibits enzymological characteristics that are different from those of other previously characterized CD39 family members.

ACKNOWLEDGMENT

We thank Dr. Alice S. Ho and Dr. Haishan Lin for their invaluable suggestions in the preparation of the manuscript.

REFERENCES

1. Valentine, M. A., Clark, E. A., Shu, G. L., Norris, N. A., and Ledbetter, J. A. (1988) *J. Immunol. 140*, 4071−4078.
2. Maliszewski, C. R., Delespesse, G. J., Schoenborn, M. A., Armitage, R. J., Fanslow, W. C., Nakajima, T., Baker, E., Sutherland, G. R., Poindexter, K., Birks, C., Alpert, A., Friend, D., Gimpel, S. D., and Gayle, R. B., III (1994) *J. Immunol. 153*, 3574−3583.
3. Handa, M., and Guidotti, G. (1996) *Biochem. Biophys. Res. Commun. 218*, 916−923.
4. Kaczmarek, E., Koziak, K., Sevigny, J., Siegel, J. B., Anrather, J., Beaudoin, A. R., Bach, F. H., and Robson, S. C. (1996) *J. Biol. Chem. 271*, 33116−33122.
5. Marcus, A. J., Broekman, M. J., Drosopoulos, J. H., Islam, N., Alyonycheva, T. N., Safier, L. B., Hajjar, K. A., Posnett, D. N., Schoenborn, M. A., Schooley, K. A., Gayle, R. B., III, and Maliszewski, C. R. (1997) *J. Clin. Invest. 99*, 1351−1360.
6. Wang, T. F., and Guidotti, G. (1996) *J. Biol. Chem. 271*, 9898−9901.
7. Bock, P. (1980) *Cell Tissue Res. 206*, 279−290.
8. Chadwick, B. P., and Frischauf, A.-M. (1998) *Genomics 50*, 357−367.
9. Todorov, L. D., Mihaylova-Todorova, S., Westfall, T. D., Sneddon, P., Kennedy, C., Bjur, R. A., and Westfall, D. P. (1997) *Nature 387*, 76−79.
10. Gayle, R. B., III, Maliszewski, C. R., Gimpel, S. D., Schoenborn, M. A., Caspary, R. G., Richards, C., Brasel, K., Price, V., Drosopoulos, J. H., Islam, N., Alyonycheva, T. N., Broekman, M. J., and Marcus, A. J. (1998) *J. Clin. Invest. 101*, 1851−1859.
11. Zimmermann, H. (1999) *Nat. Med. 5*, 987−988.
12. Enjyoji, K., Sevigny, J., Lin, Y., Frenette, P. S., Christie, P. D., Esch, J. S., II, Imai, M., Edelberg, J. M., Rayburn, H., Lech, M., Beeler, D. L., Csizmadia, E., Wagner, D. D., Robson, S. C., and Rosenberg, R. D. (1999) *Nat. Med. 9*, 1010−1017.
13. Mateo, J., Harden, T. K., and Boyer, J. L. (1999) *Br. J. Pharmacol. 128*, 396−402.
14. Smith, T. M., and Kirley, T. L. (1999) *Biochim. Biophys. Acta 1386*, 65−78.
15. Mulero, J. J., Yeung, G., Nelken, S. T., and Ford, J. E. (1999) *J. Biol. Chem. 274*, 20064−20067.
16. Wang, T. F., and Guidotti, G. (1998*) J. Biol. Chem. 273*, 11392−11399.
17. Myers, J. A., D'Andrea, M., Hunter, A. W., Mehta, P., Bernstein, S. A., and Erickson, P. A. (1995) *J. Surg. Pathol. 1*, 191−203.
18. Misumi, Y., Misumi, Y., Miki, K., Takatsuki, A., Tamura, G., and Ikehara, Y. (1986) *J. Biol. Chem. 261*, 11398−11403.
19. Plesner, L. (1995) *Int. Rev. Cytol. 158*, 141−214.
20. Grinthal, A., and Guidotti, G. (2000) *Biochemistry 39*, 9−16.
21. Flores-Herrera, O., Uribe, A., Pardo, J. P., Rendon, J. L., and Martinez, F. (1999) *Placenta 20*, 475−484.
22. Sonnhammer, E. L. L., von Heijne, G., and Krogh, A. (1998) in *Proceedings of the Sixth International Conference on Intelligent Systems for Molecular Biology* (Glasgow, J., Littlejohn, T., Major, F., Lathrop, R., Sankoff, D., and Sensen, C., Eds.) pp 175−182, AAAI Press, Menlo Park, CA.
23. Black, R. A., Rauch, C. T., Kozlosky, C. J., Peschon, J. J., Slack, J. L., Wolfson, M. F., Castner, B. J., Stocking, K. L., Reddy, P., Srinivasan, S., Nelson, N., Boiani, N., Schooley,

K. A., Gerhart, M., Davis, R., Fitzner, J. N., Johnson, R. S., Paxton, R. J., March, C. J., and Cerretti, D. P. (1997) *Nature 385*, 729−733.
24. Sadhukhan, R., Santhamma, K. R., Reddy, P., Peschon, J. J., Black, R. A., and Sen, I. (1999) *J. Biol. Chem. 274*, 10511−10516.
25. Koziak, K., Kaczmarek, E., Kittel, A., Sevigny, J., Blusztajn, J. K., Schulte Am Esch, J., II, Imai, M., Guckelberger, O., Goepfert, C., Qawi, I., and Robson, S. C. (2000) *J. Biol. Chem. 275*, 2057−2062.
26. Zhong, X., and Guidotti, G. (1999) *J. Biol. Chem. 274*, 32704−32711.
27. Sevigny, J., Robson, S. C., Waelkens, E., Csizmadia, E., Smith, R. N., and Lemmens, R. (2000) *J. Biol. Chem. 275*, 5640−5467.
28. Mulero, J. J., Yeung, G., Nelken, S. T., Bright, J. M., McGowan, D. W., and Ford, J. E. (2000) *Biochemistry 39*, 12924−12928.
29. Airaksinen, K. E., Ikaheimo, M. J., Linnaluoto, M., Tahvanainen, K. U., and Huikuri, H. V. (1998) *J. Am. Coll. Cardiol. 31*, 301−306.
30. Bates, E. R. (1998) *Curr. Opin. Cardiol. 13*, 274−279.
31. Anderson, J. L., King, G. J., Bair, T. L., Elmer, S. P., Muhlestein, J. B., Habashi, J., and Carlquist, J. F. (1999) *J. Am. Coll. Cardiol. 33*, 727−733.

BI000959Z