Site-Directed Mutagenesis of Human Endothelial Cell Ecto-ADPase/Soluble CD39: Requirement of Glutamate 174 and Serine 218 for Enzyme Activity and Inhibition of Platelet Recruitment†

Joan H. F. Drosopoulos,*,‡,§ M. Johan Broekman,‡,§ Naziba Islam,‡,§ Charles R. Maliszewski,|| Richard B. Gayle, III,|| and Aaron J. Marcus‡,§,⊥

Department of Medicine, Division of Hematology and Medical Oncology, VA New York Harbor Healthcare System, New York, New York 10010-5050, Department of Medicine and Department of Pathology, Division of Hematology and Medical Oncology, Weill Medical College of Cornell University, New York, New York 10010-5050, and Immunex Corporation, Seattle, Washington 98101

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ABSTRACT: Endothelial cell CD39/ecto-ADPase plays a major role in vascular homeostasis. It rapidly metabolizes ADP released from stimulated platelets, thereby preventing further platelet activation and recruitment. We recently developed a recombinant, soluble form of human CD39, solCD39, with enzymatic and biological properties identical to CD39. To identify amino acids essential for enzymatic/biological activity, we performed site-directed mutagenesis within the four highly conserved apyrase regions of solCD39. Mutation of glutamate 174 to alanine (E174A) and serine 218 to alanine (S218A) resulted in complete and ∼90% loss of solCD39 enzymatic activity, respectively. Furthermore, compared to wild-type, S57A exhibited a 2-fold increase in ADPase activity without change in ATPase activity, while the tyrosine 127 to alanine (Y127A) mutant lost 50−60% of both ADPase and ATPase activity. The ADPase activity of wild-type solCD39 and each mutant, except for R135A, was greater with calcium as the required divalent cation than with magnesium, but for ATPase activity generally no such preference was observed. Y127A demonstrated the highest calcium/magnesium ADPase activity ratio, 2.8-fold higher than that of wild-type, even though its enzyme activity was greatly reduced. SolCD39 mutants were further characterized by correlating enzymatic with biological activity in an in vitro platelet aggregation system. Each solCD39 mutant was similar to wild-type in reversing platelet aggregation, except for E174A and S218A. E174A, completely devoid of enzymatic activity, failed to inhibit platelet responsiveness, as anticipated. S218A, with 91% loss of ADPase activity, could still reverse platelet aggregation, albeit much less effectively than wild-type solCD39. Thus, glutamate 174 and serine 218 are essential for both the enzymatic and biological activity of solCD39.

Circulating platelets are not activated in the presence of intact endothelium. However, platelet activation in the setting of vascular injury provokes a response by endothelial cells characterized by limitation and reversal of platelet activation and recruitment (*1*−*3*). Such platelet inhibition is mainly due to an ecto-ADPase on the endothelial cell surface which rapidly metabolizes ADP released from activated platelets (*4*). This metabolic removal of ADP from the platelet releasate eliminates platelet recruitment and results in return of platelets to the resting state, thereby limiting thrombus formation (*1*, *4*). Thus, the endothelial ecto-ADPase is a critical component of thromboregulation.

Recently, we identified the vascular endothelial cell-surface ecto-ADPase (ATP diphosphohydrolase, ATPDase, apyrase, EC 3.6.1.5) as CD39 (*5*), or NTPDase-1 according to a recently proposed nomenclature (*6*). We and others have demonstrated that native and recombinant full-length CD39 possess ATPDase[1] activity (*5*, *7*−*9*). CD39 (NTPDase-1) is a membrane-associated 95 kDa glycoprotein, originally identified on activated human B lymphocytes (*10*−*13*), which metabolizes both ATP and ADP, but not AMP (*6*, *14*).

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* To whom correspondence should be addressed at the Thrombosis Research Laboratory, Room 13024W, VA New York Harbor Healthcare System, 423 E. 23rd St., New York, NY 10010-5050. Phone: (212) 686-7500, ext. 7492. FAX: (212) 951-3389. Email: jhfliess@mail.med.cornell.edu.

‡ Department of Medicine, VA New York Harbor Healthcare System.

§ Department of Medicine, Weill Medical College of Cornell University.

|| Immunex Corp.

⊥ Department of Pathology, Weill Medical College of Cornell University.

[1] Abbreviations: ACR, apyrase conserved regions; ASA, acetylsalicylic acid (aspirin); ATPDase, ATP diphosphohydrolase; BSA, bovine serum albumin; cDNA, complementary DNA; CM, conditioned medium; DEAE-dextran, diethylaminoethyl-dextran; DMEM, Dulbecco's minimum essential medium; DTT, dithiothreitol; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; HRP, horseradish peroxidase; mAb, monoclonal antibody; NTPase, nucleoside triphosphatase; PMSF, phenylmethylsulfonyl fluoride; PRP, platelet-rich plasma; PVDF, poly(vinylidene difluoride) membrane; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; solCD39, recombinant soluble human CD39.

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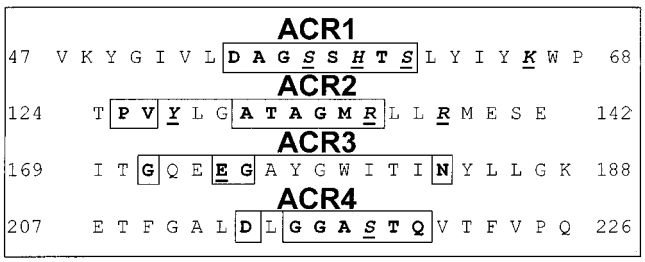

FIGURE 1: Apyrase conserved regions (ACR) of CD39. GenBank analyses of CD39 identified four regions within apyrases which are highly conserved. These have been termed "apyrase conserved regions" (ACR 1−4, boxed). Amino acid residues which we targeted for mutagenesis are underlined.

The cloned NTPDases show a high degree of similarity in amino acid sequence among each other, and to several other NTPases, particularly within four highly conserved regions, defined as "apyrase conserved regions" (ACR) (*15*−*20*). GenBank analyses have also revealed the presence of these ACR sequences in CD39. The ACR are highly conserved phylogenetically from plants to parasites to insects to mammals, suggesting their importance for the biological activity of CD39 (*15*, *16*, *18*, *21*, *22*).

Structural analyses revealed that CD39 is comprised of N- and C-terminal transmembrane-anchoring domains with short cytoplasmic tails, and a large extracellular domain, within which are the ACR sequences (*10*, *15*). To ascertain which amino acids in human CD39 are essential for enzyme activity, we carried out site-directed mutagenesis within the four apyrase conserved regions in the N-terminal portion of CD39 cDNA (Figure 1) using alanine scanning (*23*, *24*). These site-specific mutagenesis studies were performed with our recombinant, soluble form of human CD39 (solCD39) which lacks the N- and C-terminal transmembrane and cytoplasmic domains (*25*). We had previously demonstrated that solCD39 has enzymatic and biological properties identical to wild-type CD39 (*25*). Selection of amino acid residues in solCD39 targeted for mutagenesis was based on chemical modification/inhibition studies of ATPDases which suggested that Arg(R), Lys(K), Ser(S), His(H), and Tyr(Y) residues may be required for enzymatic activity (*26*−*30*). Furthermore, acidic residues, such as Asp(D) and Glu(E), are thought to be involved in metal ion coordination in ATPases (*31*−*35*) and in another class of nucleotide-utilizing enzymes, polymerases (*36*−*38*). Therefore, single alanine mutations in human solCD39 were generated at the selected Arg, His, Lys, Tyr, Ser, Asp, or Glu residues (Figure 1).

In this report, we characterized the solCD39 alanine mutants with regard to substrate specificity, divalent cation requirements, and biological capability to inhibit platelet reactivity. Our data indicate that the E174A mutation in solCD39 results in complete loss of enzymatic activity. In addition, the S218A mutation produces a 91% and 88% decrease in ADPase and ATPase activity, respectively. Wild-type solCD39 and each active mutant, except for R135A, displayed calcium over magnesium divalent cation dependence for ADPase activity. With regard to ATPase activity, no such metal ion preference was observed, except for mutants H59A and R135A, which exhibited preferences for calcium and magnesium, respectively. In parallel, alterations in enzyme activity correlate with inhibition of platelet aggregation. Our E174A mutant had no effect on platelet responsiveness, and mutant S218A, with a 91% loss of ADPase activity, still reversed platelet aggregation, although much less effectively than wild-type solCD39. Thus, glutamic acid 174 and serine 218 are essential for the enzymatic activity of solCD39 and are required for inhibition of platelet reactivity.

EXPERIMENTAL PROCEDURES

Oligonucleotide-Directed Mutagenesis. Site-directed mutagenesis was performed on solCD39 cDNA (*25*) using the Transformer Site-Directed Mutagenesis kit (Clontech) according to manufacturer's protocol. The cDNA sequence encoding solCD39, along with a C-terminal histidine (His_6) tag, was cloned into the bacterial plasmid Litmus29 (New England BioLabs) using restriction sites *Spe*I and *Bgl*II. This solCD39-C-His_6.Litmus29 plasmid was used as the template for preparation of the site-specific solCD39 alanine mutants. Alanine substitutions were generated in solCD39 targeting nine sites: Arg(R) 135 and 138; Glu(E) 174; His(H) 59; Lys(K) 66; Ser(S) 57, 61, and 218; and Tyr(Y) 127. The mutagenesis oligonucleotides were as follows with the substitution sites underlined: R135A, 5′-CATCCTGAGCAACGCCATGCCTGCCG-3′; R138A, 5′-CACTTTCCATTGCGAGCAACCGCATG-3′; E174A, 5′-ATAGGCACCTGCCTCTTGGCCAG-3′; H59A, 5′-GTATAAACTTGTGGCAGAAGAACCCG-3′; K66A, 5′-CTGCTGGCCACGCATAGATGTATA-3′; S57A, 5′-GTGTGAGAAGCACCCGCATCCAG-3′; S61A, 5′-GATGTATAAAGCTGTGTGAGAAGAAC-3′; S218A, 5′-ACTTGTGTAGCGGCTCCCCCAAG-3′; and Y127A, 5′-TGGCTCCCAGGGCAACGGGTGTCTC-3′. A 1216*Ava*I selection oligonucleotide, 5′-CCCTATCTCT**T**GGCTATTCTTTTG-3′, was also incorporated into the Litmus29 plasmid during the mutagenesis process. This selection primer mutated the unique nonessential *Ava*I restriction site on Litmus29, thus allowing primary selection against the parental plasmid by restriction digestion. The recombinant vector DNAs were transfected into competent *E. coli* host cells (Clontech). Potential mutant solCD39-containing clones were selected via *Ava*I restriction digestion, plasmid DNA isolated (Qiagen), and sequenced to confirm both the presence of the desired mutation and the absence of unwanted base changes. Wild-type solCD39 and the alanine-substituted solCD39 inserts were then restricted out of the Litmus29 plasmid and introduced into the *Spe*I/*Bgl*II restriction sites of the mammalian expression plasmid pDC206 for expression in COS-1 cells. This pDC206 vector contains an IL-2-derived leader sequence (IL-2L) (*25*) upstream of the *Spe*I restriction site, creating IL-2L-solCD39-C-His_6.pDC206. A negative control lacking solCD39, IL-2L-C-His_6.pDC206, was created by digesting IL-2L-solCD39-C-His_6.pDC206 with the restriction endonucleases *Spe*I and *Not*I. The vector DNA was then gel-purified, blunt-ended with Klenow and dNTPs, and recircularized using T4 DNA ligase. All samples were resequenced, prior to COS cell transfection, to verify that no unwanted alterations in base sequence were introduced.

Preparation of Conditioned Medium from SolCD39 Transient Transfectants. Plasmid DNA was transiently transfected into subconfluent layers of COS-1 cells (ATCC) using DEAE-dextran followed by chloroquine as previously described (*39*). Transfected cells were incubated (37 °C, 5% CO_2) in 0.5% FCS-supplemented DMEM-F12 medium in

12-well plates or 10 cm^2 Petri dishes. The IL-2 leader sequence, present in all constructs, allowed for secretion of solCD39 into the medium (*25*). After 5 days, conditioned medium (CM) from these cultures containing secreted solCD39 was collected, and cells and debris were removed by centrifugation, and sterile-filtered using MILLEX-GV 0.22 μm low protein binding sterilization filter units (Millipore). Samples of CM, from the large-scale transfections in 10 cm^2 Petri dishes, were concentrated 20-fold using Centriprep-10 centrifugal concentrators (Amicon).

Protein Determination. Protein levels of wild-type solCD39 and the alanine mutants were determined by solCD39 antibody-sandwich ELISA using the B73 monoclonal antibody (*10*), a murine IgG_1 which recognizes human CD39. Microtiter plates (Linbro) were coated with 1 μg/mL anti-CD39 mAb in PBS overnight at 4 °C (mAb73, kindly provided by Dr. Guy Delespesse, University of Montreal). Subsequent incubations were performed at room temperature for 1 h, and each step was followed by five rinses with 0.05% Tween in PBS. Plates were blocked with 5% nonfat dried milk in PBS, followed by incubation with conditioned medium samples (wild-type or mutant solCD39) titrated in 5% FBS/PBS. Purified, recombinant solCD39 was also titrated on each plate and used as internal control. To detect bound CD39, rabbit anti-human CD39 polyclonal serum (1:3000 dilution in 25% goat serum, Immunex Corp.) was subsequently added, followed by addition of HRP-conjugated donkey anti-rabbit IgG_1 (Jackson Labs), diluted 1:2500 in 10% goat serum. The ELISA was developed using the Peroxidase Substrate System (Kirkegaard Perry), analyzed on a plate reader at 650 nm, and the data were evaluated by Delta Soft version 3-1.46 software (Biometallics, Inc.).

Radio-TLC Assays for ADPase and ATPase Activities. ADPase and ATPase assays were performed in an identical manner: Conditioned medium samples (10 μL) were incubated in assay buffer [100 mM bis-tris propane, pH 8.0, 10 μM Ap5A (P^1,P^5-di(adenosine-5′) pentaphosphate), 1 mM ouabain, and 3 mM $CaCl_2$] containing 50 μM [^{14}C]ADP or [^{14}C]ATP (NEN Life Science Products) in a total volume of 50 μL for 5 min at 37 °C. Reactions were stopped by placement on ice and addition of 10 μL of "stop solution" (160 mM disodium EDTA, pH 7.0, 17 mM ADP, 0.15 M NaCl) at 4 °C to block further metabolism of ADP or ATP (*4*). Nucleotides, nucleosides, and bases were separated by TLC using 2-methyl-1-propanol/1-pentanol/ethylene glycol monoethyl ether/NH_4OH/H_2O (90:60:180:90:120) (*4*).

Radioactivity was quantitated by radio-TLC scanning ("InstantImager" Electronic Autoradiography System, Packard, Meriden, CT). Values were calculated as averages of duplicate measurements following subtraction of buffer blanks (consistently <1% of total radioactivity). Data were expressed as percent of ADP or ATP metabolized or as picomoles of ADP or ATP metabolized per minute per microliter. The enzymatic activity of each sample was corrected for background activity (vector alone) and then expressed as micromoles of ADP or ATP per minute per milligram of protein.

Radio-TLC Assays for Divalent Cation Specificity. The divalent cation requirements for ADPase and ATPase activities of wild-type solCD39 and the alanine mutants were determined using our radio-TLC assay in the presence of 3 mM $CaCl_2$ or $MgCl_2$; data were expressed as detailed above.

Proteolytic Digestion of Conditioned Medium Samples Containing Wild-Type or Mutant SolCD39. Conditioned medium samples containing wild-type or mutant solCD39 (75 ng), pH adjusted to pH 8.5 using 0.1 volume of 0.87 M Tris-HCl (pH 8.5), were subjected to either heat-treatment (95 °C, 5 min), or chymotrypsin (Sigma) digestion (room temperature, 5 min), or heat-treatment followed by chymotrypsin digestion. A chymotrypsin:total sample protein ratio of 1:100 (w/w) was used for each sample. Total protein in the CM samples was determined using the Coomassie Plus Protein Assay Reagent (Pierce) with BSA as standard (*40*). Proteolytic digestion reactions were stopped with PMSF at a final concentration of 1 mM, followed by the addition of one-fifth volume of Laemmli−SDS sample buffer containing 77.5 mg/mL DTT. Samples were immediately boiled for 5 min, and analyzed by SDS−PAGE and Western blot as detailed below.

Western Blot Analysis. Conditioned medium samples containing wild-type or mutant solCD39 (75 ng) were subjected to SDS−PAGE (*41*) on 4−20% gradient gels (Sigma) and transferred to a PVDF membrane (Hybond-P, Amersham) by electroblotting (100 V, 1 h) (*42*). Following transfer, CD39 protein was detected with a specific rabbit polyclonal anti-peptide antibody raised against amino acids 390−412 of human CD39 (GenBank accession no. S73813). Protein bands were visualized with alkaline phosphatase-conjugated goat anti-rabbit IgG using the Immun-Star chemiluminescent detection system (BioRad).

Preparation of Platelet-Rich Plasma. After obtaining informed consent from donors, blood was collected via plastic tubing using acid citrate−dextrose (citric acid, 38 mM; sodium citrate, 75 mM; glucose, 135 mM) as anticoagulant (*4*, *43*). Donors had ingested 325 mg of acetylsalicylic acid, aspirin (ASA), 18 h prior to blood collection. Platelet-rich plasma (PRP) was prepared with an initial whole blood centrifugation (200*g*, 15 min, 25 °C), followed by a second centrifugation of PRP (90*g*, 10 min) to eliminate residual erythrocytes and leukocytes. The stock suspension of PRP was maintained at room temperature under 5% CO_2−air (*4*).

Platelet Aggregation Studies. The biological activity of the solCD39 alanine mutants was characterized in our in vitro platelet aggregation test system (*4*, *5*, *25*), and compared to wild-type solCD39. ASA-PRP containing 1.22×10^8 platelets was preincubated (3 min, 37 °C) in an aggregometer cuvette (Lumiaggregometer, Chrono-Log, Havertown, PA) alone or in combination with conditioned medium samples containing wild-type or mutant solCD39. Platelet-poor plasma was used as blank to correct for light absorption by the plasma. Total volumes were adjusted to 300 μL with 100 mM bis-tris propane (pH 8.0) buffer. Following 3 min preincubation, 10 μM ADP was added, and the aggregation response was recorded for 4−5 min.

Statistical Analysis. Comparative analyses of data were performed by Student's paired, two-tailed, *t* test using Excel (Microsoft).

RESULTS

Enzymatic Characterization of Wild-Type SolCD39 and the Alanine Mutants. Conditioned medium samples of wild-type solCD39 and the alanine mutants were assayed for

Table 1: Protein Concentrations of Wild-Type and SolCD39 Mutants[a]

	protein (ng/mL)		protein (ng/mL)
wild-type solCD39	419 ± 144	wild-type solCD39	507 ± 94
S57A	581 ± 100	H59A	495 ± 79
K66A	1007 ± 149	S61A	367 ± 65
Y127A	372 ± 165	R135A	199 ± 23
R138A	675 ± 64	S218A	706 ± 90
E174A	250 ± 115		

[a] Protein levels of two groups of wild-type solCD39 and alanine mutants were determined by ELISA using the B73 monoclonal antibody, a murine IgG_1 recognizing human CD39. Values are mean ± standard deviation from 3 independent transfection experiments.

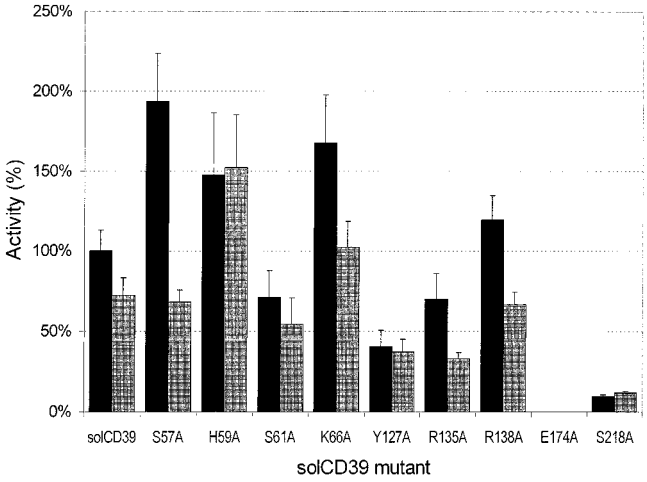

FIGURE 2: ADPase and ATPase activities of solCD39 alanine mutants. Conditioned medium samples of wild-type solCD39 and the alanine mutants were analyzed for ADPase activity (solid bars) and ATPase activity (hatched bars) by the radio-TLC assay as described under Experimental Procedures. The enzymatic activity in each sample was corrected for background activity (vector alone) and normalized with regard to protein content [μmol min^{-1} (mg of protein)$^{-1}$]. Activity was then expressed as percent activity relative to wild-type solCD39 activity [defined as 100% = 30.0 ± 3.9 μmol of ADP min^{-1} (mg of protein)$^{-1}$ and 21.7 ± 3.3 μmol of ATP min^{-1} (mg of protein)$^{-1}$]. Wild-type and mutant ATPase activity was plotted relative to ADPase activity. No measurable enzymatic activity was observed for the E174A mutant. Each bar represents the average of three independent COS-1 cell transfection experiments, each performed in duplicate.

ADPase and ATPase activity, and protein content. Importantly, the level of CD39 protein in each mutant sample was roughly similar to that of the wild-type, as measured by our sandwich ELISA using anti-CD39 mAb73 (Table 1). Activities were expressed relative to wild-type solCD39, defined as 100% [30.0 ± 3.9 μmol of ADP min^{-1} (mg of protein)$^{-1}$ and 21.7 ± 3.3 μmol of ATP min^{-1} (mg of protein)$^{-1}$]. This preference of wild-type solCD39 for the substrate ADP over ATP ($p < 0.005$) is in agreement with our previous observations (*25*). The negative control lacking solCD39, IL-2L-C-His_6.pDC206, had no measurable enzyme activity (data not shown).

Figure 2 shows that five of eight active mutants, similar to wild-type solCD39, exhibit a preference for ADP over ATP as substrate (p values < 0.005). The H59A, Y127A, and S218A mutants are the exception. S218A shows a slight preference for ATP ($p < 0.005$), while H59A and Y127A display an equal preference for ADP and ATP. Mutants S57A, H59A, K66A, and R138A had ADPase activity greater than wild-type solCD39 (p values < 0.02), whereas S61A, Y127A, and R135A had activity levels approximately 71%, 40%, and 70% of the wild-type levels, respectively. In addition, the ATPase activity of each mutant was lower than that of wild-type solCD39 with the exception of H59A and K66A which had activities 2.1- and 1.4-fold higher than that of wild-type, respectively. Interestingly, the S57A and H59A mutants exhibited the highest preference for ADP and ATP, respectively, among the mutants tested (Figure 2).

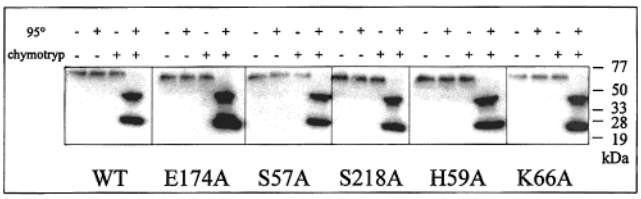

FIGURE 3: Conformational analysis of wild-type solCD39 and selected alanine mutants. Structural conformation of solCD39 and selected alanine mutants was analyzed by probing for protease accessibility via limited proteolysis. Conditioned medium samples containing wild-type or mutant solCD39 (75 ng) were subjected to either no treatment (−), or heat-treatment (+), or chymotrypsin digestion (+), or heat-treatment (+) followed by chymotrypsin digestion (+) as detailed under Experimental Procedures. Samples were subsequently boiled in Laemmli−SDS sample buffer and separated by SDS−PAGE, followed by Western blot analysis. CD39 protein was detected with polyclonal anti-peptide (aa 390−412) antibody to human CD39.

Importantly, the E174A mutation in solCD39 resulted in complete loss of enzymatic activity, and the S218A mutation in a 91% loss of ADPase and an 88% loss of ATPase activity. The monoclonal antibody B73, as used to measure CD39 protein levels, recognizes a split epitope on human CD39 (*10*), and does not bind denatured protein (unpublished observations). Since the E174A and S218A mutants are efficiently recognized by the antibody, it is likely that the overall structure of the mutants resembles that of the wild-type enzyme. Furthermore, no substantial differences were observed in protein expression levels between the mutants and wild-type enzyme (Table 1). Since misfolded proteins tend to be less stable and more rapidly degraded than those that are properly folded, the similarity in CD39 protein levels was suggestive of proper global conformation as well.

Conformational Analysis of Wild-Type SolCD39 and Selected Alanine Mutants. Limited proteolysis was used to probe for conformation-dependent protease accessibility to determine if the structural conformation of selected alanine mutants was similar to that of wild-type solCD39. As shown in Figure 3, wild-type solCD39 was resistant to chymotrypsin proteolysis, and was susceptible to digestion only upon heat denaturation. Heat-treatment alone had no degrading effect on the protein. Importantly, mutants S218A and E174A, with little or no enzymatic activity, were as resistant to proteolysis as wild-type solCD39. Moreover, as with wild-type, these mutants were equally sensitive to proteolytic digestion following thermal denaturation (Figure 3). Similar results were obtained for enzymatically active mutants, such as S57A, H59A, and K66A (Figure 3). The resistance of the alanine mutants, particularly E174A and S218A, to proteolysis substantiated the antibody binding results and provided evidence that they exhibit a global conformation similar to that of wild-type. We conclude that the loss of enzymatic activity of E174A and S218A was not due to

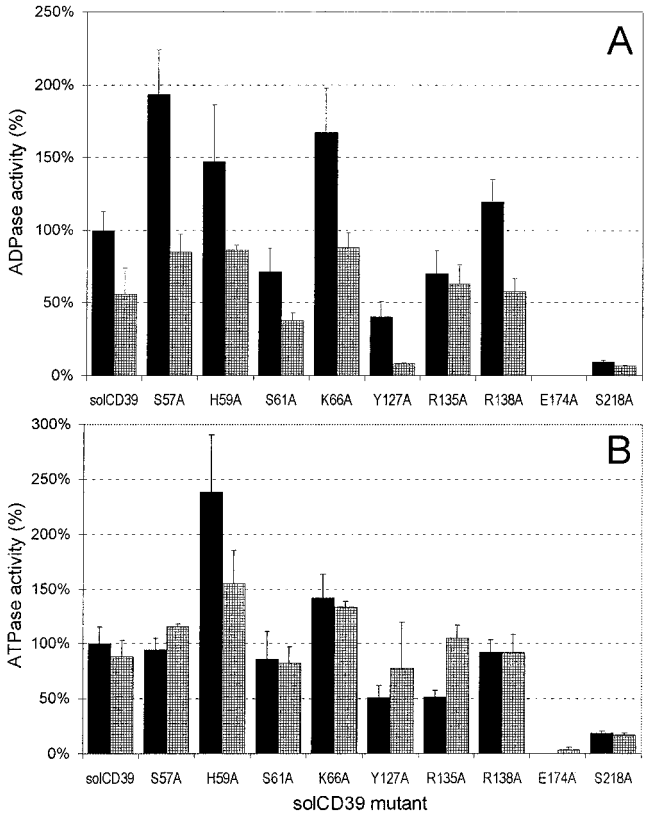

FIGURE 4: Divalent cation specificity of solCD39 alanine mutants. Radio-TLC assays were performed on conditioned medium samples of wild-type solCD39 and the alanine mutants (Experimental Procedures). The ADPase and ATPase activities of these samples were determined in the presence of calcium (solid bars) or magnesium (hatched bars), each at a concentration of 3 mM. The enzymatic activity of each sample was corrected for background (vector alone) and normalized with regard to protein content [μmol min^{-1} (mg of protein)$^{-1}$]. The activity was then expressed as percent activity relative to wild-type solCD39 (defined as 100%). In panel A, the specific activity of wild-type solCD39 was 30.0 $\pm$ 3.9 μmol min^{-1} (mg of protein)$^{-1}$ for Ca-stimulated ADPase activity and 16.8 $\pm$ 5.3 μmol min^{-1} (mg of protein)$^{-1}$ for Mg-stimulated ADPase activity. In panel B, the specific activity of wild-type solCD39 was 21.7 $\pm$ 3.3 μmol min^{-1} (mg of protein)$^{-1}$ for Ca-stimulated ATPase activity and 19.1 $\pm$ 3.2 μmol min^{-1} (mg of protein)$^{-1}$ for Mg-stimulated ATPase activity. Each bar represents the average of three independent COS-1 cell transfection experiments, performed in duplicate. (A) shows the divalent cation specificity of the ADPase activity of wild-type solCD39 and the alanine mutants. No measurable enzymatic activity was observed for the E174A mutant. (B) shows the divalent cation specificity of the ATPase activity of wild-type and mutant solCD39. Negligible ATPase activity was seen for the E174A mutant only when magnesium was used as a cofactor.

alterations in protein conformation or stability, but to modifications of amino acid residues in solCD39 critical for activity.

Divalent Cation Specificity of Wild-Type SolCD39 and the Alanine Mutants. To examine the divalent cation requirements of each alanine mutant as compared to wild-type solCD39, conditioned medium samples of wild-type and mutant solCD39 were analyzed for ADPase and ATPase activity in the presence of 3 mM $CaCl_2$ or $MgCl_2$. Wild-type solCD39 as well as each active mutant displayed a preference for calcium over magnesium for ADPase activity (p values < 0.05), with the exception of R135A (Figure 4A). The S57A, Y127A, and R138A mutants exhibited a higher preference for calcium as cofactor than wild-type solCD39 (Figure 4A). Y127A demonstrated the highest calcium:magnesium ADPase activity ratio (5:1) of the mutants tested. No measurable enzymatic activity was detected for the E174A mutant (Figure 4A). With regard to ATPase activity, wild-type solCD39 and most alanine mutants utilized calcium and magnesium with equal efficiency (Figure 4B). However, H59A exhibited a preference for calcium ($p < 0.03$), and R135A for magnesium ($p < 0.002$). Negligible ATPase activity was measured for the E174A mutant, and then only when magnesium served as cofactor. The S218A mutation in solCD39 resulted in marked loss of both ADPase and ATPase activity (Figure 4A,B).

Platelet Inhibitory Properties of Wild-Type SolCD39 and the Alanine Mutants. We previously demonstrated that full-length CD39 and solCD39 were effective inhibitors of platelet reactivity (*5*, *25*). Thus, to further characterize the solCD39 mutants and correlate their enzymatic activity with biological activity, the alanine mutants were examined in our in vitro platelet aggregation test system (*4*, *5*). Figure 5A shows that solCD39 is highly effective in reversing platelet aggregation in a dose-dependent manner. Even in the presence of only 0.05 μg/mL solCD39, platelet responsiveness to 10 μM ADP was reversed. Furthermore, each solCD39 mutant was as effective as wild-type in reversing platelet aggregation with the exception of E174A and S218A (Figure 5B). The E174A mutant, completely devoid of enzymatic activity, had no effect on platelet responsiveness since the aggregation response was indistinguishable from that of vector alone. Mutant S218A, with a 91% loss of ADPase activity, was still capable of reversing platelet aggregation, although much less effectively than wild-type solCD39 (Figure 5B). Moreover, mutants K66A, Y127A, and R135A and wild-type solCD39 at a concentration of 0.5 μg/mL were equally effective at inhibiting platelet aggregation (Figure 5C). Subsequently, dose-dependent inhibition of platelet reactivity by selected mutants was examined. Figure 6 shows that the solCD39 mutants S57A, H59A, K66A, and Y127A were similar to or more effective than wild-type in reversing platelet aggregation; however, E174A and S218A were totally and partially ineffective, respectively. Thus, the biological activity of these solCD39 mutants correlates with their enzymatic activity (Figure 2). Our data demonstrate that glutamic acid 174 and serine 218 are essential for both the enzymatic activity of solCD39 and its ability to inhibit platelet responsiveness.

DISCUSSION

Endothelial cell CD39/ecto-ADPase is a critical component of vascular homeostasis. It rapidly metabolizes ADP in the stimulated platelet releasate, thereby inhibiting further platelet activation and recruitment (*4*, *5*). Recently, we developed a recombinant, soluble form of human CD39, solCD39, with enzymatic and biological properties identical to CD39 (*25*). To ascertain which amino acid residues in solCD39 are essential for enzymatic/biological activity, site-directed mutagenesis was carried out within the four highly conserved ACR regions (ACR I−IV) (*15*) in the N-terminal portion of solCD39 via alanine scanning (*23*, *24*). The ACRs are highly conserved throughout the evolution of known nucleotidases and related proteins, including CD39 (*15*, *16*, *18*, *21*, *22*). Thus, the ACR motifs appear critical for maintaining enzymatic structure and function. Our structure−function

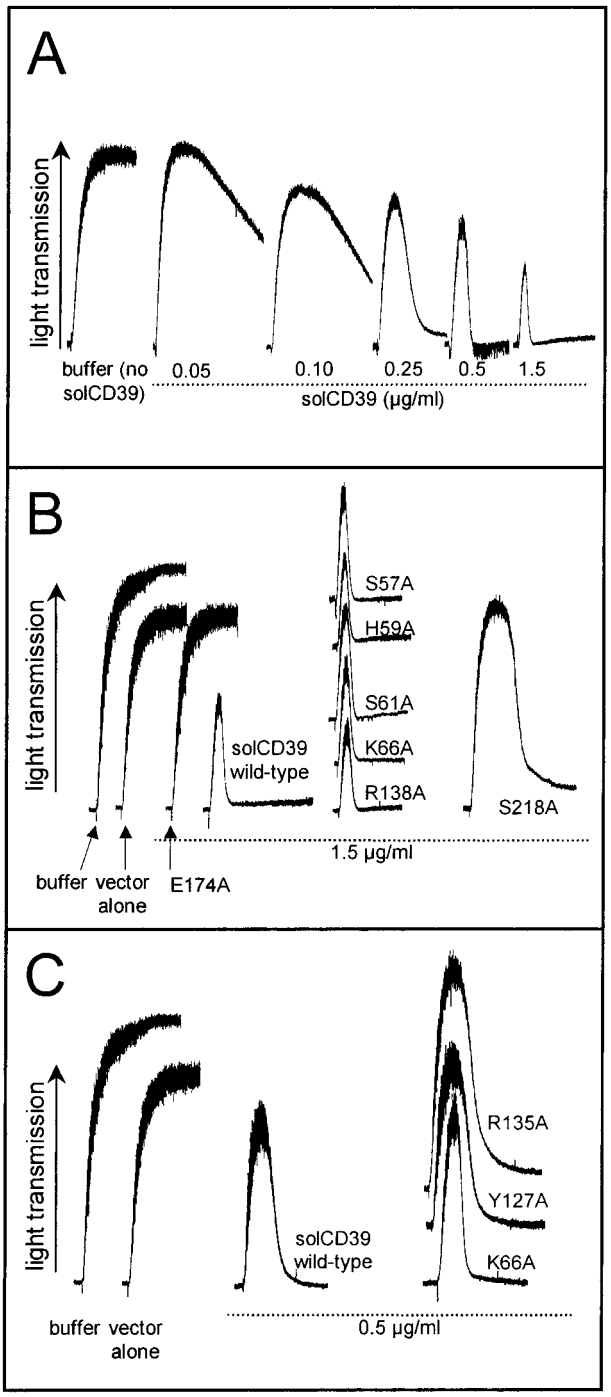

FIGURE 5: Inhibition of ADP-induced platelet aggregation by wild-type solCD39 and the alanine mutants. The inhibitory effects on platelet aggregation of conditioned medium samples containing wild-type solCD39 or the alanine mutants were analyzed following stimulation with 10 μM ADP. The effect of wild-type or mutant solCD39 on the platelet aggregation response was compared to that of buffer or vector alone. (A) Inhibition of ADP-induced aggregation by increasing quantities of solCD39 in ASA-free PRP. (B and C) Effects of wild-type solCD39 or the solCD39 alanine mutants on ASA-treated PRP. Aggregation responses were measured in 200 μL of PRP, containing 1.22×10^8 platelets, combined with 100 μL of wild-type or mutant solCD39 at a final CD39 protein concentration of 1.5 μg/mL (B) or 0.5 μg/mL (C). Data are presented as relative light transmission versus time (4 min duration). The E174A mutant, completely devoid of enzymatic activity, had no effect on platelet responsiveness, but the S218A mutant, with a 91% loss of ADPase activity, was still capable of reversing platelet aggregation, albeit less effectively than wild-type solCD39 (B).

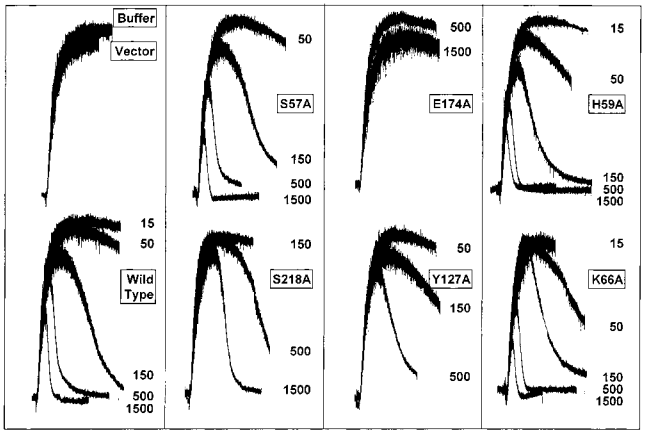

FIGURE 6: Dose-dependent inhibition of ADP-induced platelet aggregation by wild-type solCD39 and alanine mutants. The dose-dependent inhibitory effects on platelet aggregation of conditioned medium samples containing wild-type solCD39 or selected alanine mutants were analyzed following stimulation with 8 μM ADP, and compared to those of buffer or vector alone (controls). Aggregation responses were measured in 200 μL of ASA-treated PRP, containing 1.22×10^8 platelets, combined with 100 μL of wild-type or mutant solCD39 (final CD39 protein concentrations 15−1500 ng/mL). Data are presented as relative light transmission versus time (4 min duration). E174A, completely devoid of enzymatic activity, had no effect on platelet responsiveness, and S218A, with a 91% loss of ADPase activity, retained ~9% of solCD39 platelet inhibitory activity. Thus, the platelet inhibitory activity of wild-type and mutant solCD39 correlated with their enzymatic activity.

studies demonstrate that two highly conserved amino acid residues, glutamic acid 174 and serine 218, are not only essential for the enzymatic activity of solCD39 but are also required for the inhibition of platelet function. Therefore, there is a close correlation between enzyme activity and platelet inhibition.

Enzymatic Characterization of Wild-Type and Mutant SolCD39. The enzymatic characterization of wild-type solCD39 and the alanine mutants indicates that five of eight active mutants, similar to wild-type solCD39, exhibited a preference for ADP over ATP as substrate. This preference of wild-type solCD39 for ADP is in agreement with our previous observations (*25*). Changes in substrate preference by the mutants could be a reflection of alterations in the geometry of the nucleotide binding pocket. Interestingly, the S57A mutant exhibited the highest preference for ADP as substrate of the mutants tested. This preference of S57A for ADP was twice that of wild-type solCD39, while its preference for ATP remained equal to that of wild-type. The 2-fold increase in ADP preference may indicate that the S57A mutant has a greater affinity for the substrate or that it is more efficient at catalysis. Detailed kinetic analyses in the future should distinguish between these two possibilities.

The mutation of serine 218 to alanine (S218A) in ACR IV of solCD39 resulted in a 91% loss of ADPase activity and an 88% loss of ATPase activity. This loss of activity does not appear to be due to altered protein conformation, since S218A binds efficiently to mAb73 [which recognizes a split epitope on CD39 (*10*), and will not bind denatured protein]. In addition, S218A protein expression levels exceeded that of wild-type, indicating that the mutant protein is very stable. Furthermore, the proteolytic cleavage profile displayed by S218A was identical to that of wild-type solCD39, providing strong evidence that its structural

conformation is similar to that of wild-type. Thus, serine 218 is essential for the enzymatic activity of solCD39. Chemical modification/inhibition studies of ATPDases indicated that Arg(R), Lys(K), Ser(S), His(H), and Tyr(Y) residues may be required for enzymatic activity via involvement in nucleotide binding (*26*, *27*, *29*, *30*, *44*, *45*). Guidotti and colleagues showed that substitution of a serine residue in the ATP binding site of the Na,K-ATPase, five residues from the aspartyl residue which is phosphorylated during the enzyme's catalytic cycle, dramatically decreased enzyme activity (*46*). Therefore, on the basis of what has been reported for other nucleotidases, we propose that serine 218 is specifically involved in nucleotide binding.

Our mutation of glutamic acid 174 to alanine (E174A) in ACR III of solCD39 resulted in complete loss of enzymatic activity. As in the case of the S218A mutant, it is unlikely that the observed loss of activity can be attributed to alterations in the tertiary structure of the protein. E174A was efficiently recognized by mAb73, and protein expression levels were similar to that of wild-type, suggesting proper global conformation. Moreover, E174A was equally protease-resistant as wild-type solCD39, indicating proper protein folding as well. Previous studies have revealed that acidic residues, such as aspartate and glutamate, are important for enzymatic activity of ATPases and/or are involved in the cation binding process (*31*−*35*, *47*−*49*). Additional reports, involving another class of nucleotide-utilizing enzymes, the DNA polymerases (*36*−*38*), as well as calcium binding proteins (*50*, *51*), have shown that the negatively charged carboxylate groups of acidic residues provide many of the oxygen ligands for metal ions. Therefore, acidic residues may play a structural role as well as participate in metal ion coordination. Based on these studies, we hypothesize that glutamic acid 174 is essential for the enzymatic activity of solCD39 by coordinating calcium binding with substrate in the catalytic pocket of the enzyme.

Divalent Cation Specificity of Wild-Type SolCD39 and the Alanine Mutants. Wild-type solCD39, as well as each enzymatically active mutant with the exception of R135A, displayed calcium over magnesium dependence for ADPase activity. This preference for calcium as cofactor for ADPase activity of wild-type solCD39 is concordant with our previous findings for the endothelial cell ecto-ADPase/CD39 (*5*). This preference could be related to exposure of the active portion of the molecule to the extracellular space (plasma in the case of endothelial cells), where the calcium concentration is much higher than that found intracellularly. With regard to ATPase activity, wild-type solCD39 as well as each alanine mutant, with the exception of H59A and R135A, generally utilized calcium and magnesium with equal efficiency. The specific preference of wild-type solCD39 for calcium for its ADPase activity and the relaxed divalent cation preference for ATPase activity indicate that the nucleotide binding pocket accommodates calcium as cofactor more readily when the substrate is ADP than when it is ATP. Thus, magnesium may fit into the nucleotide binding pocket of solCD39 more easily when ATP is the substrate.

Mutant Y127A demonstrated the highest calcium dependence for ADPase activity, 2.8-fold higher than that of wild-type, even though its enzyme activity was greatly reduced. Perhaps the geometry of the nucleotide binding pocket in the Y127A mutant is more open than that of wild-type solCD39. This could result in greater distances between the enzyme's catalytic residues which allows for proper interaction to occur only with calcium. Site-directed mutagenesis of the vacuolar proton-translocating ATPase (V-ATPase) has shown that tyrosine 532 is involved in formation of the catalytic nucleotide binding site of the enzyme, possibly via contribution to the adenine binding pocket (*52*). Thus, tyrosine 127, in the ACR II conserved region in solCD39, may be important for enzymatic activity due to its involvement in the nucleotide binding site of the enzyme.

Platelet Inhibitory Properties of Wild-Type SolCD39 and the Alanine Mutants. Full-length CD39 and solCD39 have previously been shown to be potent inhibitors of platelet reactivity (*4*, *5*, *25*). Blockade of the platelet aggregation response occurs because CD39/solCD39 rapidly metabolizes ADP in the releasate from activated platelets. Therefore, to further characterize the solCD39 mutants and correlate their enzymatic activity with biological activity, the alanine mutants were examined in our in vitro platelet aggregation system (*4*, *5*). Importantly, alterations in the enzyme activity of solCD39 mutants correlated with inhibition of platelet aggregation. Each solCD39 mutant was highly effective in reversing platelet aggregation with the exception of E174A and S218A. The E174A mutant, completely devoid of enzymatic activity, lacked the ability to inhibit platelet reactivity, and the S218A mutant, with a 91% loss of ADPase activity, was still capable of reversing platelet aggregation, albeit much less effectively than wild-type solCD39. From these data we concluded that glutamic acid 174 and serine 218 are required for inhibition of platelet responsiveness. The direct correlation of enzyme activity with biological activity for wild-type and mutant solCD39, as well as the ability to superimpose the platelet aggregation responses for (1) buffer control, (2) conditioned medium from control (vector alone) transfections, and (3) conditioned medium containing E174A, indicates that solCD39/ecto-ADPase activity is the sole determinant of platelet inhibition.

In summary, we have identified glutamic acid 174 and serine 218 as essential for both the enzymatic activity of solCD39 and the ability to block platelet responsiveness. Our study provides a molecular biological characterization of the endothelial cell ecto-ADPase/solCD39 enzyme. Information from these structure−function studies will serve to enhance development of solCD39 as the nidus for a new class of therapeutic antithrombotic agents.

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