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Constraints imposed by transmembrane domains affect enzymatic activity of membrane-associated human CD39/NTPDase1 mutants ☆

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Abstract

Human CD39/NTPDase1 is an endothelial cell membrane-associated nucleotidase. Its large extracellular domain rapidly metabolizes nucleotides, especially ADP released from activated platelets, inhibiting further platelet activation/recruitment. Previous studies using our recombinant soluble CD39 demonstrated the importance of residues S57, D54, and D213 for enzymatic/biological activity. We now report effects of S57A, D54A, and D213A mutations on full-length (FL)CD39 function. Enzymatic activity of alanine modified FLCD39s was less than wild-type, contrasting the enhanced activity of their soluble counterparts. Furthermore, conservative substitutions D54E and D213E led to enzymes with activities greater than the alanine modified FLCD39s, but less than wild-type. Reductions in mutant activities were primarily associated with reduced catalytic rates. Differences in enzymatic activity were not attributable to gross changes in the nucleotide binding pocket or the enzyme's ability to multimerize. Thus, composition of the active site of wild-type CD39 appears optimized for ADPase function in the context of the transmembrane domains.

Keywords: E-NTPDase; Endothelial ecto-ATPDase; Ecto-nucleotidase; CD39; Site-directed mutagenesis; Apyrase conserved regions; Multimerization; Oligomer; Thrombosis

Vascular endothelial cells (EC) constitutively express a cell-surface ecto-nucleotidase (ecto-ATPDase/CD39) that plays an important role in maintenance of blood fluidity (thromboregulation). CD39 rapidly metabolizes ADP in the releasate from activated platelets, thereby inhibiting further platelet activation and recruitment [1,2]. Current nomenclature designates CD39 as NTPDase1, a member of the E-type nucleotidase family (NTPDase1–8) that hydrolyze nucleoside triphosphates (NTPs) and nucleoside diphosphates (NDPs) [3–6]. NTPDase family members 1–4, 7, and 8 are integral membrane proteins, containing N- and C-terminal transmembrane domains [3,5,7,8]. We and others have shown that native membrane-associated and recombinant full-length human CD39, an 80–95 kDa glycoprotein, possess ATPDase[1] activity—metabolizing both ATP and ADP, but not AMP [6,9–11]. This specificity

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[1] *Abbreviations used:* ACR, apyrase conserved regions; ATPDase, ATP diphosphohydrolase; BSA, bovine serum albumin; DEAE-dextran, diethylaminoethyl-dextran; DMEM, Dulbecco's minimum essential medium; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; E-NTPDase, ecto-nucleoside triphosphate diphosphohydrolase; FACS, fluorescence activated cell sorting; FCS, fetal calf serum; FLCD39, full-length CD39; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); HRP, horseradish peroxidase; mAb, monoclonal antibody; MOPS, 3-(*N*-morpholino)propanesulfonic acid; NTPase, nucleoside triphosphatase; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonylfluoride; PVDF, poly(vinylidene difluoride); SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; solCD39, recombinant soluble human CD39.

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provides the basis for the sequence of events following exposure of an ADP-containing activated platelet releasate to CD39 on the endothelial cell surface.

Importantly, NTPDases demonstrate a high degree of amino acid sequence similarity amongst each other, particularly within five highly conserved regions known as "apyrase conserved regions" (ACR 1–5) [12–19]. Structural analyses revealed that the ACR sequences occur exclusively within the large extracellular domain of CD39 [12,20]. They are strongly conserved phylogenetically from plants to parasites to insects to mammals, implicating these motifs as important contributors to the activity of NTPDases, including CD39 [12,13,15,16,18,21]. Mutational analyses of individual residues within the ACRs as well as ACR deletion analysis have confirmed their central role in the enzymatic and biological activity of CD39 and other E-type NTPDases [22–26]. It is thought that the ACRs line the catalytic pocket of these enzymes [25,27]. In addition, NTPDase1–3 have been reported to form oligomeric complexes from dimers to tetramers, and alterations in this quarternary structure may affect the interactions of the ACRs that are involved in substrate binding and hydrolysis [5,25,28,29].

The conservation of amino acids within the ACRs provided the basis for our previous site-directed mutagenesis studies aimed at identifying amino acids essential for enzymatic and biological activity of CD39 [22,23]. In these studies, a recombinant, soluble form of human CD39 (solCD39) (66 kDa) with enzymatic and biological properties similar to native CD39 was utilized [30]. These analyses identified several amino acid residues important for human solCD39 activity, including S57, D54, and D213 [22,23]. In addition, these studies revealed that certain substitutions of catalytically key residues led to enhanced solCD39 activity. Specifically, D54A and D213A solCD39 mutants exhibited increased enzymatic activity as compared to wild-type solCD39, and kinetic analyses indicated this was due to an increase in their rates of catalysis. The participation of these aspartate residues in catalysis appears to be through their involvement in calcium utilization, potentially aiding cation coordination with the substrate in the catalytic pocket of solCD39 [23]. Furthermore, substitution of alanine at serine 57 yielded an enzyme with a 2-fold increase in ADPase activity without changing its ATPase activity, effectively increasing the enzyme's preference for ADP [22].

SolCD39 and FLCD39 differ in that solCD39 is composed of only the extracellular domain of CD39 and lacks the N- and C-terminal transmembrane domains [22,23,30]. In addition to anchoring CD39 to the cell membrane, the transmembrane domains function as mediators of multimerization [25,31]. Hence, solCD39, lacking the transmembrane domains, acts as a monomer. Studies have shown that interaction between the transmembrane domains and the active site of CD39 modulates both total enzymatic activity and substrate specificity [29,31–33].

In this study, the effects of the S57A, D54A, and D213A mutations on the function of FLCD39 were examined. We found that, unexpectedly, the enzymatic activity of S57, D54, and D213 alanine modified FLCD39s was less than that of wild-type, in contrast to the enhanced activity of their soluble counterparts. Furthermore, conservative substitution of D54 and D213 with glutamate led to enzymes with ADPase activities greater than the alanine modified FLCD39s, but not to the level of wild-type. The observed reductions in mutant enzyme activity were primarily associated with reduced catalytic rates. The differences in enzymatic activity were not attributable to gross changes in the nucleotide binding pocket or the enzyme's ability to multimerize. We conclude that, in the context of transmembrane domain localization in the cells' membrane, the composition of the active site of wild-type CD39/NTPDase1 is optimal for enzymatic and biological function in the regulation of purinergic signaling.

Materials and methods

DNA construction

Wild-type human FLCD39 cDNA was PCR amplified with primers encoding an N-terminal Flag epitope sequence (DYKDDDDK) and inserted into the *Pst*I and *Hin*dIII sites of pAAV-MCS (Stratagene). This generated the parent plasmid, pAAV-FLCD39-N-Flag, wherein expression of N-terminal Flag-tagged FLCD39 is driven by the strong cytomegalovirus (CMV) promoter. pAAV-FLCD39-N-Flag mutants (S57A, D54A, and D213A) were generated by exchanging restriction fragments containing the specific mutation from corresponding mutant solCD39 plasmids [22,23]. To create the D54E- and D213E-pAAV-FLCD39-N-Flag plasmids, the CD39 cDNA sequence was PCR amplified in two overlapping segments, a 5′ and a 3′ fragment. Primers used generated common *Bsa*I sites on the 3′ end of the 5′ fragment and the 5′ end of the 3′ fragment, within the overlapping sequence in these fragments. The primer that was used to produce the 5′ fragment also contained the desired mutation, 3′ of the *Bsa*I sequence. The *Bsa*I sites were located such that digestion with *Bsa*I, a Type IIs restriction endonuclease, would generate cohesive ends at a common position within the native sequence in the overlapping segment. The fragments were digested with *Bsa*I and each fragment additionally digested with a restriction endonuclease that cut at a unique site within the fragment. These fragments were then ligated into the pAAV-FLCD39-N-Flag plasmid backbone previously digested with both of the unique cutting restriction endonucleases. The recombinant vector DNAs were transfected into competent *Escherichia coli* host cells (Clontech). Potential mutant FLCD39-containing clones were selected, plasmid DNA isolated (Qiagen) and sequenced to confirm both the presence of the desired mutation and the absence of unwanted base changes.

COS-1 cell expression of human wild-type and mutant FLCD39

Plasmid DNA was transiently transfected into subconfluent layers of COS-1 cells (ATCC) using DEAE-dextran followed by chloroquine as described [22,23] or via lipofection using the GenePORTER 2 Transfection Reagent (Gene Therapy Systems) according to manufacturers recommendations [22]. Transfected cells were incubated (37 °C, 5% CO_2) in 10% FCS-supplemented DMEM in 24-well plates or 10 cm^2 Petri dishes. After 48–72 h, cells were harvested and used for analyses. Cell membranes were also prepared from the transfectants and used for analyses (see below). COS-1 cells transfected with empty vector served as negative control.

Total membrane preparation

Cell membranes from transfected COS-1 cells ((2–4) 10 cm^2 Petri dishes each) were prepared as described [31,34,35]. Briefly, cell monolayers