Biochemical Characterization of CD39L4

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ABSTRACT: Nucleotides are involved in regulating a number of important processes ranging from inflammation to platelet aggregation. Enzymes that can modulate levels of nucleotides in the blood therefore represent important regulatory components in these physiological systems. CD39L4 is a soluble E-nucleoside triphosphate dephosphohydrolase (E-NTPDase) with specificity for nucleotide diphosphates (NDPs). In this study, stable mammalian and insect cell lines were generated expressing CD39L4 protein to purify and characterize the recombinant protein. We demonstrate that recombinant CD39L4 protein expressed in human embryonic carcinoma 293 cells is glycosylated by comparing the molecular masses before and after glycosidase treatment. Activity measurements of CD39L4 isolated from tunicamycin-treated, transiently transfected COS-7 cells indicate that glycosylation is not required for full ADPase activity. Recombinant human CD39L4 protein isolated from stable insect cells was glycosylated differently, but also demonstrated relative activity comparable to that of the mammalian protein. When denatured by SDS under nonreducing conditions, a fraction of the CD39L4 protein migrates as a 110 kDa disulfide-linked dimer. We determined that the monomer is the most active form of CD39L4 by measuring the activity of sucrose density gradient fractions of monomers and partially purified dimers. The physiological significance of the biochemical and enzymatic characterization is discussed.

Platelet aggregation is accompanied by a rapid release of ADP, serotonin, thromboxane A2, and other signaling molecules which act in an autocrine fashion on additional platelets arriving at the site of original aggregation (*1*, *2*). We are attempting to develop new approaches toward reduction of platelet activation and recruitment. To this end, we and others have identified and characterized genes encoding soluble as well as cell-associated proteins capable of hydrolyzing nucleotides. CD39L4 has previously been characterized as a soluble E-NTPDase[1] with substrate specificity toward NDPs and relatively low specificity toward NTPs (*3*). Recently, the enzymatic characterization of another soluble E-NTPDase, CD39L2, also revealed a specificity toward the hydrolysis of NDPs (*4*), thus defining a subclass of human E-NTPDases with unique substrate specificities and soluble properties. CD39 is the most extensively characterized member of the family, with a rate of hydrolysis of nucleotide triphosphates comparable to that of the diphosphates (*5*−*7*). CD39 has been shown to be expressed in vascular endothelial cells and to inhibit ADP-induced platelet aggregation in vitro (*6*−*8*). It has been postulated that this enzyme may be involved in the control of platelet reactivity due to its ability to hydrolyze nucleotides promoting platelet activation and recruitment. This prediction is supported by evidence derived from mouse gene deletion studies (*9*).

CD39 is an integral membrane protein with two transmembrane domains (one at each end of the protein). A small portion of the amino terminus of the protein appears to be cytoplasmic, but the largest portion of the molecule is extracellular, including the portion responsible for its enzymatic activity. Maximal enzymatic activity of CD39 is attained when the enzyme exists as a membrane-bound tetramer (*10*).

CD39L4 has three potential glycosylation sites, and on the basis of its apparent mobility on SDS−PAGE, we have hypothesized that the protein is glycosylated (*3*). In this study, we tested the effect of deglycosylation on enzymatic activity. Furthermore, the formation of dimers and their effect on enzymatic activity were studied. We demonstrate that neither glycosylation nor dimerization is necessary for enzymatic activity of CD39L4. These features distinguish CD39L4 from the other proteins in this family, and may suggest an important role for this soluble enzyme in platelet biology.

EXPERIMENTAL PROCEDURES

CD39L4 Expression Vectors. The construction of the mammalian expression construct CD39L4-His_6 pcDNA3.1 with a Gly-Ser-His_6 epitope tag immediately following Arg^{24} has been described elsewhere (*3*). The insect expression plasmid CD39L4 pIZ was constructed by digesting CD39L4 in pcDNA3.1 with *Hin*dIII and *Xho*I and inserting the open reading frame into the polylinker of the vector pIZ/V5-His (Invitrogen). This construct did not include the Gly-Ser-His_6 epitope tag following Arg^{24}. Additionally, the CD39L4 stop codon was included in the sequence, thereby preventing the inclusion of the detection tags, V5 and His_6.

Cell Culture. Human embryonic kidney 293 cells were obtained from the American Type Culture Collection and

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[1] Abbreviations: Ap5A, P^1,P^5-di(adenosine)-5′-pentaphosphate; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; E-NTPDase, E-nucleoside triphosphate dephosphohydrolase; FBS, fetal bovine serum; NDP, nucleotide diphosphate; NEM, *N*-ethylmaleimide; NTP, nucleotide triphosphate.

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grown in DMEM/F12 medium (Gibco) supplemented with 10% FBS (Gibco) and 100 units/mL penicillin G and 100 μg/mL streptomycin sulfate at 37 °C in 10% CO_2. Transfections were performed at 60% confluency in a 10 cm plate with Fugene-6 (Roche) according to the instructions of the manufacturer. In summary, cells in 8 mL of medium were incubated with 16 μL of Fugene-6 (Roche) and 4 μg of CD39L4-His_6 pcDNA3.1. After 20 h, the cells were transferred to a T-150 flask and the medium was replaced with 293 medium containing 800 μg/mL Geneticin (G418, Life Technologies Inc.). The cells were fed every 2 days with selection medium for 20 days.

High Five insect cells were grown in serum-free High Five medium (Invitrogen) at 27 °C. Transfections were performed at 60% confluence in a 6 cm plate with Insectin-Plus liposomes (Invitrogen). In summary, the transfection mixture was generated by incubating10 μg of CD39L4 pIZ with 20 μL of Insectin-plus liposomes and 1 mL of High Five serum-free medium for 15 min. The cells were incubated with the transfection mixture for 4 h. The transfection was terminated by adding 2 mL of High Five serum-free medium, and the cells were incubated for an additional 48 h before being transferred to 96-well plates containing 400 μg/mL Zeocin. After selection for 3 weeks, the insect cell medium from 96-well plates was assayed for the secreted protein by Western blotting of a slot blot. The high overproducer clone was rescreened by Western blot analysis of medium separated on a 10% SDS−polyacrylamide gel.

Antibodies. Anti-human CD39L4 polyclonal antibodies were generated in rabbits immunized with a 14-amino acid peptide (EVAKDSIPRSHWKK) corresponding to amino acids 109−122 (Animal Pharm). The antibodies were affinity purified using the peptide sequence shown above with an extra Cys residue added at the C-terminus so that it could be coupled to the Sulfo link coupling gel (Pierce).

Affinity Purification of the CD39L4-His_6 Protein. Low-serum medium (1% FBS) from a 293 stable transfected cell line or transiently transfected COS-7 cells was collected and centrifuged to clear any cell debris. Protease inhibitors (0.5 μg/mL leupeptin, 0.7 μg/mL pepstatin, and 0.2 μg/mL aprotinin) were added to the medium before purification through a Ni−NTA column (Qiagen). The column was washed with 10 column volumes of PBS buffer and the protein eluted with 0.1 M sodium acetate (pH 4.5) and 0.3 M NaCl. The eluted protein was collected as fractions and neutralized with Tris-HCl (pH 9) added to a final concentration of 70 mM. The fractions with the most ADPase activity were pooled and concentrated further with Microcon concentrators (Amicon).

Western Blot. The affinity-purified mammalian-derived protein or medium samples from transfected High Five cells expressing CD39L4 were separated by SDS−polyacrylamide gel electrophoresis (10%) under reducing (143 mM β-mercaptoethanol) or nonreducing conditions. The proteins were transferred onto an Immobilon-P (Millipore) membrane and incubated with a CD39L4 specific antibody (1 μg/mL) and with a horseradish peroxidase-conjugated anti-mouse antibody (Pierce). The bands were visualized using a chemiluminescent reagent (ECL, Amersham Pharmacia). Quantitative information was extracted with the Image Master 1D primer software (Amershan-Pharmacia) and an Epson expression 800 scanner with a transparency unit.

Tunicamycin Treatment. The transient transfection of COS-7 cells has been described elsewhere (*3*). The tunicamycin stock was prepared in DMSO (Sigma).

The culture medium was replaced 24 h after transfection with either 1% FBS−DMEM medium containing 5 μg/mL tunicamycin (Sigma) or medium containing DMSO alone (control cells). The following day, the culture medium was replaced again with medium containing the same amount of tunicamycin or with DMSO alone (control cells), and 48 h later, the cells were harvested.

Density Gradient Ultracentrifugation. The centrifugation studies using a sucrose gradient were carried out in a Beckman L8-M (Fullerton, CA) ultracentrifuge equipped with a SW 41 rotor. Samples were centrifuged at 39 000 rpm for 15 h at 4 °C without using the brake. The protein was layered on top of a 5 to 15% (w/v) sucrose density gradient (10 mL) in 10 mM Tris (pH 7.5), 150 mM NaCl, and 5 mM $CaCl_2$. Individual fractions were then collected from the bottom of the tube and processed for UDPase and ADPase activity assays, immunoblotting, Coomassie staining, and refractive index measurements. The mass of CD39L4 was estimated by comparison of its mobility with those of standard proteins. The molecular mass standards that were employed were cytochrome *c* (12 400 Da), carbonic anhydrase (29 000 Da), bovine serum albumin (66 000 Da), alcohol dehydrogenase (150 000 Da), and β-amylase (200 000 Da).

ADPase Assay. Enzyme activity was determined by measuring the amount of inorganic phosphate released from ADP as previously described (*3*) using the technique of Daly and Ertingshausen (*11*). The protein was assayed by the addition of ADP or UDP to a final concentration of 15 mM and incubated at 37 °C for 15 min. The determination of kinetic parameters for ADP hydrolysis was carried out in the presence of the apyrase assay buffer (15 mM $CaCl_2$, 1 mM ouabain, 10 mM NEM, and 10 μM Ap5A) and varying concentrations of ADP, at 37 °C for 15 min. The reaction was stopped by adding 100 volumes of phosphorus reagent (Sigma). The amount of phosphate released from each reaction was quantitated by comparing the absorbance at 340 nm with that of a calcium/phosphorus standard (Sigma).

Curve fitting of the Michaelis−Menten equation [$V_0 = (V_{max}[S])/(K_m + [S])$] to the data points was performed by DeltaGraph 4.0 software (SPSS Inc., Chicago, IL), and the resulting R^2 value was 0.994.

RESULTS

Expression of the CD39L4 Protein in Human 293 Cells. To study the biochemical properties of the CD39L4 protein, a stable human 293 embryonic kidney cell line was established. The construct contained a Gly-Ser-His_6 epitope immediately after Arg^{24}, allowing for purification via the His_6 tag at the amino terminus as described in Experimental Procedures. The protein was analyzed under nonreducing as well as reducing conditions using Western blotting (Figure 1, lanes A and C). The predicted molecular mass is 46 kDa, but under both nonreducing and reducing conditions, we detected a major species with a mobility of approximately 51 kDa. We hypothesized that this shift in mobility was due to glycosylation because of three potential N-glycosylation sites in the predicted amino acid sequence.

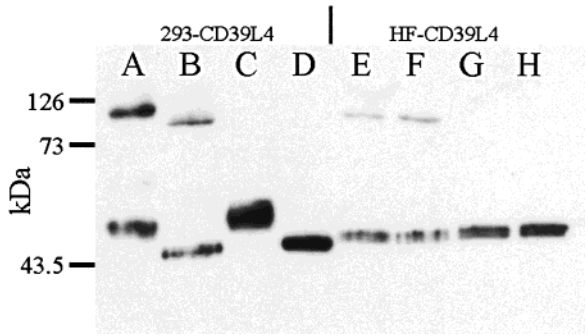

FIGURE 1: Immunoblot analysis of CD39L4 protein treated with peptide *N*-glycosidase F. Protein was isolated from transfected 293 and High Five cells as described in Experimental Procedures. The samples were incubated in either the presence or absence of peptide *N*-glycosidase F. The samples were denatured in Laemmli buffer in either the absence or presence of the reducing agent β-mercaptoethanol (143 mM). Molecular size markers are indicated. Lanes A−D correspond to protein isolated from transfected 293 cells: (A) nonreduced, (B) nonreduced and deglycosylated, (C) reduced, and (D) reduced and deglycosylated. Lanes E−H correspond to protein derived from transfected High Five insect cells: (E) nonreduced, (F) nonreduced and deglycosylated, (G) reduced, and (H) reduced and deglycosylated.

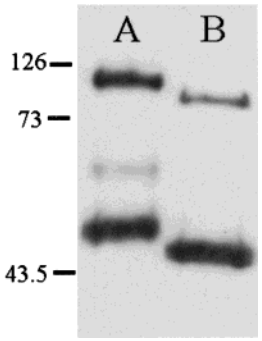

FIGURE 2: Mammalian- vs insect-produced CD39L4 activity. The protein was purified as described in Experimental Procedures. ADPase activity was determined for several dilutions of the protein. When equal activities were matched, the samples were loaded on a nonreducing SDS−PAGE gel and the relative amounts of protein estimated from the intensity of the band with a scanning unit: (A) 293-derived CD39L4 protein and (B) High Five-derived CD39L4 protein.

To show that the protein is glycosylated, we incubated the 293 cell derived protein in the presence of peptide *N*-glycosidase F (PNGase F) (Figure 1, lanes B and D). When the protein is treated with the glycosidase, protein migrates at approximately 46 kDa. Interestingly, under nonreducing conditions, we also detected a second species with a mobility of approximately 110 kDa when glycosylated and approximately 100 kDa when deglycosylated. These species could correspond to dimers held together via disulfide bonds (Figure 1, lanes A and B).

Production of Recombinant Human CD39L4 in High Five Insect Cells. In general, insect cells glycosylate recombinant proteins differently than mammalian cells (*12*). Post-translational modifications in many cases impart new properties to proteins that affect their solubility as well as intrinsic properties. To test whether modifications in glycosylation are important for the enzymatic activity of CD39L4, we expressed the protein in High Five insect cells. The recombinant CD39L4 protein was detected as a major band of approximately 46 kDa in the culture medium, indicating that CD39L4 is efficiently secreted in insect cells. The observed molecular mass of recombinant CD39L4 in insect cells was in very close agreement with that of the deglycosylated mammalian protein (Figure 1, lane E). A minor band was also detected at around 100 kDa which could correspond to disulfide-linked dimers (Figure 1, lanes E and F). The recombinant protein was also treated with PNGase F, but no change in mobility was observed, demonstrating that the insect derived CD39L4 protein is not glycosylated to the same extent as that of the protein derived from 293 cells (Figure 1, lanes E and F). Reduction of the disulfide bonds also resulted in the disappearance of the 100 kDa species (Figure 1, lanes G and H).

Human CD39L4 Recombinant Proteins Produced from Insect and Mammalian Cell Lines Have Similar Activities. An important goal of this study is to identify the most active ADPase form of the CD39L4 protein. To determine this, we compared the activities of the minimally glycosylated insect protein with that of the glycosylated mammalian protein. By using a fixed amount of insect-produced protein and varying amounts of mammalian-derived protein, we determined matching amounts of ADPase activities. Relative amounts of protein with comparable activities were then determined by immunoblotting with anti-CD39L4 antibody. In Figure 2, the amounts of proteins required to yield comparable activities are quantitatively similar in the monomers, but the amount of the disulfide-linked dimer contribution in the mammalian fraction is twice as much as that of the insect fraction. Since the amounts of monomers are similar and the amounts of the dimers are not, it is possible that the dimers do not contribute significantly to the enzymatic activity of the enzyme. Nevertheless, the main conclusion of this experiment is that the insect-produced protein (Figure 2, lane B) although glycosylated differently is at least as active as the mammalian-derived protein (Figure 2, lane A).

COS-7 cells expressing CD39L4 protein were cultured in the presence of the glycosylation inhibitor, tunicamycin, which prevents the addition of N-linked glycans to the nascent polypeptide chain (*13*). As shown in Figure 3, cells treated with tunicamycin exclusively produced a protein with a molecular mass of 46 kDa, which corresponds to CD39L4 lacking all the N-linked glycans. Treatment with tunicamycin also affected the amount of protein found in the medium, reducing by approximately 90% the amount of protein found in the medium when compared to the control cells (data not shown). Different amounts of proteins were tested until matching ADPase activities were found. Relative amounts of protein with comparable activity were then determined by immunoblotting with anti-CD39L4 antibody. This analysis demonstrates that both the deglycosylated and glycosylated forms of CD39L4 protein have equal activities (Figure 3, lanes B and D).

The CD39L4 Disulfide-Linked Dimer Is Inactive. Native CD39L4 protein was subjected to ultracentrifugation in a 5 to 15% sucrose density gradient, and the sedimentation of CD39L4 protein was compared with that of marker proteins with known native molecular masses. As shown in Figure 4, most of the CD39L4 activity was observed in the 7.25% region of the sucrose gradient, the peak of CD39L4 immunoreactivity also colocalized with the peak of activity yielding an estimated molecular mass of approximately 50 kDa (Figure 4). The regression plot for the standards (r =

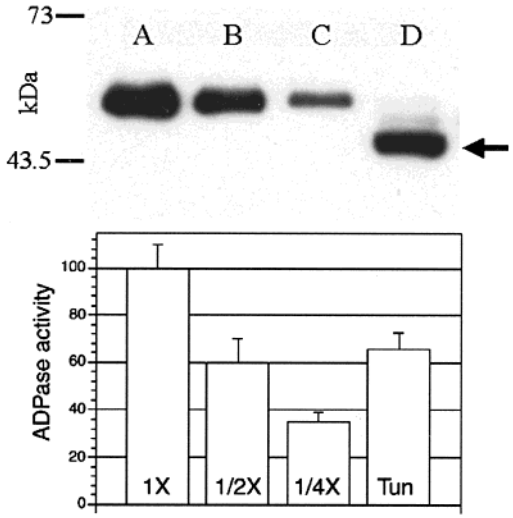

FIGURE 3: Effect of tunicamycin on CD39L4 activity. COS-7 cells transiently transfected with CD39L4-His_6 pcDNA3.1 were incubated in either the presence or absence of tunicamycin. The protein derived from the tunicamycin-treated cells was compared to several dilutions of the control protein, and their activities were measured. Lanes A−C correspond to 2-fold dilutions of the control protein, and lane D corresponds to the tunicamycin-treated sample.

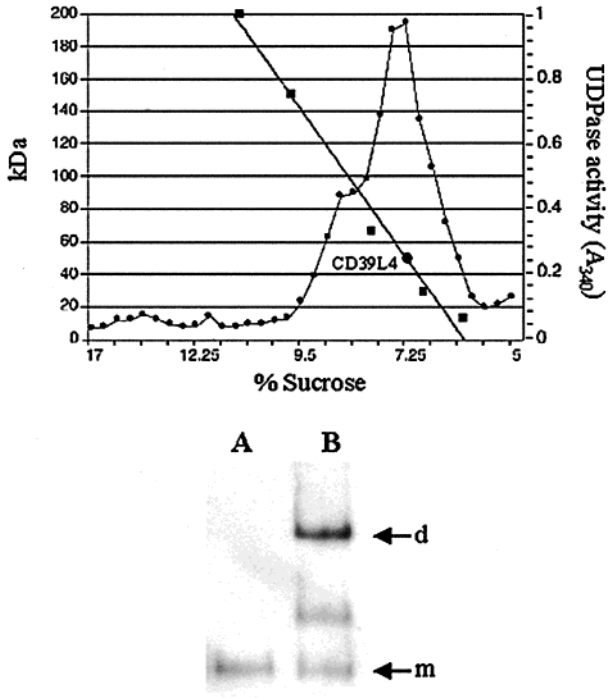

FIGURE 4: Sucrose density gradient centrifugation of CD39L4. CD39L4 protein was purified from 293 cells transfected with a CD39L4-His_6 construct. The protein was isolated from the medium and purified with Ni−NTA resin. Each fraction was assayed: (1) for UDPase activity, (2) with Coomassie blue-stained gels to determine the position of the standards, (3) by immunoblotting with a CD39L4 specific antibody, and (4) by refractometry to determine the percentage of sucrose. The calibration curve was obtained by plotting the percentage of sucrose vs the molecular mass of the standards. The immunoblot panel represents fraction 27 (lane A) and fraction 17 (lane B) after matching ADPase activities. Monomers (m) and dimers (d) are shown by arrows.

0.985) is also shown in Figure 4. The peak of activity was not fully symmetrical, containing a smaller amount of activity sedimenting at a higher sucrose concentration between 8 and 9%. This smaller peak has an estimated molecular mass of approximately 100 kDa. Western blot analysis of these fractions demonstrated that they were enriched for dimers although not fully devoid of monomers. Different amounts of proteins were tested until matching ADPase activities were found for fraction 17 (9.4% sucrose; Figure 4, lane B) and fraction 27 (7.0% sucrose; Figure 4, lane A). Relative amounts of proteins with comparable activity were determined by immunoblotting with anti-CD39L4 antibody. The analysis showed equivalent amounts of monomeric CD39L4 after quantitative scanning. However, in fraction 17 the dimer concentration was the predominant form, yet did not appear to contribute to ADPase activity, suggesting that the dimers are inactive.

Characterization of the ADPase Activity of CD39L4. Previous data (*3*) showed that CD39L4 exhibits a higher affinity for nucleoside diphosphates than for nucleoside triphosphates. In particular, it has been shown that its ADPase activity is at least 20-fold greater than its ATPase activity. A more detailed characterization of the ADPase activity has been carried out by determining the kinetic parameters for ADP hydrolysis. The rate of product release was found to be linear within the first 20 min of reaction; therefore, the initial velocity V_0 was taken to be the rate of reaction over the first 15 min. Initial velocities plotted as a function of ADP concentration revealed normal Michaelis−Menten kinetics (Figure 5). Curve fitting of the Michaelis−Menten equation [$V_0 = (V_{max}[S])/(K_m + [S])$] to the data points resulted in a calculated V_{max} of 1191 pmol/min and a K_m of 12.7 mM. The amount of partially purified CD39L4 protein in each reaction mixture was estimated to be 60 ng, and when a molecular mass of 46 000 g/mol is assumed for CD39L4 protein, the turnover number, k_{cat}, of CD39L4 with ADP as a substrate was determined to be 913 min^{-1}.

DISCUSSION

Experiments in the current study provide evidence that human recombinant CD39L4 protein expressed in 293 embryonic carcinoma cells is glycosylated and capable of forming disulfide-linked dimers. Interestingly, neither dimer formation nor glycosylation appears to contribute significantly to its ADPase activity.

CD39L4 forms dimers held together via a disulfide bond. The predicted mature protein encodes five cysteine residues. Therefore, one of the residues is potentially available for dimer formation. The dimers and monomers can be detected with nonreducing SDS gels, although the predominant species is the monomeric form. We also measured the size of the native protein by sucrose density gradient centrifugation. Under these conditions, the peak of activity and protein concentration colocalized with the monomeric form of CD39L4. Comparable amounts of ADPase activities from monomer- and dimer-enriched fractions from the sucrose density gradient demonstrated that the disulfide-linked dimer does not possess any significant level of enzymatic activity (Figure 4). In Figure 2, we observed equal amounts of monomers from the mammalian- and insect-derived protein samples. However, the amount of disulfide-linked dimer in the mammalian-derived sample was twice as much as that found in the insect-derived protein. On the basis of these observations, we propose that the monomer is the active form of the protein and that the disulfide-linked dimer represents

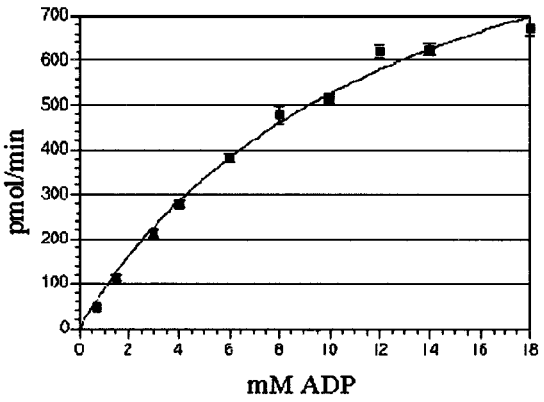

FIGURE 5: Determination of K_m and V_{max} of CD39L4 ADPase activity. Michaelis−Menten data representation of the ADP concentration from 0.5 to 18 mM. V_0 was determined over a range of ADP concentrations, and each data point represents an average of three separate experiments. Curve fitting of the data points resulted in an r^2 value equal to 0.994.

an inactive form due to the oxidation of the odd-numbered cysteine residues found in CD39L4. Examination of the CD39, CD39L2, and CD39L3 amino acid sequences revealed an odd number of cysteine residues. Interestingly, CD39L2, the family member most like CD39L4, does not show dimer formation via disulfide bonds (*4*). CD39 also encodes an odd number of cysteine residues. However, the first cysteine residue has been shown to be palmitoylated (*14*), a post-translational modification that may facilitate targeting of the protein to caveolae (*15*).

Other members of the human E-NTPDase family such as CD39L3 (*16*) and CD39 (*10*) appear to form higher oligomeric structures. More recently, positive cooperativity for ADPase activity displayed by the CD39L2 protein indicates that this protein also forms multimers (*4*). Interestingly, CD39L4 appears to be unique in that its monomeric form has higher enzymatic activity than its dimeric form. Tetrameric forms by sucrose density gradient centrifugation were not detected, and the kinetic data do not support an allosteric model, often displayed by multimeric proteins.

The cDNA encoding CD39L4 predicts three potential N-glycosylation sites (*17*). Treatment of CD39L4 with peptide *N*-glycosidase F results in a dramatic shift in the mobility of the protein (Figure 1), demonstrating that the enzyme is glycosylated. Interestingly, the mobility of the human CD39L4 expressed in insect cells matches that of the deglycosylated protein and does not shift mobility upon treatment with peptide *N*-glycosidase F. This result suggests that if the protein is glycosylated, it either is resistant to hydrolysis by PNGase F or may not be glycosylated to the same extent as the mammalian-derived protein. Since the two proteins are glycosylated differently, we compared their respective activities to determine whether different levels of glycosylation would affect the enzymatic activity. The results demonstrate that the two proteins appear to be equally active. The effects of preventing N-linked glycosylation on the activity of the mammalian-derived CD39L4 protein were studied with transiently transfected cells cultured in the presence and absence of tunicamycin. As shown in Figure 3, tunicamycin treatment resulted in a shift in the mobility of the protein but also had no effect on enzymatic activity. This result differs from findings for other members of the CD39 family. CD39L3 protein appears to partially lose its enzymatic activity upon treatment with *N*-glycosidase F. A complete loss of CD39L3 activity was obtained from the enzyme isolated from tunicamycin-treated cells (*16*). The same authors treated membranes from COS-7 cells transfected with CD39 with PNGase F, resulting in a time-dependent inactivation of the nucleotidase activity. In contrast, Schulte et al. (*18*) reported no changes in the biochemical activity of CD39-deglycosylated protein.

The kinetic data for the CD39L4 protein show a K_m value in the millimolar range for ADP. However, the levels of ADP in the circulation appear to be in the low micromolar range. Interestingly, CD39L2 also exhibits a similar K_m value (*4*), indicating a potential overlapping function for these two extracellular enzymes.

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