Mutagenesis of Lysine 62, Asparagine 64, and Conserved Region 1 Reduces the Activity of Human Ecto-ATPase (NTPDase 2)†

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Received January 8, 2007; Revised Manuscript Received April 4, 2007

ABSTRACT: The human ecto-ATPase (NTPDase 2) contains conserved motifs including five apyrase conserved regions (ACRs) and four conserved regions (CRs) as well as conserved lysine and arginine residues that are also present in other cell surface E-NTPDases. Some of the positively charged amino acids may be involved in ATP binding. The protein also contains six potential N-linked glycosylation sites. Results obtained with seven lysine and six arginine mutants indicate the importance of K62 that is located in CR1, K182, which is downstream of ACR3, and R155, which immediately follows CR3. Mutation of asparagine at the six potential N-linked glycosylation sites individually to glutamine established the importance of N64 in CR1 and N443 in ACR5 in protein function and expression. Mutation of N64, which is conserved in all cell surface NTPDases, results in the expression of an unstable protein, the activity of which is only manifested in the presence of concanavalin A. Both K62 and N64 reside in CR1 that is conserved in all cell surface NTPDases. In the sequence of the CR1 of human ecto-ATPase, ^{58}WPADKENDTGIV^{69}, ^{65}DTG^{67} is similar to the phosphate-binding motif (DXG) in ACR1 and 4. The D65A and G67A mutants have reduced protein expression and activity. Mutations of other residues in CR1 to alanine led to partial to complete loss of protein expression and activity except for P59. The alanine mutants of the three acidic amino acid residues, D61, E63, and D65, all have decreased affinity for divalent ions. D61 can be substituted by glutamate, but E63 appears to be invariable. Taken together, these results indicate that CR1, which follows ACR1 in the cell surface NTPDases, is an essential structural element in these enzymes.

Nucleoside triphosphate diphosphohydrolases (NTPDases[1]) are glycosylated enzymes that hydrolyze nucleoside triphosphates and diphosphates in the presence of Mg^{2+} or Ca^{2+} and have an exoplasmic active site. Eight members of NTPDases have been established according to sequence homology (*1*). Among these, NTPDases 1, 2, 3, and 8 are cell surface enzymes; NTPDases 4, 5, and 7 are intracellular enzymes; and NTPDase 6 consists of both cell surface and intracellular enzymes. NTPDases 1−4, 7, and 8 have two membrane-spanning domains, whereas NTPDases 5 and 6 have one membrane-spanning domain and can exist as both membrane bound and soluble forms. All NTPDases have five highly conserved regions called the apyrase conserved regions (ACRs) because they were first identified in the potato apyrase (*2*, *3*), a related NTPase. We identified four other conserved regions (CRs) among the cell surface NTPDases (*4*). Apart from the ACRs and the CRs, the sequences of the different NTPDases vary greatly.

The human ecto-ATPase, an NTPDase 2, preferentially hydrolyzes nucleoside triphosphates with little activity toward nucleoside diphosphates (*5*−*9*). The functional human ecto-ATPase was first cloned from bladder tumor cells by Mateo et al. (*7*) and subsequently from hepatoma and small lung cell carcinoma cells in our laboratory (*9*). The functional splice variant of the human ecto-ATPase contains 495 amino acids and has a native molecular mass of 66 kDa (*7*, *9*, *10*). The enzyme has two transmembrane domains at the N- and C-termini, two short cytoplasmic tails, a large extracelluar domain that contains the conserved regions, and 10 conserved cysteine residues.

Thus far, the three-dimensional structure and active site of the human ecto-ATPase have not been elucidated. Most enzymes that utilize ATP contain positively charged residues in their nucleotide binding sites. The most common binding motifs among ATPases are the Walker A and B binding motifs, which are (G,A)X_4(G,A)K(S,T) and (R,K)X_{1-4}G$X_{2-4}\phi$-Xϕ(D,E), respectively, where ϕ is a hydrophobic amino acid, and X is any amino acid (*11*, *12*). Both motifs contain the positively charged lysine and arginine. However, the Walker sequences are absent in the NTPDases, which belong to the actin-hexokinase-hsp70 protein family (*13*−*15*). Of the five motifs defined for nucleotide binding in this family (*13*), the phosphate 1 motif, DXG, is found in ACR1 of all NTPDases, whereas the phosphate 2 motif, DXXG, corresponds to ACR4 in these proteins (*2*, *3*, *15*). Although both motifs lack positively charged residues, it is important to note that of the 16 sequences of sugar kinases, prokaryotic cell cycle proteins, actin, and hsp70 proteins examined by Bork et al., a lysine is often found to be close to the

† This work was supported by the California Metabolic Research Foundation.

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[1] Abbreviations: NTPDase, nucleoside triphosphate diphosphohydrolase; ACR, apyrase conserved region; CR, conserved region; ConA, concanavalin A; NBT/BCIP, nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate.

10.1021/bi700036e CCC: $37.00
Published on Web 05/10/2007

phosphate 1 motif (*13*). The kinase-1a motif designated for human actin by Traut contains a lysine residue downstream of the phosphate 1 motif as follows: ^{11}DN GSGL CKAG FA (*12*). Thus far, the roles of only a few of the conserved lysine and arginine residues have been examined in human NTPDase 3. Mutation of two conserved arginine residues, one in ACR1 and one in ACR2, did not result in loss of activity when corrected for protein expression level (*16*). Mutation of K79 in CR1 resulted in moderate reduction of protein expression but ~80% of the activity (*17*).

In this study, five lysine and five arginine residues were chosen for site-directed mutagenesis after examining the sequences of 22 members of the NTPDase family. In addition to conserved lysine and arginine residues, the human ecto-ATPase has six putative N-linked glycosylation sites. A previous study by Mateo et al. showed that mutation of N443 to aspartate caused the retention of the mutant protein in the endoplasmic reticulum (*10*). The roles of the other five asparagine residues in the proper processing or function of the human ecto-ATPase were not assessed. The results obtained from mutagenesis of the residues mentioned above revealed the importance of K62 and N64, both located in the previously unexamined conserved region 1 (CR1) with the sequence, ^{58}WPADKENDTGIV^{69}. Site-directed mutagenesis of other residues in this region indicated that it is an essential structural element in human ecto-ATPase.

EXPERIMENTAL PROCEDURES

Materials. The QuikChange site-directed mutagenesis kit was purchased from Stratagene. Oligonucleotides were synthesized at the Microchemical Core Facility at San Diego State University, GenBase (San Diego, CA), and IDT (San Diego, CA). *Pfu* Turbo DNA polymerase, Epicurian *coli* XL1-Blue competent cells, and SURE competent cells were purchased from Stratagene. DH5α *E. coli* cells, Dulbecco's modified Eagle's media (DMEM), OptiMEM, Lipofectamine, trypsin-EDTA, and antibiotics were purchased from Invitrogen. Fetal calf serum was purchased from Invitrogen and Gemini-Bio Products (Calabasas, CA). Newborn calf serum was purchased from Gemini-Bio Products. ATP, ConA, and streptavidin immobilized on 4% beaded agarose were purchased from Sigma Chemical Co. Prestained protein ladder was purchased from Fermentas and Invitrogen. PVDF membrane was purchased from Perkin-Elmer Life Sciences Inc. and Millipore Corporation. Goat anti-rabbit IgG conjugated with alkaline phosphatase was purchased from Promega. Alkaline phosphatase substrate tablets (NBT/BCIP) were purchased from Roche. SDS−PAGE reagents and Bio-Rad Dc protein assay reagents were purchased from Bio-Rad Laboratories. EZ-link sulfo-NHS-biotin was purchased from Pierce. Polyclonal antibody against the C-terminus (LRQVHSAKLPSTI-COOH) of the human ecto-ATPase was a kind gift from Dr. Terence Kirley of the Department of Pharmacology and Cell Biophysics at the University of Cincinnati.

Site-Directed Mutagenesis of the Human Ecto-ATPase. The human ecto-ATPase cDNA, cloned from human small cell lung carcinoma and inserted into the mammalian expression vector pcDNA3 (*9*), was used as the template in PCR reactions using the QuikChange site-directed mutagenesis kit. The 50 μL PCR mixture contained 5 μL of 10× reaction buffer, 1−2 μL of human ecto-ATPase cDNA (50 ng/μL), 1−3 μL of forward and reverse mutagenic primers (100 ng/μL), 1 μL of dNTP mixture (10 mM), and 2.5 units of the *Pfu* Turbo DNA polymerase. The sequences of the forward mutagenic primers (sense oligonucleotides) used in this study are shown in Table 1. The following primer, 5′-GAGAACTTCATCAGGTACGGCTGGGTG-3′, was used initially for generating the K182R mutant but proved to be problematic. The correct mutation for the K182R mutant was generated with the oligonucleotide primer containing the codon AGA for arginine (Table 1). Double asparagine mutants were generated using the single asparagine mutant cDNA as the template in the PCR mixture and the appropriate forward and reverse mutagenic primers in order to introduce mutation of the second N-linked glycosylation site. The PCR cycle contained the following steps: (1) 95 °C for 30 s, (2) 95 °C for 30 s, (3) 55 or 60 °C for 1 min, and (4) 68 °C for 14 min. Steps 2, 3, and 4 were repeated 16 times. The PCR mixture was then incubated at 37 °C for 1 h with Dpn I to digest the parent template. After verifying that the PCR reactions were successful by agarose gel electrophoresis, 2 μL of the PCR reaction mixture were transformed into Epicurian *coli* XL1-Blue competent cells, SURE competent cells, or DH5α *E. coli* cells. Plasmid DNA was isolated and sequenced to confirm the presence of the mutation and absence of any other alterations in the DNA. DNA sequenc-

Table 1: Sense Oligonucleotides Used for the Generation of Mutants in This Study[a]

mutation	sense oligonucleotides
	lysine mutants
K39A	5′-CGCCCGCCCTCGCGTATGGCATCGTCCTGG-3′
K57A	5′-CCATGTTTATCTACGCGTGGCCGGCAGACAAG-3′
K62A	5′-CCGGCAGACGCGGAGAACGACAC-3′
K62R	5′-CCGGCAGACAGGGAGAACGACAC-3′
K182A	5′-GAGAACTTCATCGCGTACGGCTGGGTGG-3′
K182R	5′-GAGAACTTCATCAGATACGGCTGGGTG-3′
K428A	5′-CGTGATCTTCCAGAAGGCGGCCGCGGACACTGC-3′
	arginine mutants
R155A	5′-CCCTTTGACTTCGCAGGTGCACGCATC-3′
R155K	5′-CCCTTTGACTTCAAGGGTGCACGCATC-3′
R158A	5′-CTTCCGGGGTGCAGCAATCCTCTCGGGC-3′
R245A	5′-CTCTGCTATGGCGCAGACCAGGTCCTC-3′
R311A	5′-CCCCACCTCTGCGCAGATCTGGTTTC-3′
R394A	5′-GGGCAACGGGCCGCACTGGCCGACTAC-3′
	mutants of N-glycosylation sites
N64Q	5′-GCAGACAAGGAGCAGGACACAGGCATTGTG-3′
N88Q	5′-CAGCTATGCAGACCAGCCTTCTGGGGCCAGC-3′
N129Q	5′-GTATGCGCCTGCTCCAGCTGACCAATCCAGA-3′
N294Q	5′-CCCCAGAACTTCCAGAGCAGTGCCAGGGTC-3′
N378Q	5′-GTGAATGTCTGCCAGCAGACCTGGGCTCAG-3′
N443Q	5′-GGCTACATGCTGCAGCTGACCAACCTGATC-3′
	CR1 mutants
W58A	5′-GTTTATCTACAAGGCGCCGGCAGACAAG-3′
P59A	5′-GATCTACAAGTGGGCGGCAGACAAGGAG-3′
D61A	5′-GTGGCCGGCAGCAAAGGAGAACGA-3′
D61E	5′-GTGGCCGGCAGAAAAGGAGAACGAC-3′
E63A	5′-CCGGCAGACAAGGCAAACGACACAG-3′
E63D	5′-CCGGCAGACAAGGACAACGACACAGGC-3′
D65A	5′-CAAGGAGAACGCAACAGGCATTG-3′
D65E	5′-CAAGGAGAACGAGACAGGCATTG -3′
T66A	5′-CAAGGAGAACGACGCAGGCATTGTGGGCG-3′
G67A	5′-GAGAACGACACAGCAATTGTGGGC-3′
I68A	5′-CGACACAGGCGCTGTGGGCCAGC-3′
V69A	5′-GACACAGGCATTGCGGGCCAGCAC -3′

[a] The codon of the introduced mutation is underlined in each of the sense oligonucleotides.

ing service was provided by the Microchemical Core Facility at San Diego State University.

Transient Transfection in HEK293 Cells. The HEK293 cells were maintained in DMEM containing 1% penicillin/streptomycin, 5% fetal calf serum, and 5% newborn calf serum at 37 °C in an atmosphere of 5% CO_2/95% air. Unless stated otherwise, cells grown in 6-well plates were used for transfection. After reaching 50−70% confluency, the cells in each well were transfected with 1 μg of vector or wild-type and mutant human ecto-ATPase cDNAs using 5 μL of Lipofectamine in 1 mL of OptiMEM according to the manufacturer's instructions. Four hours after transfection, 1 mL of DMEM containing serum was added back to each well. Fresh media were added after 24 h. Forty-eight to 72 h after transfection, the cells were harvested by trypsinization, washed twice with 1.5−3 mL of isotonic buffer (0.1 M NaCl, 0.01 M KCl, and 25 mM TrisCl at pH 7.5), and resuspended in a small volume (200−400 μL) of the same buffer for determination of ATPase activity and protein concentration.

ATPase Assay. ATPase activities of transiently transfected cells were determined in 0.5 mL of reaction mixture containing 50 mM TrisCl at pH 7.5, 2.5 mM $MgCl_2$, 0.1 mM NaN_3, 2.5 mM ATP, and 10−25 μL of cells (13−100 μg of protein) in duplicates. ATPase reactions were initiated with the addition of ATP and allowed to proceed at 37 °C for 10 min and then stopped with the addition of 0.1 mL of 10% trichloroacetic acid. Denatured proteins were removed by centrifugation and an aliquot (0.1−0.3 mL) of the supernatant was used for the determination of Pi by adding 2 mL of a colorimetric reagent consisting of 1 volume of 10 mM ammonium molybdate, 1 volume of 5 N H_2SO_4, and 2 volumes of acetone as described previously (*9*). Absorbance was determined at 355 nm. The method measures Pi in the range of 0−0.25 μmol.

Preparation of Membranes from Transfected HEK293 Cells for Determination of Affinity for ATP and Divalent Cations. Cell membranes were prepared from transiently tranfected HEK293 cells grown on 10-cm plates. Cells at 50−70% confluency were transfected with 5 μg of wild-type or mutant human ecto-ATPase cDNAs mixed with 30 μL of Lipofectamine in 6.5 mL of OptiMEM. After 4 h, 6.5 mL of DMEM containing serum were added to the plate. The transfection media were replaced with 10 mL of fresh media after 24 h. Forty-eight hours after transfection, cells were harvested by trypsinization. The cell pellets were resuspended in 3 mL of 0.25 M sucrose, 30 mM MOPS at pH 7.5, and 2 mM EDTA. After homogenation in a Potter-Elvehjem homogenizer, the cell homogenate was centrifuged at 48,000 rpm in a Beckman TY 65 rotor. The pellet was resuspended in 1 mL of 0.25 M sucrose/10 mM TrisCl at pH 8, and centrifuged for 5 min at 3,000 rpm in a microfuge to remove cell debris and nuclear material. The supernatant that contained cell membranes was used for ATPase determination.

For the determination of *K*m for MgATP and CaATP, the reaction mixture (0.25 mL) containing 50 mM TrisCl at pH 7.5, 0.1 mM azide (to inhibit mitochondrial ATPase), and varying concentrations of MgATP or CaATP were brought to 37 °C before the reaction was initiated by the addition of membrane (10−25 μg of protein). [γ-^{32}P]ATP (1−2 × 10^6 cpm/μmol) was used as the substrate, and the Mg or Ca to ATP ratio was maintained at 1. The reaction was terminated after 1 min by the addition of 0.05 mL of 1.8 N $HClO_4$. The amount of ^{32}Pi in an aliquot (0.15 mL) of the reaction mixture was determined after extraction with isobutanol/benzene as previously described (*5*).

For the determination of the binding of Mg^{2+} and Ca^{2+} by the wild-type and mutant human ecto-ATPases, the reaction mixture (0.25 mL) contained 50 mM TrisCl at pH 7.5, 0.1 mM azide, 2.5 mM ATP, and varying concentrations of $MgCl_2$ or $CaCl_2$. The reaction was initiated by the addition of membranes (10−25 μg of protein). After incubation at 37 °C for 10 min, the reactions were terminated by the addition of 0.05 mL of 10% trichloroacetic acid. After centrifugation to remove denatured membrane proteins, an aliquot (0.1−0.15 mL) of the supernatant was used for the determination of Pi with 1 mL of the colorimetric reagent as described above.

Protein Determination. Protein concentrations of cell lysates and membranes were determined using Bio-Rad Dc Reagent with bovine serum albumin as the standard. The absorbance was measured at 750 nm.

SDS−PAGE and Western Blot Analysis. Samples of the cell lysate to be analyzed were mixed with 4× SDS gel sample buffer without reducing agents and heated at 100 °C for 3 min. SDS−PAGE was carried out with 25 μg of proteins in 7.5% or 10% polyacrylamide gel according to the method of Laemmli (*18*). After transfer to a PVDF membrane, immunoblotting was carried out as previously described (*9*) using a polyclonal antibody directed against the C-terminus (LRQVHSAKLPSTI-COOH) of the human ecto-ATPase as the primary antibody (5000-fold dilution) and goat anti-rabbit IgG conjugated to alkaline phosphatase as the secondary antibody (5000-fold dilution).

Cell Surface Biotinylation. For cell surface biotinylation, HEK293 cells were grown in10-cm plates and transfected with 5 μg of the wild-type and mutant human ecto-ATPase cDNAs mixed with 30 μL of Lipofectamine as described above. The harvested cells were collected in 10 mL of media and divided into two 5 mL aliquots, one for biotinylation and one as a control. The cells in the two aliquots were centrifuged, resuspended in 1 mL of phosphate-buffered saline (PBS), and transferred to a microfuge tube and centrifuged. The cell pellets were washed two more times with PBS and resuspended in 1 mL of PBS. EZ-Link Sulfo-NHS-Biotin (0.5−0.8 mg) was added to the cells, and the tubes were rotated at room temperature for 30 min. The biotinylated cells were pelleted by brief centrifugation, washed twice with 1 mL of isotonic buffer (0.1 M NaCl, 0.01 M KCl, and 25 mM TrisCl at pH 7.5), and once with 1 mL of PBS. The cells were solubilized in 0.5 mL of RIPA solution (0.15 M NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, and 50 mM TrisCl at pH 8). After incubation on ice for 30 min with vortexing at 5-min intervals, the cell suspension was centrifuged at 4 °C for 20 min at 13,000 rpm to remove insoluble cell debris. To the supernatant was added 100 μL of a 50% slurry of streptavidin-agarose beads, and the tubes were rotated for 20 h at 4 °C. The streptavidin-agarose beads were washed three times with 1 mL of 1% Triton X-100 in Tris-buffered saline, resuspended in 200 μL of 1× SDS sample buffer, and boiled for 5 min. After centrifugation at 13,000 rpm for 4 min to sediment the agarose beads, aliquots of the supernatant were used for

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  1 MAGKVRSLLPPLLLAAAGLAGLLLLCVPTR
 31 DVREPPALKYGIVLDAGSSHTSMFIYKWPA
 61 DKENDTGIVGQHSSCDVPGGGISSYADNPS
 91 GASQSLVGCLEQALQDVPKERHAGTPLYLG
121 ATAGMRLLNLTNPEASTSVLMAVTHTLTQY
151 PFDFRGARILSGQEEGVFGWVTANYLLENF
181 IKYGWVGRWFRPRKGTLGAMDLGGASTQIT
211 FETTSPAEDRASEVQLHLYGQHYRVYTHSF
241 LCYGRDQVLQRLLASALQTHGFHPCWPRGF
271 STQVLLGDVYQSPCTMAQRPQNFNSSARVS
301 LSGSSDPHLCRDLVSGLFSFSSCPFSRCSF
331 NGVFQPPVAGNFVAFSAFFYTVDFLRTSMG
361 LPVATLQQLEAAAVNVCNQTWAQLQARVPG
391 QRARLADYCAGAMFVQQLLSRGYGFDERAF
421 GGVIFQKKAADTAVGWALGYMLNLTNLIPA
451 DPPGLRKGTDFSSWVVLLLLFASALLAALV
481 LLLRQVHSAKLPSTI
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FIGURE 1: Domain structure of the human ecto-ATPase (NTPDase 2) and amino acid residues selected for mutagenesis in this study. The primary structure of the human ecto-ATPase is shown. The two transmembranous domains are underlined. The five apyrase conserved regions (ACR) are shaded in dark gray. The four conserved regions (CR) are shaded in light gray. Lysine (K), arginine (R), and CR1 residues mutated in this study are shown in bold. The asparagine (N) residues mutated in this study are boxed and shown in bold.

Table 2: Ecto-ATPase Activities of HEK293 Cells Transiently Transfected with the pcDNA3 Vector, Wild-Type Human Ecto-ATPase, and Lysine Mutant cDNAs[a]

cDNA	% WT ecto-ATPase activity
pcDNA3	0.23 ± 0.14
WT	100
K39A	80.0 ± 10.5
K57A	84.2 ± 12.6
K62A	0.24 ± 0.32
K62R	0.65 ± 0.50
K182A	0.83 ± 1.07
K182R	24.4 ± 10.9
K428A	95.5 ± 23.5

[a] The specific ecto-ATPase activity of HEK293 cells transfected with the wild-type human ecto-ATPase cDNA ranged between 1.12 and 2.89 μmol min^{-1} mg of protein^{-1}. The values reported represent the average of at least three separate transfections ± standard deviations.

Western blot analysis with the antibody against the C-terminus peptide of human ecto-ATPase.

RESULTS

Mutagenesis of Conserved Lysine Residues in Human Ecto-ATPase. Seven human ecto-ATPase lysine mutant cDNAs (K39A, K57A, K62A, K62R, K182A, K182R, and K428A) were generated. The lysine residues as well as the arginine, asparagine, and CR1 residues that were mutated in this study are shown in bold in Figure 1. Arginine mutants were generated for those lysine to alanine mutations which resulted in a loss of more than 95% of wild-type human ecto-ATPase activity. The data in Table 2 show that the K39A, K57A, and K428A mutants lost little activity, displaying 80%, 84%, and 96% of the wild-type enzyme activity, respectively. Expression of the K39A, K57A, and K428A mutant proteins was also comparable to that of the wild-type enzyme (Figure 2). In contrast, enzyme activity was abolished upon mutation of K62 and K182 to alanine, which correlated with a lack of protein expression (K62A and K182A in Figure 2). Protein expression was somewhat increased relative to the K62A mutant when K62 was replaced with arginine (K62R in Figure 2); however, the ATPase activity of the K62R mutant remained similar to that of mock-transfected cells. Substitution of K182 by arginine resulted in protein expression similar to that of the wild-type enzyme (K182R in Figure 2); however, its activity was only 24% of that of the wild-type enzyme. These results indicate that although a positively charged amino acid residue at position 182, which occurs after ACR3, is sufficient for protein expression, the presence of lysine is necessary for full activity. However, although substitution of K62 by arginine also increased protein expression relative to the K62A mutant, there was negligible increase in ATPase activity.

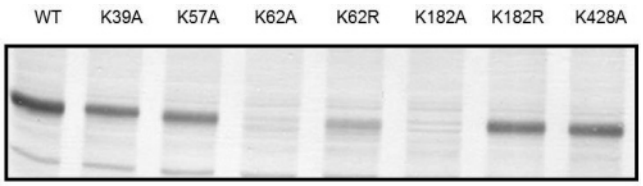

FIGURE 2: Western blot analysis of wild-type and lysine mutant ecto-ATPases. Twenty-five micrograms of lysates of cells transiently transfected with recombinant plasmids containing wild-type and mutant human ecto-ATPase cDNAs were electrophoresed in a 10% polyacrylamide gel. Western blot analysis was conducted as described in Experimental Procedures.

Table 3: Ecto-ATPase Activities of HEK293 Cells Transiently Transfected with the Wild-Type Human Ecto-ATPase and Arginine Mutant cDNAs[a]

cDNA	% WT ecto-ATPase activity
WT	100
R155A	22.2 ± 8.9
R155K	87.0 ± 8.5
R158A	118.2 ± 29.0
R245A	47.3 ± 12.7
R311A	101.7 ± 28.7
R394A	56.7 ± 10.3

[a] The specific ecto-ATPase activity of HEK293 cells transfected with the wild-type human ecto-ATPase cDNA ranged between 1.48 and 3.71 μmol min^{-1} mg of protein^{-1}. The values reported represent the average of at least three separate transfections ± standard deviations.

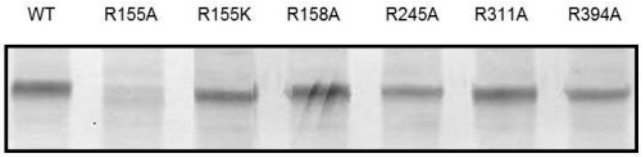

FIGURE 3: Western blot analysis of wild-type and arginine mutant ecto-ATPases. Twenty-five micrograms of lysates of cells transiently transfected with recombinant plasmids containing wild-type and mutant human ecto-ATPase cDNAs were electrophoresed in a 10% polyacrylamide gel.

Mutagenesis of Conserved Arginine Residues in Human Ecto-ATPase. Five arginine residues in the human ecto-ATPase were mutated to alanine. The ATPase activities of the mutants R158A, R245A, R311A, and R394A were 118%, 47%, 102%, and 57%, respectively, of the activity of the wild-type enzyme (Table 3) and correlated with protein expression (Figure 3). The low activity of the R155A mutant also correlated with the markedly diminished protein expres-

Table 4: Ecto-ATPase Activities of HEK293 Cells Transiently Transfected with Wild-Type Human Ecto-ATPase and Mutant cDNA in Which the Asparagines Residues of Potential N-Linked Glycosylation Sites Are Mutated to Glutamine

cDNA	% WT ecto-ATPase activity
	A
WT	100
N64Q	4.02 ± 2.63
N88Q	39.1 ± 15.6
N129Q	66.1 ± 15.0
N294Q	45.2 ± 10.1
N378Q	86.2 ± 20.2
N443Q	21.6 ± 10.2
	B
WT	100
N88Q/N129Q	15.3 ± 3.6
N88Q/N294Q	13.8 ± 4.5
N88Q/N378Q	41.9 ± 15.3
N129Q/N294Q	49.5 ± 5.7
N129Q/N378Q	60.3 ± 18.1
N294Q/N378Q	22.7 ± 5.2

[a] The specific ecto-ATPase activity of HEK293 cells transfected with the wild-type human ecto-ATPase cDNA ranged between 1.5 and 3.0 μmol min^{-1} mg of protein^{-1}. The values reported represent the average of at least four separate transfections ± standard deviations.

sion of the mutant. Substitution of R155 with lysine restored both enzymatic activity and protein expression to levels similar to that of the wild-type enzyme (Table 3 and Figure 3). These results indicate that a positively charged amino acid at position 155, which immediately follows CR3 and precedes ACR3 (Figure 1), is important for both protein expression and enzyme activity.

Mutagenesis of Potential N-Linked Glycosylation Sites in Human Ecto-ATPase. The polypeptide encoded by the human ecto-ATPase cDNA has a predicted molecular mass of 54 kDa, whereas the mature protein has an observed molecular mass of 66 kDa, indicating that the protein is glycosylated. We next determined if N-linked glycosylation is necessary for the expression and function of human ecto-ATPase. Human ecto-ATPase has six potential N-linked glycosylation sites, that is, N64, N88, N129, N294, N378, and N443, because these asparagine residues are found in the NXT/S consensus sequence (Figure 1). When the individual N-linked glycosylation site was removed by substituting asparagine with glutamine, the most deleterious effects were obtained with the N64Q and N443Q mutants, which displayed only 4% and 22%, respectively, of the wild-type enzyme activity (Part A in Table 4). Protein expression of the N64Q mutant was similar to that of the wild-type protein, but the N443Q mutant was poorly expressed (Figure 4). The N88Q, N129Q, and N294Q mutants retained 40−70% ATPase activity, whereas the N378Q mutant displayed full activity (Part A in Table 4). Except for N88Q, all of the asparagine mutant proteins showed reduced molecular mass (Figure 4), indicating that the other five potential N-linked glycosylation sites are utilized in the native protein in the HEK293 cells. The N378Q mutant ecto-ATPase, which was fully active, also showed the highest protein expression, whereas the reduced activities of the N88Q, N129Q, N294Q, and N443Q mutants correlated with reduced protein expression (Figure 4). Three important conclusions obtained from this series of mutants are (1) the N64Q mutant ecto-ATPase protein, although synthesized, appears to be inactive, (2) the N88Q mutant shows reduced protein expression even though N88 is not glycosylated in the native protein, and (3) glycosylation of N378 is not essential for activity, although it is glycosylated in the native protein.

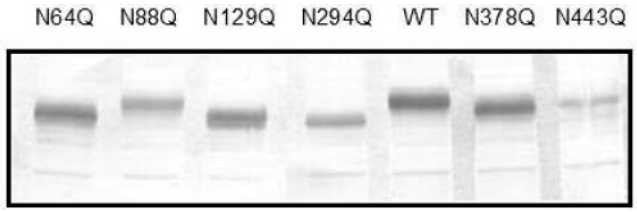

FIGURE 4: Western blot analysis of wild-type and asparagine mutant ecto-ATPases. Twenty-five micrograms of lysates of cells transiently transfected with recombinant plasmids containing wild-type and N-linked glycosylation mutant human ecto-ATPase cDNAs were electrophoresed in a 10% polyacrylamide gel. The N-mutants had variable expression. N88Q had a molecular mass similar to that of the wild-type enzyme, indicating that N88 was not utilized for glycosylation in HEK293 cells.

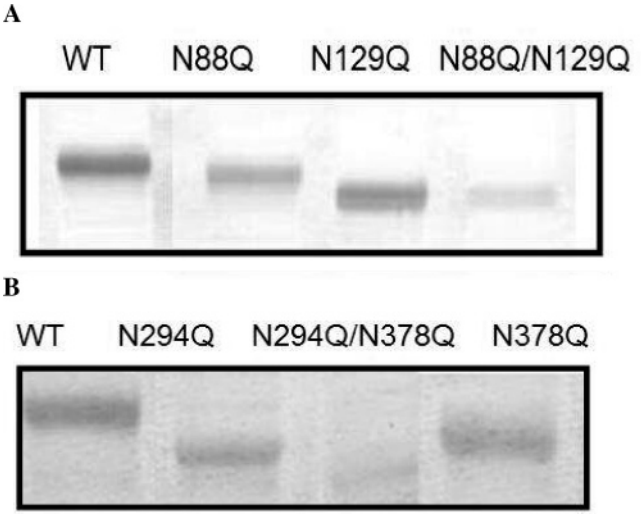

FIGURE 5: Western blot analysis of wild-type, single, and double asparagine mutant ecto-ATPases. Twenty-five micrograms of lysates of cells transiently transfected with recombinant plasmids containing wild-type, single, and double N-linked glycosylation mutant human ecto-ATPase cDNAs were electrophoresed in 10% polyacrylamide gel. (A) Single asparagine mutants, N88Q and N129Q, and their double mutant N88Q/N129Q. (B) Single asparagine mutants, N294Q and N378Q, and their double mutant N294Q/N378Q. The double mutants had markedly decreased protein expression.

Double mutation of the four non-essential N-glycosylation sites resulted in mutants whose activities followed two patterns. The N88Q/N378Q, N129Q/N294Q, and N129Q/N378Q mutants had activities similar to that of the single mutant with the lower activity (Part B in Table 4). However, the N88Q/N129Q, N88Q/N294Q, and N294Q/N378Q double mutants had lower ATPase activity than the activities of either of the individual mutants. The greater loss of activity of these double asparagine mutants correlated with diminished protein expression as shown for N88Q/N129Q (Figure 5A) and N294Q/N378Q (Figure 5B) when compared to the single asparagine mutants from which they were derived. The N88Q/N129Q mutant protein had the same molecular mass as that of the N129Q mutant protein (Figure 5A), supporting the conclusion that N88 of the human ecto-ATPase was not glycosylated in HEK293 cells. However, the molecular mass of the double mutant N294Q/N378Q was less than that of either of the single N294Q and N378Q mutants, as was expected (Figure 5B).

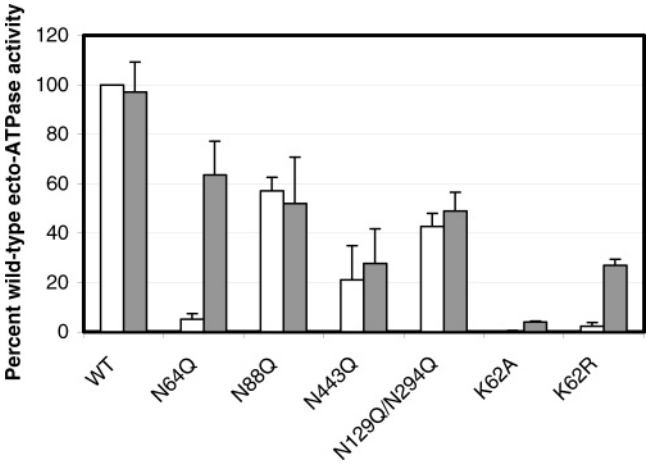

FIGURE 6: Ecto-ATPase activities of wild-type, several N-linked glycosylation mutants, and the K62A and K62R mutants obtained in the absence and presence of ConA. Ecto-ATPase activities of HEK293 cells transiently transfected with wild-type and mutant human ecto-ATPase cDNAs were determined in 0.5 mL reaction mixture containing 0.1 M NaCl, 0.01M KCl, 25 mM TrisCl at pH 7.5, 0.1 mM NaN_3, 5 mM $MgCl_2$, and 5 mM ATP with and without ConA. ConA (125 μg) was added to the reaction mixture (without ATP) containing 60−90 μg of cell protein. After 5 min of preincubation at 23 °C, the reaction was initiated by the addition of ATP and allowed to proceed at 37 °C for 10 min. The white bars represent values obtained in the absence of ConA and the gray bars represent values obtained in the presence of ConA ± standard deviations.

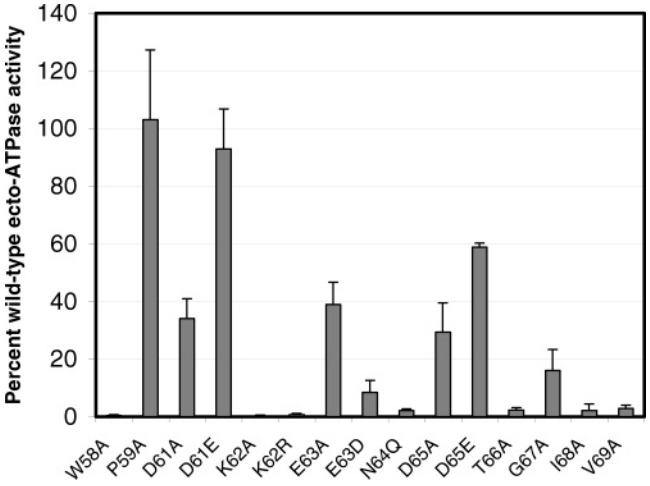

FIGURE 7: Ecto-ATPase activities of wild-type human ecto-ATPase and mutants of CR1. Ecto-ATPase activities of HEK293 cells transiently transfected with the wild-type human ecto-ATPase and mutant cDNAs of the CR1 region were determined as described in Experimental Procedures. Specific ecto-ATPase activity of HEK cells transfected with wild-type human ecto-ATPase cDNA ranged between 1.2 and 2.5 μmol min^{-1} mg of protein $^{-1}$. The values reported represent the average of at least three separate transfections ± standard deviations.

We were particularly interested in the N64Q mutant, which was expressed (Figure 4) but showed little activity (Table 4, Part A). Cell surface biotinylation of HEK293 cells transfected with the N64Q mutant ecto-ATPase cDNA also indicated that the protein was delivered to the cell surface (data not shown). These results suggest that the N64Q mutant protein was incapacitated. Nevertheless, the protein was resistant to limited proteolysis (data not shown), indicating maintenance of global structure. More interestingly, significant ATPase activity was obtained in the presence of ConA. The experiment was conducted because (1) previous reports showed that the ATPase activities of cell membrane fractions containing human ecto-ATPase (NTPDase 2) and human NTPDase 3 are increased 2−3 fold when assayed in the presence of the lectin, ConA (*9*, *19*), and (2) when N81 of the human NTPDase 3, which is equivalent to N64 of the human ecto-ATPase, was mutated to aspartate, the ATPase activity of the N81D mutant was no longer stimulated by ConA (*19*). We found that, in contrast to ATPase activity in the membranes, there was little stimulation of the ecto-ATPase activity of intact cells transfected with wild-type human ecto-ATPase cDNA by ConA (Figure 6), probably because the enzyme already exists in an oligomeric form that is optimal for enzyme activity. In contrast, cells tranfected with N64Q showed <5% of the wild-type enzyme activity when assayed in the absence of ConA but 50−75% of the wild-type enzyme activity when assayed in the presence of ConA, that is, a greater than 10-fold stimulation of ecto-ATPase activity by ConA (Figure 6). None of the other single or double N-linked glycosylation mutants showed similar enhancement of activity in the presence of ConA as shown by the data obtained with the N88Q, N443Q, and N129Q/N294Q mutants in Figure 6. These results indicated that the N64Q mutant protein was synthesized and delivered to the cell membrane in a conformation that is not suitable for catalysis. The marked stimulation of the ATPase activity of the N64Q mutant by ConA suggests that ConA-induced oligomerization altered the protein conformation so that the enzyme regained its activity.

Mutagenesis of Amino Acid Residues in Conserved Region 1 (CR1) of Human Ecto-ATPase. The results described above indicate that K62 in CR1 is invariant and that mutagenesis of N64 results in an unstable protein. Both K62 and N64 are located in conserved region 1 (CR1). CR1 and three other conserved regions (CR2−4) shown in Figure 1 are not present in all of the NTPase sequences originally selected for the identification of the five apyrase conserved regions (*2*, *3*). These four conserved regions were identified upon alignment of the sequences of the cell surface NTPDases, that is, NTPDases 1, 2, 3, and 8 (*4*). CR1, which is located between ACR1 and a region designated as ACR1a (*19*), is absent from NTPDases 4−7 as shown in the sequence alignment of human NTPDases 1−8 in Supporting Information.

A closer examination of the CR1 sequence revealed the presence of ^{65}DTG^{67}, a motif similar to the phosphate 1 (DXG) and phosphate 2 (DXXG) motifs that are located in ACRs 1 and 4 of the NTPDases. The DTG sequence is also present in the CR1 of NTPDases 1, regardless of species, and murine NTPDase 8 but not in NTPDase 3. To explore the possibility that ^{65}DTG^{67} in the human ecto-ATPase constitutes an alternative or additional phosphate binding site, D65A and G67A mutants were generated. Both mutants suffered significant loss of activity with the D65A mutant displaying ∼30% and the G67A mutant displaying only ∼16% of the wild-type enzyme activity (Figure 7). The activity loss of the G67A mutant was greater than the reduction in protein expression (Figure 8A). A more conservative substitution of D65 by glutamate resulted in a mutant, D65E, whose protein expression was similar to that of the wild-type enzyme but only ∼60% activity (Figure 7).

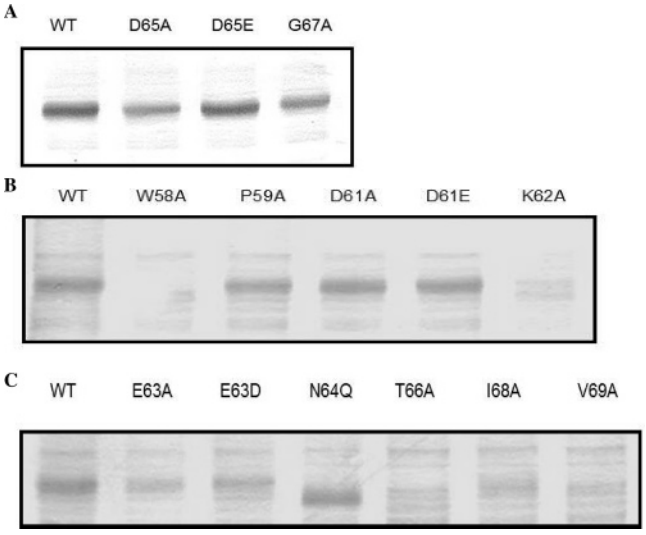

FIGURE 8: Western blot analysis of wild-type human ecto-ATPase and mutants of CR1. Twenty-five micrograms of lysates of cells transiently transfected with recombinant plasmids containing wild-type and mutant human ecto-ATPase cDNAs were electrophoresed in a 7.5% polyacrylamide gel. (A) Samples were lysates of cells transfected with the wild-type enzyme, D65A, D65E, and G67A mutant ecto-ATPase cDNAs. (B) Samples were lysates of cells transfected with the wild-type, W58A, P59A, D61A, D61E, and K62A mutant ecto-ATPase cDNAs. (C) Samples were lysates of cells transfected with wild-type, E63A, E63D, N64Q, T66A, I68A, and V69A mutant ecto-ATPase cDNAs.

We subsequently individually mutated the remaining seven amino acid residues in CR1 to alanine, except for A60. The W58A, T66A, I68A, and V69A mutant proteins suffered the greatest loss in ecto-ATPase activity, having values that were 0.48%, 3.7%, 2.1%, and 2.9%, respectively, of the wild-type enzyme activity (Figure 7). Western blot analysis indicated that all four mutants had minimal protein expression (Figure 8B and C) with a lower molecular weight band detected for T66A (Figure 8C). This was expected because the N-linked glycosylation consensus sequence, ^{64}NDT^{66}, was replaced by ^{64}NDA^{66} in the T66A mutant, and N64 could no longer be glycosylated. However, in contrast to N64Q, the protein expression of which was not affected, the T66A mutant was poorly expressed, and its activity was not enhanced when assayed in the presence of ConA (data not shown).

Surprisingly, the mutation of P59 to alanine had no effect on either the expression or activity of the enzyme. Mutation of D61 and E63 to alanine resulted in a loss of ~70% activity. D61 could be substituted by glutamate because the D61E mutant had activity similar to that of the wild-type enzyme. However, E63 could not be substituted by aspartate because the E63D mutant had even less activity (8.8%) than that of the E63A mutant (38.9%) (Figure 7). Protein expression of the E63D mutant was comparable to that of the E63A mutant (Figure 8C).

In summary, W58, T66, G67, I68, and V69 in CR1 are important for protein expression. Of the three acidic residues present in the region, E63 cannot be replaced by aspartate, whereas a negatively charged amino acid residue suffices for position 61. Only partial activity (~60%) was obtained upon substitution of D65 by glutamate.

Effect of Mutation of Acidic Residues in CR1 on the Binding of Divalent Ions and ATP. Mutation of the three acidic residues in CR1, D61, E63, and D65 to alanine all resulted in ~70% loss of activity. To investigate whether cation or substrate binding is altered in these mutants, we determined the ATPase activities of these mutant proteins by varying Mg^{2+}, Ca^{2+}, and ATP concentrations using membranes prepared from transiently transfected HEK293 cells.

Table 5: *K*m Values for MgATP, CaATP, Mg^{2+}, and Ca^{2+} of Wild-Type Human Ecto-ATPase and Mutants D61A, D61E, E63A, and D65A[a]

	MgATP (mM)	CaATP (mM)	Mg^{2+} (mM)	Ca^{2+} (mM)
WT	0.37 ± 0.029	0.499 ± 0.128	0.73 ± 0.054	0.38 ± 0.042
D61A	0.304 ± 0.096	0.388 ± 0.087	>15	6.58 ± 2.53
D61E	0.375 ± 0.133	0.482 ± 0.102	0.31 ± 0.057	0.15 ± 0.00
E63A	0.206 ± 0.059	0.362 ± 0.06	>15	>15
D65A	0.127 ± 0.047	0.139 ± 0.035	5.72 ± 1.35	5.51 ± 1.15

[a] The *K*m values for MgATP, CaATP, Mg^{2+}, and Ca^{2+} were determined using membranes prepared from HEK293 cells transiently transfected with wild-type human ectco-ATPase and the four mutant ecto-ATPase cDNAs at the same time. For the determination of *K*m for MgATP and CaATP, the Mg/ATP and Ca/ATP ratios were maintained at 1. For the determination of *K*m for Mg^{2+} and Ca^{2+}, ATP was maintained at 2.5 mM, and $MgCl_2$ and $CaCl_2$ concentrations were varied from 0.08−15 mM. The *K*m values were the average of three separate determinations of the same membrane ± standard deviations.

*K*m values obtained with either MgATP or CaATP, in which the cation/ATP ratios were maintained at 1, indicate that substrate binding to the mutant proteins are similar to that of the wild-type enzyme (Table 5), with the D65A mutant showing lower *K*m values. However, binding of Mg^{2+} or Ca^{2+} decreased markedly in the D61A, E63A, and D65A mutants when compared to that in the wild-type enzyme as illustrated by the D61A mutant (Figure 9). The *K*m value estimated using total Mg^{2+} and Ca^{2+} concentrations in the presence of 2.5 mM ATP indicated that there was a 10−20-fold increase in *K*m for the divalent cations for the D61A, E63A, and D65A mutants. However, the affinities of Mg^{2+} and Ca^{2+} for the D61E mutant, which had ATPase activity comparable to that of the wild-type enzyme, were actually higher than that of the wild-type enzyme (Figure 9 and Table 5).

DISCUSSION

The amino acid residues involved in substrate binding and catalysis of the NTPDases have not been established. An early study of the rat brain NTPDase 1/CD39 utilized protein modifying reagents to identify the amino acid residues that are important for enzymatic activity. It was shown that phenylmethylsulfonyl fluoride and 5,5′-dithiobis(2-nitrobenzoic acid) had no effect on enzyme activity, but reaction of the enzyme with phenylglyoxal, Woodward's reagent, Koshland's reagent, and maleic anhydride led to 70−90% decrease in ADPase and ATPase activity. When preincubated with substrate and then individually treated with the group modifying reagents, the decline in activity in the presence of Woodward reagent and phenylglyoxal was significantly attenuated. Wink et al. concluded that arginine, tryptophan, amino groups, and carboxylic groups are implicated in the active site (*21*).

Since the molecular cloning of various NTPDases, extensive mutagenesis studies have been conducted on the human NTPDase 3 by Kirley's group, and the results were sum-

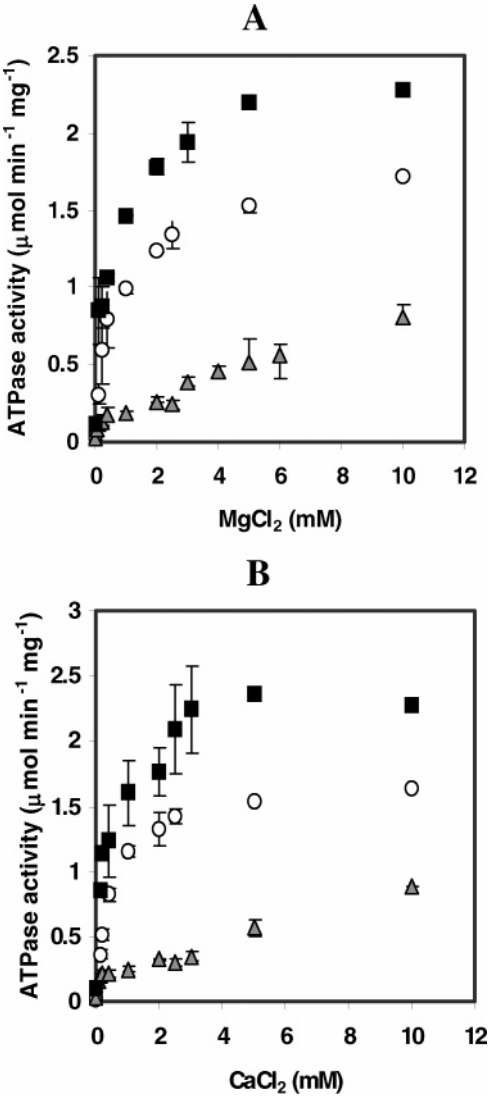

FIGURE 9: Dependence of ATPase activities of the wild-type, the D61A, and the D61E mutant human ecto-ATPases on Mg^{2+} and Ca^{2+}. ATPase activities were determined using membranes (10−25 μg of protein) prepared from HEK293 cells transiently transfected with wild-type human ecto-ATPase (○−○), D61A (Δ−Δ), and D61E (■−■) mutant ecto-ATPase cDNAs at the same time. The membrane containing the D61E mutant protein displayed higher ATPase activity than that containing the wild-type enzyme. The reaction mixtures contained 2.5 mM ATP and 0−10 mM $MgCl_2$ (A) or $CaCl_2$ (B). Data shown are the average of three separate determinations with the same membrane ± standard deviations.

marized in a recent review (*22*). These and the mutagenesis studies of other NTPDases focused mostly on residues within the ACRs. Smith and Kirley established that aspartate and glycine residues of the phosphate 1 and 2 motifs, DXG, located in ACR1 and ACR4, respectively, are important for catalytic activity of the human NTPDase 3 (*15*). Drosopoulos et al. reported that mutation of E174 (in ACR3) and S218 (in ACR4) in the soluble human NTPDase 1/CD39 to alanine resulted in an inactive enzyme (*23*). The same glutamate and serine residues in the membrane-bound human NTPDase 3 were found to be also essential for activity (*16*), thus establishing the importance of these two residues in the function of most NTPDases. It was postulated that the specific serine and glutamate residues are respectively involved in nucleotide and Ca^{2+} binding (*23*). However, the results of a later study suggested that D54 and D213 in the two DXG motifs of the soluble NTPDase 1/CD39 are also implicated in calcium binding (*24*). Interestingly, despite their reduced affinity for Ca^{2+}, the ATPase activities of the two aspartate mutants of the soluble CD39 were actually enhanced (*24*) in contrast to the loss of activity seen with the membrane-bound human NTPDase 3 that had the same mutations (*15*). Mutation of various other conserved residues in the ACRs of NTPDase 3 led to protein misfolding, disruption of trafficking to the cell membrane, altered nucleotide binding, and ADPase/ATPase ratios (*16*, *20*, *25*).

Except for two previous reports on the mutagenesis of R146 of the human NTPDase 3 (*16*), K66A of the soluble human NTPDase1/CD39 (*23*), and a recent report on the mutagenesis of K79 in the human NTPDase 3 (*17*), which correspond, respectively, to R126 (in ACR2), K57 (preceding CR1), and K62 (in CR1) of the human ecto-ATPase, no systematic study has been undertaken to investigate the roles of the conserved lysine and arginine residues of the NTPDases. Their importance in nucleotide binding is clearly seen in the numerous ATP-utilizing enzymes whose 3D structures have been elucidated (*14*, *26*−*29*). In this study, five lysine and five arginine residues in the human ecto-ATPase/NTPDase 2 were mutated.

Despite the close proximity of K39 and K57 to the phosphate 1 motif, ^{45}DAG^{47}, in ACR1, alanine mutants of these two lysine residues still retained >80% of ATPase activity. However, K62, which occurs in CR1, cannot be replaced. K182, which occurs after ACR3, is also required for protein expression. Replacement by arginine restored protein expression but only 20% activity. In contrast, the presence of a positively charged residue at position 155 following CR3 suffices for protein expression and activity. These results indicate that of the 10 conserved lysine and arginine residues, only K62 in CR1 and K182, which is not in a conserved region, appear to be essential for protein expression and function. It is interesting that mutation of most of the conserved arginine and lysine residues did not affect enzyme function, and ADPase/ATPase ratios of the mutants remain below 0.1. Similar results were obtained with another cell surface protein with an extracellular ATP binding site, the $P2X_1$ receptor, which is an ATP-gated ion channel. Ennion et al. mutated 11 positively charged conserved residues of the human $P2X_1$ receptor and found that mutation of only 2 of these residues resulted in marked decrease in ATP potency (*30*). It was concluded that most of these positively charged residues are present at the active site to attract the negatively charged ATP, whereas specific lysine residues are the most critical in contacting the β or γ phosphate of ATP (*30*, *31*).

The lysine residue corresponding to K62 in the human ecto-ATPase was previously mutated in human NTPDase 3. However, in contrast to the K62A mutant of the human ecto-ATPase, which was not expressed, and the K62R mutant which was expressed and resistant to limited proteolysis (data not shown) but had little activity, the K79A and K79R mutants of the human NTPDase 3 retained ~70% protein expression and ~20% of the MgATPase activity of the wild-type enzyme (*17*). Nevertheless, a positively charged amino acid residue at position 79 appeared to be required for the enhanced activity of NTPDase 3 obtained in the presence of ConA, a tetravalent lectin that promotes oligomerization

and stimulates the activity of NTPDase 3 and NTPDase 2 from several species (*9*, *19*, *32*−*34*). It was suggested that K79 in human NTPDase 3 may be important in stabilizing the tertiary or quaternary structure of the enzyme (*17*). In contrast, we found that the activities of both K62A and K62R mutants of the human ecto-ATPase were stimulated approximately 10-fold by ConA to ∼4% and ∼28% of the wild-type enzyme activity (Figure 6). Thus, mutation of K62 in the human ecto-ATPase did not interfere with ConA-induced quaternary structure formation. The different results obtained with mutagenesis of this conserved lysine in human NTPDase 2 and NTPDase 3 may be related to the different cells used for transfection but also may reflect a different role of the particular lysine residue in the two NTPDases that have different primary sequences and substrate specificities.

We also determined if glycosylation is important for human ecto-ATPase function. Determination of the 3D structure of the NTPDases would be facilitated if they did not require glycosylation for function and the proteins could be expressed in bacteria. Different members of the NTPDases have variable numbers of N-linked glycosylation sites. Those in human NTPDase 5 and 6 appear not to be essential for proper folding or activity because functional proteins of these two enzymes could be obtained after expression in *E. coli* (*35*, *36*). However, mutation of N81 of human NTPDase 3 to aspartate or glutamate (*19*), N443 of human NTPDase 2/ecto-ATPase to aspartate (*10*), and N73 and N458 of rat NTPDase1/CD39 to serine (*37*) resulted in marked to total loss of activity even though the mutant proteins were expressed in all cases. N64 in the human ecto-ATPase, which is equivalent to N73 in the rat NTPDase 1 and N81 in the human NTPDase 3, is conserved in all the cell surface NTPDases, whereas the asparagine residue in ACR5, that is, N443 in human ecto-ATPase and N458 in rat NTPDase 1, is conserved in NTPDases 1, 2, and 8, but not in NTPDase 3. We found that, of the six N-linked glycosylation sites, mutation of N64 and N443 in the human ecto-ATPase had the most deleterious effects. The N64Q mutant is unique in that its activity in the transfected cells is stimulated more than 10-fold by ConA to 50−75% of that of the wild-type enzyme. This result suggests that although the conformation of the N64Q mutant protein is unsuitable for catalysis, it has evaded the quality control mechanism in the ER. Once expressed at the plasma membrane, cross-linking with ConA may have converted the mutant protein to a conformation that is more favorable for catalysis. The stimulation of the N64Q mutant ecto-ATPase activity by ConA differed markedly from the lack of stimulation of the activity of the N81D mutant of human NTPDase 3 by the same lectin (*19*). The result obtained with the N443Q mutant also differed from that of a previous study by Mateo et al., who showed that the N443D mutant of the human ecto-ATPase had comparable expression in the CHO-K1 cells but that most of it was retained in the endoplasmic reticulum (*10*). In the present study, we found that expression of the N443Q mutant was significantly reduced, but the mutant protein was targeted to the plasma membrane as the cells displayed ecto-ATPase activity. It is not known whether the different expression of the N443 mutant protein in the two studies is due to the different amino acid that was used to substitute asparagine or whether it is due to the different cells used for transfection. In our laboratory, we found that protein expression and glycosylation patterns of the same asparagine mutants in HeLa cells were different from that obtained in the HEK293 cells, yet both results indicate the critical roles of N64 and N443 for human ecto-ATPase function and protein expression (*38*).

Of the six potential N-linked glycosylation sites, N88 does not appear to be glycosylated in the human ecto-ATPase expressed in HEK293 cells as shown by the molecular mass of the N88Q mutant protein, which is similar to that of the wild-type protein (Figures 4 and 5A). Because N88 is followed by a proline, this result agreed with previous reports showing that glycosylation is often impaired when X in the NXS/T motif is a proline (*39*). In spite of the lack of glycosylation of N88, mutation of the residue to glutamine nevertheless resulted in reduced protein expression and ATPase activity, which is exacerbated by the simultaneous mutation of other asparagine residues that are used for N-linked glycosylation, as seen in the N88Q/N129Q and N88Q/N294Q double asparagine mutants (Part B in Table 4).

We noted with interest that K62 and N64, the mutation of which resulted in severe loss of protein expression in the former and activity in the latter, are both located in the CR1, a region which has not been examined previouly. Subsequent mutagenesis of the individual residues in CR1 to alanine showed that except for P59, all are important either for protein expression or function (Figures 7 and 8). There was little protein expression of the W58A, K62A, T66A, I68A, and V69A mutants, most likely due to misfolding of these proteins and their elimination before reaching the plasma membrane. Partial activity was retained in the D61A, E63A, D65A, and G67A mutants. ^{65}DTG^{67} constitutes another phosphate-1 like motif found in many ATP-utilizing enzymes. The aspartate residues in the phosphate motifs are important in interacting with Mg^{2+} and Ca^{2+} that are complexed to ATP, which are essential to ATP binding as well as presenting a suitable conformation of ATP for the catalytic groups at the active site (*13*, *27*, *41*). At present, the roles of the two DXG motifs in ACR1 and ACR4 in NTPDase catalysis are uncertain because the mutation of the two aspartate residues of the soluble human CD39 resulted in proteins with enhanced activities (*24*). Nevertheless, it is unlikely that ^{65}DTG^{67} in the human ecto-ATPase binds phosphate because of the location of CR1. In the linear peptide sequence, CR1 immediately follows ACR1 (Figure 1). In the recently proposed 3D structures of the NTPDases (*42*), which were obtained from computer modeling based on the structure of the crystallized exopolyphosphatase/guanosine pentaphosphate phosphohydrolase (PPX/GPPA) of *Aquifex aeolicus* (*43*), ACR1 and ACR4 are shown to form the substrate binding site, whereas CR1 is located near the top of the substrate binding cleft. Secondary structure analysis also indicated that the ^{59}PADKENDTG^{67} peptide has relatively high solvent accessibility. While it needs to be recognized that PPX/GPPA and other members of the actin-hexokinase-hsp70 family are soluble proteins (*41*, *43*, *44*), whereas the cell-surface NTPDases are membrane-bound proteins, the proposed structures (*22*, *42*) serve as useful models. Our experimental data suggest that CR1 provides a cluster of acidic amino acid residues for cation binding. In addition to D65, mutation of the other two acidic residues,

D61 and E63, to alanine also resulted in loss of activity. Although binding to the MgATP or CaATP substrate complexes was not affected in these three mutants, their divalent cation binding was markedly decreased (Table 5). In the presence of the same concentration of ATP (2.5 mM), maximal activity of the wild-type enzyme was obtained with ~2 mM Mg^{2+} or Ca^{2+}, whereas the activity of the three mutants was still increasing linearly at 10−15 mM divalent ion concentrations (Figure 9). Of these three mutants, D61 can be substituted by glutamate because the D61E mutant has activity and divalent ion binding similar to those of the wild-type enzyme. The fact that the alanine mutants displayed higher activity at divalent ion concentrations that far exceed that of ATP suggest that there may be a separate divalent ion binding site in addition to the divalent ion−ATP complex binding site. Grinthal and Guidotti have observed biphasic dependence of the activities of rat NTPDase 1/CD39 and human ecto-ATPase on calcium, inhibitory in the former and activating in the latter (*8*). The presence of a second Mg^{2+} or Ca^{2+} binding site in the human ecto-ATPase and its importance in the reaction mechanism will be determined in future investigations.

In summary, we have conducted the first extensive mutagenesis study of the human ecto-ATPase and identified a hitherto unexamined conserved region, CR1, as being an important element of the enzyme. The major findings are that (1) several of the residues in CR1 are required for protein expression, (2) K62 and E63 appear to be invariable, (3) glycosylation of N64 is required to produce a stable protein, and (4) substitution of the acidic residues in CR1 by alanine results in decreased divalent ion binding.

ACKNOWLEDGMENT

We thank Dr. Terence L. Kirley for his generous gift of the anti-C-terminus antibody of the human ecto-ATPase. Nicole Pelletier is the recipient of a 2005 Pfizer summer undergraduate research fellowship. We thank Robin Chang for generating the D61A mutant and other assistance during this study.

SUPPORTING INFORMATION AVAILABLE

Amino acid sequence alignment of human NTPDases 1−8. This material is available free of charge via the Internet at http://pubs.acs.org.

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BI700036E