Site-Directed Mutagenesis of a Human Brain Ecto-Apyrase: Evidence That the E-Type ATPases Are Related to the Actin/Heat Shock 70/Sugar Kinase Superfamily†

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ABSTRACT: On the basis of sequence homologies observed between members of the E-type ATPases and the phosphate binding motifs of the actin/heat shock protein 70/sugar kinase superfamily, a human ecto-apyrase was analyzed by site-directed mutagenesis of conserved amino acids in apyrase conserved regions (ACR) I and IV. The expressed proteins were analyzed to assess the significance of these amino acids. A conserved aspartic acid residue in ACR IV was mutated to alanine, asparagine, and glutamic acid, and the relative activity and K_m for ATP and ADP were determined. Mutation of this Asp 219 to Ala or Asn yielded an enzyme severely reduced in ATP hydrolyzing activity (>90%) and completely devoid of ADPase activity, along with a similar extent of inhibition of hydrolysis of other nucleoside di- and triphosphates. Interestingly, mutation of Asp 219 to Glu completely restored the ability of the enzyme to hydrolyze nucleoside triphosphates at levels above that of the wild-type enzyme, while the ability to hydrolyze nucleoside diphosphates was slightly reduced. Mutation of a second conserved aspartic acid in ACR I (Asp 62) and two invariant glycine residues in both ACR I (Gly 64) and ACR IV (Gly 221) also severely disrupted nucleotidase activity. These results demonstrate that the E-type ATPases contain the nucleoside phosphate binding domains present in the actin/heat shock protein/sugar kinase superfamily. Together with analysis of computer-predicted secondary structures, the results suggest that the ecto-ATPases and ecto-apyrases are part of, or closely related to, the actin superfamily of proteins.

The E-type ATPases,[1] consisting of the ecto-ATPases and ecto-ATPDases (ecto-apyrases), are a family of glycosylated enzymes characterized by a nucleotide binding and hydrolysis active site situated on the exterior of the cell (*1*, *2*). These enzymes are found as both soluble (*3*−*5*) and membrane-associated proteins (*6*−*9*). Recently, several studies have focused on the E-type ATPases of nervous tissue (*10*−*13*), since extracellular ATP has been established as a neurotransmitter in the nervous system (*14*−*16*). There is convincing evidence to suggest that ATP is stored within neuronal synaptic vesicles and often coreleased along with other neurotransmitters such as acetylcholine (*17*−*19*). Two families of nucleotide purinoceptors (designated P2X and P2Y) have been discovered that serve as receptors for such nucleotide ligands (*20*, *21*). ATP-induced effects have been shown to be transduced through ligand-gated ion channels and G proteins (*22*−*24*), and mechanisms for the inactivation of the nucleotide ligand after the appropriate response has been generated must be in place. Analogous to acetylcholine breakdown by the ecto-enzyme acetylcholinesterase, ecto-ATPases and ATPDases are postulated to serve a similar functional role by regulating the concentrations of agonists and antagonists around the sites of the purinoceptors (*25*, *26*).

One distinguishing hallmark of the E-type ATPases is their ability to hydrolyze a range of nucleotide substrates at extremely high turnover rates. Turnover numbers for the ecto-ATPases have been estimated to be 500 000 molecules/min (*1*), 50 times higher than the value of the Na^+,K^+ ATPase and 2 orders of magnitude higher than the ATPase activity of the plasma membrane Ca^{2+} pump (*1*, *27*). The K_{cat} of these enzymes approaches that of acetylcholinesterase. This unique feature makes elucidation of amino acids comprising the active nucleotide hydrolysis site very interesting. It has been noted that many of the E-type ATPases lack the consensus Walker ATP binding sequence motifs (*28*); instead, the deduced amino acid sequences of these nucleotidases have been shown to possess a putative ATP β-phosphate 1 binding motif (*4*, *29*−*32*, *52*). This "fingerprint" sequence {(I/L/V) X (I/L/V/C) D X G (T/S/G) (T/S/G) X X (R/K/C)}, where X is any amino acid and strictly conserved residues are

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[1] Abbreviations: ADP, adenosine 5′-diphosphate; AMP, adenosine 5′-monophosphate; ATP, adenosine 5′-triphosphate; CAPS, 3-(cyclohexylamino)propanesulfonic acid; cDNA, complementary DNA; CD39, lymphoid cell activation antigen (ecto-apyrase); E-type ATPase, ecto-ATPase, ecto-apyrase; ECL, enhanced chemiluminescence; ecto-apyrase, ecto-ATPDase, ecto-ATP diphosphohydrolase; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HB6, human brain E-type ATPase clone; MOPS, 3-(*N*-morpholino)propanesulfonic acid; NDP, nucleoside diphosphate; NTP, nucleoside triphosphate; PVDF, poly(vinylidene difluoride) membrane; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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underlined (*33*), can be found in the apyrase conserved region (ACR) I of all of the E-type ATPases sequenced to date, and is also found in proteins as diverse as actin, HSP70 heat shock proteins, and sugar kinases. All of these proteins have in common the ability to bind and hydrolyze ATP, despite their diverse primary structures and functions.

The ATP β-phosphate 1 binding motif is one of five sequence motifs common to the actin/HSP70/sugar kinase superfamily (for a review, see ref *34*). Each of these five motifs (phosphate 1, connect 1, phosphate 2, adenosine, and connect 2) consist of conserved residues and secondary structural elements that interact in the three-dimensional structure of the enzyme to form a nucleotide binding site. The secondary structure topology of this conserved core is of the form $\beta\beta\beta\alpha\beta\alpha\beta\alpha$. A common structural feature for all of these protein members is that of two domains with a similar fold on either side of a large cleft, with an ATP binding site at the bottom of the cleft (*35*). ATP hydrolysis results in a large conformational change in these domains.

In this report, we have used site-directed mutagenesis of a human brain ecto-apyrase (HB6) in an attempt to elucidate amino acids important in nucleotide hydrolysis. Multiple sequence alignments of the phosphate binding motifs from the E-type ATPases and the actin/HSP70/sugar kinases guided selection of potentially important amino acids, in particular, two strictly conserved aspartic acid and glycine residues. Mutations of these amino acids dramatically reduced ATP and ADP hydrolysis rates, similar to those determined in mutagenesis studies performed on the sugar kinases. These mutations also had significant effects on the ability of the enzyme to hydrolyze other nucleotide di- and triphosphates, confirming the importance of these amino acids for the function of the enzyme. The predicted secondary structure of the HB6 ecto-apyrase also suggests that the enzyme is related to the actin/HSP70/sugar kinase superfamily. This report represents the first site-directed mutagenesis study of an E-type ATPase and provides the first experimental evidence supporting the hypothesis that the E-type ATPases contain the phosphate binding motifs of the actin/HSP70/sugar kinase superfamily.

EXPERIMENTAL PROCEDURES

Materials. The QuikChange site-directed mutagenesis kit and Epicurian coli ultracompetent bacteria were purchased from Stratagene. Synthetic oligonucleotides were produced by the DNA Core Facility at the University of Cincinnati. Plasmid purification kits were purchased from Qiagen, Inc. Lipofectamine Plus Reagent, Dulbecco's Modified Eagle's Medium (DMEM), calf serum, goat anti-rabbit horseradish peroxidase-conjugated secondary antibody, and antibiotics and antimycotics were all obtained from Life Technologies. Falcon tissue culture-treated plates were from Becton-Dickinson. The mammalian expression vector pcDNA3 was obtained from Invitrogen. *Not*I and *Kpn*I restriction endonucleases and T4 DNA ligase were purchased from Promega. Sequenase Version 2.0 kits were obtained from U.S. Biochemical Corp., while ^{35}S-labeled dATP was from Dupont NEN. Ampicillin, nucleotides, and other reagents were from Sigma.

Construction of the HB6 cDNA in pcDNA3. The isolation of the cDNA for the human brain ecto-apyrase (designated HB6) was described in a previous publication (*31*). The 2.8 kb HB6 cDNA (GenBank accession number AF034840) was excised from the pCMV-SPORT plasmid and cloned into the 5.4 kb mammalian expression vector pcDNA3 using *Not*I and *Kpn*I restriction endonucleases. This construct (designated HB6 wild type) was used as the basis for all site-directed mutagenesis experiments.

Site-Directed Mutagenesis of HB6. Mutagenesis of the HB6 wild type was performed by following the instructions provided with the QuikChange site-directed mutagenesis kit from Stratagene with the exception that Epicurian coli ultracompetent cells were used for all bacterial transformations instead of the Epicurian coli supercompetent cells provided with the kit. The sense oligonucleotides (26 or 27 nucleotides in length) used for mutagenesis are as follows: 5′-GGTATTGTGCTGGCTGCCGGGTCTTC-3′ for Asp 62 Ala, 5′-GTGCTGGATGCCGCGTCTTCAAGAACC-3′ for Gly 64 Ala, 5′-CGGGTGCCCTGGCCTTAGGTGGTGCC-3′ for Asp 219 Ala, 5′-CGGGTGCCCTGAACTTAGGTG-GTGCC-3′ for Asp 219 Asn, 5′-CGGGTGCCCTGGAGT-TAGGTGGTGCC-3′ for Asp 219 Glu, and 5′-CCCTGGA-CTTAGCTGGTGCCTCCACC-3′ for Gly 221 Ala, where the altered codons are underlined. The complementary antisense oligonucleotides also necessary for the mutagenesis are not shown. The mutagenized HB6 cDNA was then used to transform ultracompetent cells as described by the manufacturer (Stratagene). Mutagenized HB6 plasmid DNA was purified from 5 mL overnight cultures using the Qiagen miniprep system. The presence of the correct mutation was confirmed in all constructs by dideoxy DNA sequencing using Sequenase Version 2.0. The mutagenized insert was then excised from the pcDNA3 vector using *Not*I and *Kpn*I restriction endonucleases, purified on a 0.8% low-melting agarose gel, and religated into non-PCR-amplified pcDNA3 expression vector using T4 DNA ligase (to eliminate any potential mutations in the plasmid DNA caused by the Pfu amplification reaction). Identical results were obtained without ligating the mutagenized insert cDNA into a non-PCR-amplified, "virgin" plasmid.

COS-1 Cell Expression of Human Brain Ecto-Apyrase (HB6). COS-1 cells (a generous gift from L. Lane, University of Cincinnati) were grown in complete DMEM (Dulbecco's Modified Eagle's Medium supplemented with 10% calf serum and 100 units/mL penicillin G, 100 μg/mL streptomycin sulfate, and 0.25 μg/mL amphotericin B as Fungizone) at 37 °C in 10% CO_2. Cells were grown in this manner and passaged every 3−4 days. Cells were seeded the day prior to transfection in 100 mm plates so as to be approximately 75% confluent when transfected 20−24 h later with Lipofectamine Plus Reagent. Briefly, the cells were incubated for 5 h in DMEM (serum and antibiotic and antimycotic free) containing Lipofectamine Plus Reagent and either 4 μg of wild-type HB6 cDNA, 4 μg of mutagenized HB6 cDNA, or no cDNA for the control mock-transfected COS-1 cells. At the end of transfection for 5 h, an equal volume of DMEM containing 20% calf serum and double the normal concentrations of antibiotics and antimycotics was added. Twenty-four hours later, the culture medium was removed and replaced with complete DMEM. Approximately 72 h post-transfection, the COS-1 cells were used for analyses. The COS cell expression system was chosen to permit more direct comparison to previous studies on expressed E-type ATPases (*31, 36*−*40*).

Cell Membrane Preparations. For cell membrane preparations, cell monolayers were washed three times with ice-cold isotonic wash buffer (IWB) containing 20 mM MOPS/NaOH (pH 7.4), 140 mM NaCl, and 5 mM KCl, harvested by scraping the culture plates in tissue homogenization buffer (THB) containing 30 mM MOPS/NaOH (pH 7.4), 250 mM sucrose, and 2 mM EDTA, and homogenized in a glass hand homogenizer (Thomas Scientific, Swedesboro, NJ). The cell homogenate was centrifuged at 48 000 rpm in a 50Ti rotor (150000*g*) for 60 min at 4 °C. The pellet was resuspended in a small volume of THB, homogenized again, and used for biochemical assays.

Nucleotidase Assays. Nucleotidase activity was determined by measuring the amount of inorganic phosphate released from nucleotide substrates at 37 °C using a modification (*41*) of the technique of Fiske and Subbarow (*42*). Briefly, 1−3 μg of cell membranes were added to assay buffer containing either 20 mM MOPS/NaOH, (pH 7.4) and 1 mM EGTA for cation-independent nucleotidase activity or 20 mM MOPS/NaOH (pH 7.4), 1 mM EGTA, and 5 mM $MgCl_2$ for cation-dependent nucleotidase activity. The enzyme assay was initiated by the addition of the respective nucleotide to final concentrations ranging from 9 μM to 2.5 mM and incubated at 37 °C for 30−60 min. The reaction was stopped, and the amount of inorganic phosphate released was determined colorimetrically. The cation-stimulated nucleotidase activity was determined by subtracting values obtained with EGTA alone from those with 5 mM $MgCl_2$ with chelator. The minor nucleotidase activities that could be detected in control COS cell membranes (3−7% of wild-type HB6-transfected cell membranes) were subtracted from all HB6 wild-type- and mutant-transfected COS cells. Nucleotide hydrolyzing units are expressed in micromoles of P_i liberated per hour.

Protein Assay. Protein concentrations were determined using the Bio-Rad CB-250 dye binding technique according to the modifications of Stoscheck (*43*, *44*) using bovine serum albumin as the standard.

Polyclonal Antibody Production. Anti-peptide polyclonal antiserum was generated in rabbits by Lampire Biological Laboratories (Pipersville, PA) using a synthetic peptide designed from the HB6 carboxy-terminal amino acid sequence. The peptide sequence synthesized (KRHSE-HAFDHAVDSD-COOH) represents amino acids 515−529 of the HB6 protein, predicted to be intracellular. The synthetic peptide was both generated and coupled to keyhole limpet agglutinin by QCB, Inc. (Hopkinton, MA).

Electrophoresis and Western Blot Analysis. SDS−PAGE was performed following the method of Laemmli (*45*). Control or HB6-transfected COS-1 cell membrane preparations (10 μg per lane) were boiled for 5 min in SDS sample buffer containing 30 mM dithiothreitol and 8 M urea and were resolved on a 4 to 15% linear gradient SDS−polyacrylamide gel. After electrophoresis, the separated proteins were transferred to poly(vinylidene difluoride) (PVDF) membrane by electroblotting for 3 h at 33 V in cold 10 mM CAPS (pH 11) (*46*). After transfer, the PVDF membrane was blocked for 1 h in 5% nonfat dry milk in Tris-buffered saline containing 0.02% sodium azide and incubated overnight at room temperature in a 1:2000 dilution of polyclonal antisera in blocking buffer. The PVDF membranes were subsequently washed, incubated in goat anti-rabbit IgG horseradish peroxidase secondary antibody, and washed again, and immunoreactivity was detected by chemiluminescence with the Amersham ECL reagents as described by the manufacturer.

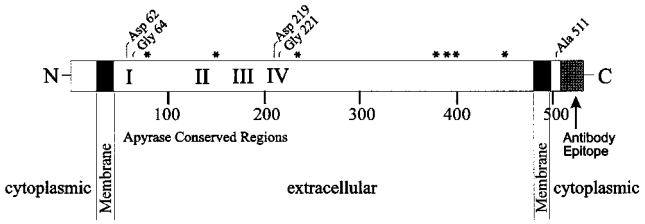

FIGURE 1: Schematic diagram of the structure of wild-type HB6 ecto-apyrase. The human brain HB6 E-type ATPase consists of 529 amino acids. The cytosolic amino and carboxy termini are denoted with an N and C, respectively. The two transmembrane regions at either end of the protein are black. The four extracellular apyrase conserved regions are denoted with Roman numerals I−IV, and the 15-amino acid C-terminal portion of the protein for which peptide antisera were generated is denoted with dark shading. The seven potential N-glycosylation sites are denoted with asterisks (*). Those amino acids selected for site-directed mutagenesis are indicated above the drawing.

RESULTS

Expression of Wild-Type and Mutant HB6 cDNA in COS-1 Cells. The isolation and characterization of the 2809-base human brain ecto-apyrase cDNA (HB6) was described in a previous publication (*31*). The deduced amino acid sequence of HB6 consists of short intracellular N and C termini, two membrane-spanning domains, and a large extracellular region containing the nucleotide binding and hydrolysis site (Figure 1). Seven potential N-glycosylation sites exist in this protein. In addition, the four apyrase conserved regions (I−IV) described by Handa and Guidotti (*4*) are found in this enzyme sequence.

Identification of the expressed ecto-apyrase protein product was performed using polyclonal antisera generated against the cytosolic C-terminal sequence. In Western blot analyses of control (mock-transfected) and wild-type HB6-transfected COS cells, an approximately 79 kDa broad protein band, typical of glycoproteins, was identified in the transfected cells (Figure 2). No immunoreactive proteins were detected at this molecular mass in control COS cells. The unidentified faint protein band at approximately 110 kDa present in both the control and HB6-transfected cells was observed when probing with the preimmune sera as well (data not shown).

To ensure that the site-directed cDNA mutants were expressed in COS cells at levels similar to that of the HB6 wild-type protein, cell membrane preparations of transfected cells were electrophoresed and Western blot analysis was conducted on the membrane proteins using the anti-peptide antisera. As shown in Figure 3, the approximately 79 kDa ecto-apyrase was detected in six mutants at levels similar to that of the wild-type protein. In contrast, no immunoreactivity was detected in mock-transfected COS cell membranes or in a truncation mutant (A511STOP) in which the 19 cytosolic C-terminal amino acids of the protein were deleted (and therefore the epitope recognized by the anti-peptide antibody was absent). This A511STOP mutant served as a negative control to demonstrate the specificity of the anti-peptide antibody used to quantitate and normalize the nucleotidase data, correcting for slight variations in transfection efficiency and expression levels (Figure 3).

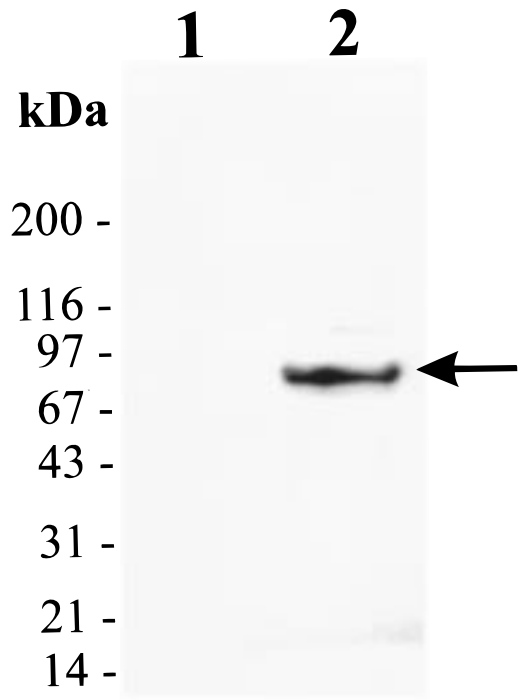

FIGURE 2: Western blot analysis of expressed wild-type ecto-apyrase using a polyclonal anti-peptide antibody generated against the 15 C-terminal amino acids. Control (lane 1) and HB6 wild-type (lane 2)-transfected COS cell membranes were electrophoresed on a 4 to 15% linear gradient gel, transferred to PVDF membrane, and analyzed by Western blotting. The ecto-apyrase is an approximately 79 kDa broad protein band (denoted with an arrow), not present in mock-transfected COS cell membranes. The apparent molecular mass of 79 kDa was calculated by nonlinear curve fitting of the molecular mass standards indicated at the left.

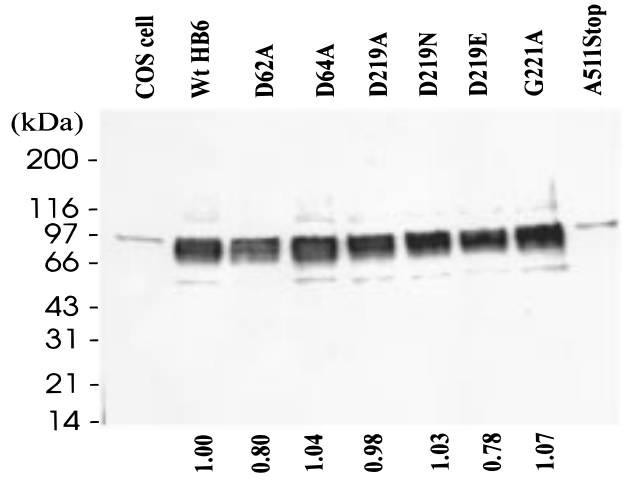

FIGURE 3: Western blot analysis of expressed wild-type and mutant ecto-apyrase. Ten micrograms of control, wild-type HB6, and mutant HB6-transfected COS cell membranes were electrophoresed on a 4 to 15% linear gradient gel and analyzed by Western blotting with the C-terminal anti-peptide antibody. The ecto-apyrase protein is seen in wild-type HB6 as well as in six of the seven mutants under examination. The A511STOP mutant represents a truncation mutant missing the cytoplasmic C-terminal tail (and thus the region to which the anti-peptide antibody binds). The numbers at the bottom of the blot indicate the normalization values used to correct nucleotidase assay data for the slight differences in expression levels. Values were obtained by scanning densitometry and quantitation using the AlphaEase version 3.21 program from Alpha Innotech Corp. Molecular mass standards are indicated.

Enzymatic Characterization of the Wild-Type HB6 and Mutant Ecto-Apyrase Proteins. To examine the effects of these single amino acid changes on the activity of the HB6 ecto-apyrase, wild-type and mutant-transfected COS cells were harvested as described and the cell membrane preparations examined for nucleotide hydrolysis activity. Examples of the computer fit to experimental data used to calculate V_{max} and K_m for the wild type and one mutant (D219E) are shown in Figure 4. The results obtained by fitting the data for all of the mutants are shown in Figure 5 and Table 1. Several mutations had deleterious effects on the ATP and ADP hydrolyzing activity of the ecto-apyrase. The mutation of Asp 219 to Ala or Asn resulted in a loss of greater than

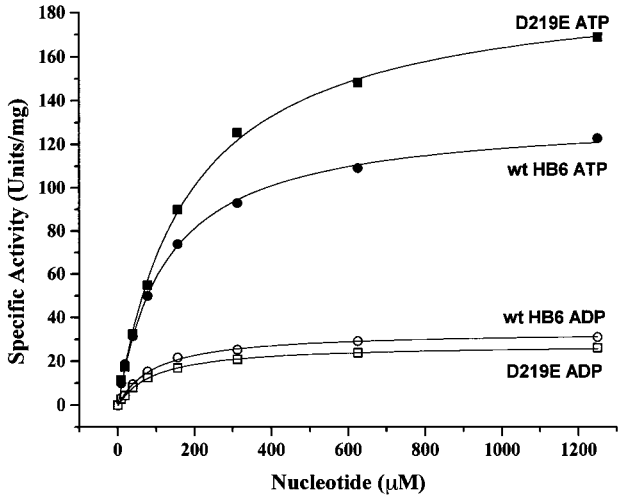

FIGURE 4: Nucleotide concentration curve for HB6 wild-type ecto-apyrase and the Asp 219 Glu mutant. Wild-type HB6- and D219E mutant-transfected COS cell membranes were assayed as described in Experimental Procedures and corrected for both background COS cell activity and expression levels. Values are displayed as specific activity units (micromoles of P_i per hour per milligram) vs ATP or ADP concentration. The data for wild-type ecto-apyrase are represented by black circles (ATP) and white circles (ADP). The data for the D219E mutant are represented by black boxes (ATP) and white boxes (ADP). All values in Table 1 were obtained by fitting data similar to that shown in this figure for wild-type HB6 and each mutant.

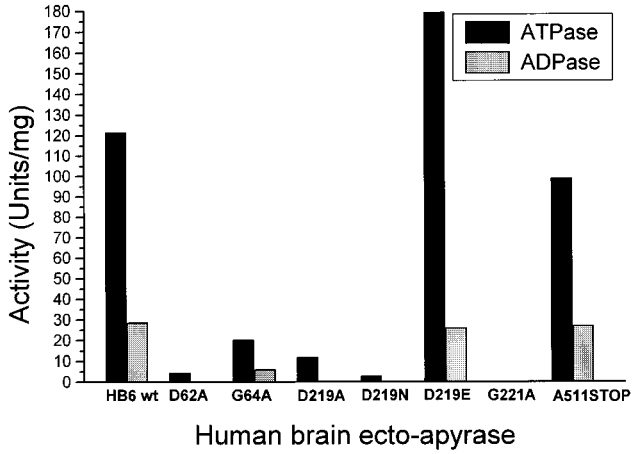

FIGURE 5: Specific activities of the ATP and ADP hydrolyzing activity of wild-type HB6 ecto-apyrase and mutants. The wild-type HB6- and mutant-transfected COS cell membranes were assayed as described in Experimental Procedures using 2.5 mM substrate and corrected for both background COS cell activity (3−7% of the wild-type activity) and expression levels (see Figure 3). V_{max} values (see Table 1) are displayed as specific activity units (micromoles of P_i per hour per milligram) for ATPase (black bars) and ADPase (gray bars). No bars are visible in some positions since several mutants had no measurable activity.

Table 1: Enzymatic Characterization of the Wild-Type and Mutant Forms of HB6 Ecto-Apyrase[a]

HB6 ecto-apyrase	ATPase K_m (μM)	ATPase V_{max} (units/mg)	ADPase K_m (μM)	ADPase V_{max} (units/mg)	ATPase:ADPase ratio
wild-type	128 ± 4	122 ± 1	96 ± 6	29 ± 0.6	4.2:1.0
Asp 62 Ala	96 ± 13	5 ± 0.6	inactive	inactive	
Gly 64 Ala	48 ± 6	20 ± 0.9	36 ± 4	6 ± 0.2	3.3:1.0
Asp 219 Ala	85 ± 6	12 ± 0.5	inactive	inactive	
Asp 219 Asn	57 ± 9	3 ± 0.6	inactive	inactive	
Asp 219 Glu	185 ± 8	179 ± 2	97 ± 4	26 ± 0.2	6.9:1.0
Gly 221 Ala	inactive	inactive	inactive	inactive	
Ala 511 STOP	123 ± 6	99 ± 2	92 ± 6	27 ± 0.5	3.6:1.0

[a] The values shown are the parameters determined ± the standard deviation of data fit to a Michaelis−Menten plot (see Figure 4 for an example of the fit data). Nucleotide hydrolyzing units are expressed as micromoles of P_i released per hour. All values are corrected for small differences in expression levels by quantitation of the 79 kDa HB6 band in the Western blot shown in Figure 3.

Table 2: Human Brain Wild-Type and Mutant Ecto-Apyrase Hydrolysis of Nucleoside Mono-, Di-, and Triphosphates[a]

HB6 apyrase	ATP (139)	CTP (95)	ITP (106)	GTP (108)	ADP (34)	IDP (35)	GDP (33)	AMP (0.0)
wild-type	100	100	100	100	100	100	100	0
Asp 62 Ala	6.2	9.7	14	11	0	0	0	0
Gly 64 Ala	16	11	25	20	11	11	10	0
Asp 219 Ala	9.5	7.6	11	9.1	0	0	0	0
Asp 219 Asn	2.4	2.4	2.2	0.7	0	0	0	0
Asp 219 Glu	133	135	136	120	74	66	92	0
Gly 221 Ala	0	0	0	0	0	0	0	0

[a] Enzymatic assays were performed using a 2.5 mM final concentration for each nucleoside phosphate. Values are expressed as a percentage of the HB6 wild-type activity for each nucleotide under examination. The specific activities for the wild-type ecto-apyrase are given in parentheses and expressed in units per mg. The activities are both corrected for background COS cell nucleotidase activity and normalized for expression levels.

90% of the ATP hydrolyzing activity, when compared to the wild-type HB6 ecto-apyrase activity. The ability to hydrolyze ADP was lost completely with these two mutations. In contrast, mutation of Asp 219 to Glu restored both the ATP and ADP hydrolyzing activities, although to different extents. The extent of ATP hydrolysis actually increased slightly with this substitution, while the extent of ADP hydrolysis decreased somewhat (see Figure 4). This caused the ATPase:ADPase ratio to change somewhat, from approximately 4.2:1.0 in the wild-type HB6 ecto-apyrase to 6.9:1.0 in the Asp 219 Glu mutant (Table 1). Two other mutants (Asp 62 Ala and Gly 64 Ala) also lost significant amounts of ATP and ADP hydrolyzing activity. The ability to hydrolyze ADP was lost with the Asp 62 Ala mutation. A Gly 221 Ala mutation resulted in a complete loss of both ATP and ADP hydrolyzing activity. The K_m for substrate for each of these mutations varied somewhat when compared to that of the wild type (Table 1), from 48−185 μM for ATP to 36−97 μM for ADP, not nearly as dramatic a change when compared to the change in V_{max} for each of these mutants.

Deletion of the cytoplasmic tail had little effect on the ADP and ATP hydrolyzing rate (5−20% reduction) compared to that of the wild-type HB6 enzyme (Figure 5). The K_m values for both ATP and ADP were virtually unchanged (Table 1).

Nucleotide Hydrolysis Ratios for the Wild-Type HB6 Ecto-Apyrase and Mutants. To examine what effect these mutations had on the ability of the enzyme to hydrolyze other nucleoside tri- and diphosphates (NTPs and NDPs) in addition to ATP and ADP, wild-type and mutant-transfected COS cell membranes were assayed in the presence of various substrates. Characteristic of the E-type ATPases, the HB6 wild-type ecto-apyrase was able to hydrolyze a broad range of nucleoside tri- and diphosphates (Table 2), while the ability to hydrolyze nucleoside monophosphates (AMP) was absent. ATP was the preferred substrate under these conditions. Interestingly, several mutations had significant effects on the ability to hydrolyze nucleoside diphosphates versus triphosphates. For example, mutation of Asp 219 to Ala and Asn caused similar decreases in the extent of nucleoside triphosphate hydrolysis and complete loss of nucleoside diphosphate hydrolysis. Mutation of this residue to Glu actually increased the rates of NTP hydrolysis (120−136% of that of the wild type) while reducing the amount of NDP hydrolysis (66−92% of that of the wild type). One mutation (Gly 221 Ala) caused a complete loss of both NTP and NDP hydrolyzing activity.

Secondary Structure Predication of the HB6 Ecto-Apyrase. To further test the hypothesis that the E-type ATPases contain nucleotide binding domains very similar to those of the actin/HSP70/sugar kinase superfamily, the secondary structure of the HB6 enzyme (predicted by computer analysis using the SSP secondary structure prediction program, version 2, Baylor College of Medicine) was compared with the computer analyses of the actin superfamily members, as well as the secondary structures known from their crystal structures (Table 3). From the known crystal structures of the superfamily members, the active sites of these proteins are characterized by a secondary structure topology of the form $\beta\beta\beta\alpha\beta\alpha\beta\alpha$ (*34*). Shown in Table 3 are the primary and secondary structures of the regions comprising the nucleotide binding site in the superfamily (*34*), along with the sequence and predicted secondary structure of the human brain HB6 E-type ATPase. As is evident from the sequences, except for the invariant Asp and Gly residues, there is little primary structure conservation either between the members of the superfamily or between the superfamily members and the E-type ATPases. However, as has been noted previously for the actin/HSP70/sugar kinase superfamily, the secondary (and tertiary) structure of the nucleotide binding site is completely conserved.

DISCUSSION

Despite the lack of global sequence similarity at the amino acid level between the E-type ATPases, actins, sugar kinases, and HSP70 heat shock proteins, we have used site-directed mutagenesis on the basis of analogies with the actin superfamily as a tool to examine the functional importance of conserved amino acids in a human brain E-type ATPase. This was made possible due to the fact that each family member possesses local nucleotide binding motifs consisting of a set of common conserved amino acids. As shown earlier by Flaherty et al. (*33*), multiple sequence alignments of one of these motifs from several protein members display a pattern of amino acid properties characteristic to each position (Table 3). Referred to as the phosphate 1 binding motif (*35*), the β-phosphate amino acid consensus sequence for this region is (I/L/V) X (I/L/V/C) D X G (T/S/G) (T/S/

Table 3: Comparison and Alignment of Primary and Secondary Structures of the HB6 E-Type ATPase with the Actin/HSP 70/Sugar Kinase Superfamily[a]

ACTIN SUPERFAMILY DOMAIN IA

Crystal 2° structure	β1	β2	β3	α1	β4	α2	β5	α3
	⇒⇒⇒⇒	⇒⇒⇒⇒	⇒⇒	»»»»»»»»»»»»»»»»»»	⇒⇒⇒⇒	»»»»»»»»»»	⇒⇒⇒	»»»»»»»»»»
Human Hexokinase	[529]LALDLGGTNFRVLLV	[557]NKIY	[570]TGEELFDHIVSCISDFLDYM	[597]PLGFTFS	[634]VTLLRDAIK	[652]VAVVNDTVGTMMTCA		
Human HSC70	[7]VGIDLGTTYSCVGVF	[36]RTTP	[116]PEEVSSMVLTKMKEIAEAYL	[143]NAVTVPY	[157]ATKDAGTIA	[170]LRIINEPTAAAIAYG		
Human Actin	[8]LVCDNGSGLVKAGFA	[29]AVFP	[78]NWDDMEKIWHHTFYNELRVA	[102]PTLLTEA	[114]ANREKMTQI	[132]MYVAIQAVLSLYASG		
Computer Predict	+	-	-	+	+	+	+	+
Computer Predict HB6	+	+	+	+	-	+	+	+
Human HB6	[59]IVLDAGSSRTTVYVY	[89]QTFK	[113]FEECMQKVKGQVPSHLHGST	[135]HLGATAG	[142]MRLLRLQNE	[184]VYGWITANYLMGNFL		

ACTIN SUPERFAMILY DOMAIN IIA

Crystal 2° structure	β1	β2	β3	α1	β4	α2	β5	α3
	⇒⇒⇒⇒	⇒⇒⇒	⇒⇒	»»»»»»»»»»	⇒⇒⇒⇒	»»»»»»»	⇒⇒	»»»»»»»»»»»»
Human Hexokinase	[673]EVGLIVGT-GSNACY	[706]NMEW	[835]GAGMAAVVDK	[858]VGVDGT	[871]FSRIMH	[889]SFLL	[895]DGSEKGAALITAV	
Human HSC70	[195]VLIFDLGGGTFDVSI	[222]TAGD	[313]TLDPVEKALR	[334]IVLVGG	[348]KLLQDF	[358]ELNK	[367]EAVAYGAAVQAAI	
Human Actin	[150]GIVLDSGDGVTHNVP	[175]IMRL	[274]IHETTYNSIM	[297]NVMSGG	[311]DRMQKE	[328]KIIA	[337]YSVWIGGSILASL	
Computer Predict	+	+	-	+	+	+	+	-
Computer Predict HB6	-	+	+	+	+	+	+	-
Human HB6	[215]TGALDLGGASTQISF	[242]IMQV	[410]LPKFDEVYAR	[431]LFVNGY	[446]IHFEKE	[466]SLTN	[470]QIPAESPLIRLPI	

[a] The human hexokinase, HSC70, and actin protein sequences were aligned according to the method of Hurley (*34*) using the known crystal structure to distinguish helices (double arrowheads) from strands (arrows). The secondary structure prediction program SSP, version 2, was used to analyze the primary sequences for the presence of secondary structural elements which were compared to the secondary elements of the known crystal structure. Those regions that were correctly predicted in at least two out of three of the superfamily members are denoted with a plus (+) below the three sequences, while those regions that were not predicted are denoted with by a minus (−). The HB6 E-type ATPase primary sequence was also analyzed for secondary structural elements and denoted with a plus or minus above the HB6 sequence. The conserved aspartic acid and glycine residues are underlined.

G) X X (R/K/C), where strictly conserved residues are underlined. As seen from the above consensus sequence, the invariant Asp 62 and Gly 64 of HB6 ACR I make particularly attractive residues for mutagenesis as these amino acids are also found in all E-type ATPases sequenced to date (Table 4). From studies performed on hexokinase, the conserved aspartic acid is thought to bind the metal ion of the nucleotide−metal complex, while the glycine residue is thought to be involved in a β-turn, necessary to avoid steric hindrance from the β- or γ-phosphate (*33*, *47*).

Bork et al. (*35*) combined the use of three-dimensional crystal structure alignments of actin, HSP 70, and hexokinase with multiple sequence alignments of these proteins and many other members of these families to identify a set of five sequence motifs involved in ATP binding. Each of these five sequence motifs (consisting of approximately 20 amino acids and termed phosphate 1, connect 1, phosphate 2, adenosine, and connect 2) possesses distinguishing amino acid properties and was used to identify those invariant amino acids thought to play critical roles in nucleotide binding. The phosphate 1, phosphate 2, and adenosine motifs are loop structures interacting with the β- and γ-phosphate and adenosine base of ATP, respectively. Site-directed mutagenesis studies on several invariant amino acids in the phosphate 1 and phosphate 2 motifs of human brain hexokinase and *Escherichia coli* glycerol kinase have shown the importance of these amino acids for the functioning of these enzymes (*47*−*49*). In particular, the invariant D and G of phosphate 1 (corresponding to Asp 62 and Gly 64 of HB6 ACR I) were shown to be absolutely essential for hexokinase activity. Mutation of either of these residues decreased the K_{cat} of the hexokinase enzyme 1000- and 4000-fold, respectively. Only small changes were observed in the K_m for ATP for both of these mutants however, and the authors therefore proposed that the interactions between the aspartic acid and the metal ion of the nucleotide−metal complex were likely not responsible for the ATP binding affinity (*47*, *48*); rather, they were thought to be intimately involved in ATP hy-

Table 4: Alignment of Adenine Nucleotide β-Phosphate (Phosphate 1) and Phosphate 2 Binding Sequences[a]

PROTEIN	PHOSPHATE 1 BINDING MOTIF		PHOSPHATE 2 BINDING MOTIF	
Human HSP70	6	AVGI**D**L**G**TTYSC	195	VLIF**D**L**G**GGTFD
Human Actin	7	ALVC**D**N**G**SGLVK	150	GIVL**D**S**G**DGVTH
Human Hexokinase	528	FLAL**D**L**G**GTNFR	675	GLIV**GT**GSNACY
E. coli glycerol kinase	6	IVAL**D**Q**G**TTSSR	261	KNTY**GT**GCFMLM
Human CD39 (ACR I, ACR IV)	50	GIVL**D**A**G**SSHTS	209	FGAL**D**L**G**GASTQ
Human CD39L1 (ACR I, ACR IV)	41	GIVL**D**A**G**SSHTS	197	LGAM**D**L**G**GASTQ
Human golgi UDPase (ACR I, ACR IV)	91	GIVV**D**C**G**SSGSR	268	AGIL**D**M**G**GVSTQ
Human brain HB6 (ACR I, ACR IV)	58	GIVL**D**A**G**SSRTT	215	TGAL**D**L**G**GASTQ
PHOSPHATE MOTIF FINGERPRINT SEQUENCE (*35*)		IXI**D**X**G**TTXXR L L SS K V V GG C C		

[a] Alignments of the ATP phosphate-binding sequences of human HSP70, human actin, human hexokinase, and *E. coli* glycerol kinase were determined according to refs *33* and *35*. Apyrase conserved regions I and IV from human CD39 ecto-apyrase (*8*), human brain ecto-apyrase HB6 (*31*), human CD39L1 ecto-ATPase (*51*), and human uridine diphosphatase (*38*) were then aligned with the superfamily sequences (all other cloned and sequenced E-type ATPases also contain the conserved DXG in both ACR I and ACR IV). Strictly conserved residues are boldface. The consensus sequence of phosphate 2 for all of the sugar kinases is GT; for actin, HSP70, and the E-type ATPases, it is DXG. Note that the only completely conserved residues in the phosphate motif fingerprint sequence are the aspartic and glycine residues mutated in this work.

drolysis. The glycine to alanine mutation was thought to have disrupted the β-turn, such that unfavorable steric interactions with the nucleotide phosphates would occur. Studies with *E. coli* glycerol kinase involved mutation of Asp 10 (equivalent to Asp 62 of HB6) to Asn; this decreased enzymatic activity to 3% of that of the wild-type enzyme. Mutation of Asp 62 and Gly 64 to Ala in the human brain ecto-apyrase caused a dramatic decrease in the ATP and ADP V_{max} values of the mutants compared to that of the wild-type HB6 (Table 1), similar to the results observed with the sugar kinases. Only slight changes in the K_m for these substrates were observed however, suggesting that, like the case for the actin superfamily enzymes, these residues are likely to be more important in the hydrolysis, rather than the binding of the substrate.

The binding motif consisting of phosphate 2 also has several conserved amino acids, albeit different ones depending upon the particular protein family being studied. The actin and HSP70 proteins, for example, both have an invariant DXG sequence in this motif, while the sugar kinases have a GTX sequence (Table 4). In this regard, the E-type ATPases more closely resemble actin and HSP70 family members than the sugar kinases since ACR IV contains the invariant DXG sequence (not found in the kinases). In the HB6 ecto-apyrase, mutation of both the D and G of HB6 (Asp 219 and Gly 221 in ACR IV) shows the critical roles that these amino acids play in enzyme activity. Mutation of Asp 219 to Ala or Asn caused the loss of 90−98% of the ATPase activity and 100% of the ADPase activity (Table 1). Interestingly, changing the Asp residue to a Glu residue restored much of the ADP hydrolyzing activity and actually increased the ATP hydrolyzing activity (Table 2). This suggests that although the charge of the amino acid is critical for hydrolysis of the substrate, other factors, such as the size of the functional group, are also important. Mutation of Gly 221 to Ala caused a complete loss of both ATP and ADP catalysis, consistent with the superfamily members.

As shown in Table 3, the predicted secondary structure of the HB6 ecto-apyrase is consistent with the conserved secondary structures of the superfamily members, strengthening the hypothesis that the E-type ATPases either are members of the actin/HSP70/sugar kinase superfamily or are derived from the same ancestor as the superfamily members. This should apply to all E-type ATPases, since they have similar primary and secondary structures and the HB6 E-type ATPase appears to be the ancestor from which both the ecto-ATPases and CD39 ecto-apyrases are descended (*31*). Also consistent with this hypothesis, it should be noted that polyclonal antibodies raised against the size-selected, monoclonal antibody affinity-purified, 66 kDa chicken gizzard ecto-ATPase cross-react with actin (*50*).

The results of this site-directed mutagenesis study demonstrate that the human brain ecto-apyrase HB6 (and presumably all other members of the E-type ATPase family) possesses the consensus ATP phosphate binding motifs present in the actin superfamily. In addition, comparison of the predicted secondary structures of the actin/HSP70/sugar kinase superfamily with the E-type ATPases supports the hypothesis that the two families are structurally related.

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