Glycosylation Is Essential for Functional Expression of a Human Brain Ecto-apyrase†

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ABSTRACT: The importance of N-linked glycosylation for the function and oligomerization of an E-type ATPase was examined by using tunicamycin and peptide N-glycosidase F, two agents used to prevent and remove glycosylations, respectively. The cDNA encoding a human ecto-apyrase (HB6), predicted to have seven N-linked glycosylation sites, was transiently expressed in mammalian COS cells and the resulting membrane preparations were treated with peptide N-glycosidase F (PNGase-F). PNGase-F caused a decrease in the apparent molecular weight of the protein (consistent with glycan removal) and a decrease in enzymatic activity over time. The ecto-apyrase was also expressed in the presence of tunicamycin, which completely prevented N-linked glycosylation, resulting in a nonglycosylated core protein devoid of ATP and ADP hydrolyzing activity. However, control and tunicamycin-treated cells expressed the enzyme to similar levels and localization. Interestingly, the quaternary structure of this E-type ATPase appears to be dependent upon the presence of glycan chains. The glycosylated ecto-apyrase exists as a homodimer in situ as assessed by both size-exclusion chromatography of detergent-solubilized ecto-apyrase and cross-linking of membrane-bound ecto-apyrase, in contrast to the enzymatically deglycosylated ecto-apyrase and the tunicamycin-treated ecto-apyrase. These results suggest that glycosylation is necessary for homooligomerization and nucleotide hydrolyzing activity, but not for expression and plasma membrane localization of the E-type ATPase. Similar results were obtained with another human ecto-apyrase, CD39, suggesting that the importance of glycosylation may be generalized to all membrane-bound E-type ATPases.

N-Linked glycosylation is a common cotranslational modification found in many plasma membrane proteins, occurring on asparagine residues in the consensus sequence Asn-X-(Ser)(Thr), where X is any amino acid except proline or aspartic acid (*1*). The role of N-glycosylation in the proper functioning of some proteins has been examined in great detail and many different theories have been advanced concerning the biological roles of the oligosaccharides in glycoproteins (*2*). Of the available methods for modifying oligosaccharide containing proteins to study structure and function, two compounds have proven to be quite useful in the analyses of glycoproteins: tunicamycin, which blocks the formation of N-glycosidic linkages by inhibiting the transfer of *N*-acetylglucosamine 1-phosphate to dolichol monophosphate, and peptide N-glycosidase F, which cleaves all N-linked glycans at the glycosylamine linkage, removing the intact oligosaccharides from the glycoprotein (*3*, *4*).

The recently described class of ATPases known as the E-type ATPases[1] are glycosylated proteins (*5*−*9*), and some work has been published concerning the glycan constituents and types of oligosaccharide attachment (i.e., O− versus N-linked) on one E-type ATPase, CD39 (*10*). However, little research has been targeted at determining directly the role played by the glycans in the structure and activity of the enzymes. The effect of carbohydrate-binding lectins on the regulation of E-type enzymatic activity (*11*−*13*) suggests that the oligosaccharides may play important roles in the maintenance and regulation of enzymatic activity. A recent report examined the glycosylation of an ATP diphosphohydrolase in a limited manner (*14*). In the present study, using several methodologies, we report findings demonstrating the importance of N-linked glycosylation for the enzymatic function and quaternary structure of a human E-type ATPase closely related to both the CD39 ecto-apyrases and the ecto-ATPases (*15*).

EXPERIMENTAL PROCEDURES

Materials. Lipofectamine Plus reagent, Dulbecco's modified Eagle medium (DMEM), calf serum, goat anti-rabbit

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[1] Abbreviations: ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; CAPS, 3-(cyclohexylamino)propanesulfonic acid; cDNA, complementary DNA; CD39, lymphoid cell activation antigen (an ecto-apyrase); DMSO, dimethyl sulfoxide; DSS, disuccinimido suberate; E-type ATPase, ecto-adenosinetriphosphatase, 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; PBS, phosphate-buffered saline; PNGase F, peptide N-glycosidase F; PVDF, poly(vinylidene difluoride) membrane; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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horseradish peroxidase-conjugated secondary antibody, and antibiotics/antimycotics were all obtained from Life Technologies. Goat anti-rabbit CY3-conjugated secondary antibody was from Jackson Immunoresearch. 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. The noncleavable cross-linker disuccinimido suberate (DSS) was from Pierce. Peptide N-glycosidase F (0.2 unit/μL) was obtained from Boehringer Mannheim. The size-exclusion gel-filtration standards were obtained from Bio-Rad, and all other reagents were from Sigma Chemical Co.

Construction of the HB6 cDNA in pcDNA3. The isolation and characterization of the cDNA for the human brain ecto-apyrase (designated HB6) was described previously (*15*). 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 by use of *Not*I and *Kpn*I restriction endonucleases. This construct was used as the basis for all ecto-apyrase glycosylation studies.

COS-1 Cell Expression of Human Brain Ecto-apyrase (HB6). For transient transfection, COS-1 cells (a generous gift from Lois Lane, University of Cincinnati) were grown in complete DMEM (Dulbecco's modified Eagle 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 as specified by the manufacturer. Briefly, the cells were incubated for 5 h in DMEM (serum and antibiotic/antimycotic free) containing Lipofectamine and Plus reagent and 4 μg of HB6 cDNA. At the end of the 5 h transfection, an equal volume of DMEM containing 20% calf serum and double the normal concentrations of antibiotics/antimycotics was added. For studies utilizing tunicamycin-treated cells, tunicamycin prepared in sterile DMSO was added immediately following transfection to the indicated final concentration. HB6 control cells received DMSO alone. The following day, the culture medium was removed and replaced with complete DMEM containing the same concentration of tunicamycin. Approximately 48 h posttransfection, the COS-1 cells were harvested and cell membranes were prepared.

Total Membrane Preparation. For cell membrane preparations, cell monolayers were washed 3 times with ice-cold isotonic wash buffer (IWB) consisting of 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; 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.

Peptide N-Glycosidase F Deglycosylation of HB6 Ecto-apyrase. For removal of all N-linked glycosylations under denaturing conditions, HB6-expressing COS cell membranes were acetone-precipitated (*16*) and dissolved in 0.2% SDS. After sonication and boiling for 3 min, the SDS concentration was adjusted to 0.1% by the addition of reaction buffer containing peptide N-glycosidase F (*4*, *17*). The deglycosylation reaction was incubated at 37 °C overnight and subsequently analyzed by SDS−PAGE. For analysis of the effects of deglycosylation on HB6 ecto-apyrase activity as a function of time, HB6-expressing COS cell membranes were resuspended in 30 mM MOPS−NaOH, pH 7.4, 2 mM EDTA, and 0.02% saponin, and the deglycosylation reaction was started by the addition of peptide N-glycosidase F. The control sample was treated in an identical manner with the exception that the peptide N-glycosidase F enzyme that was added was heat-inactivated (boiled for 15 min) prior to initiation of the reaction. The samples were incubated at 37 °C, and aliquots were taken at the indicated time points and either boiled in reducing SDS sample buffer for western analysis or analyzed for ATP and ADP hydrolyzing activity.

Nucleotidase Assays. Nucleotidase activity was determined by measuring the amount of inorganic phosphate released from nucleotide substrates at 37 °C by a modification (*18*) of the technique of Fiske and Subbarow (*19*). Briefly, 1−3 μg of cell membranes was 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 a final concentration of 2.5 mM and incubated at 37 °C for 30 min. The reaction was stopped and the 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 $MgCl_2$ plus chelator. Nucleotide hydrolyzing units are expressed in micromoles of P_i liberated per hour.

Protein Assay. Protein concentrations were determined by the Bio-Rad CB-250 dye binding technique according to the modifications of Stoscheck (*20*, *21*) using bovine serum albumin as the standard.

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

Electrophoresis and Western Blot Analysis. SDS−PAGE was performed according to Laemmli (*22*). HB6-transfected COS-1 cell membrane preparations (typically 3−5 μg/lane) were boiled for 5 min in SDS sample buffer containing 30 mM dithiothreitol and 8 M urea and were resolved on an SDS linear gradient polyacrylamide gel (4−15%). 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 (*23*). 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 antiserum in blocking

buffer. The PVDF membranes were subsequently washed, incubated in a 1:20 000 dilution of goat anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody, washed again, and immunoreactivity was detected by chemiluminescence with the Amersham ECL reagents as described by the manufacturer.

Preparation of Cells for Immunocytochemistry. COS-1 cells were transiently transfected with HB6 cDNA as described above and incubated with or without 2 μg/mL tunicamycin. Approximately 16 h posttransfection, cells were removed from the respective plates by incubation with trypsin and diluted into a Lab-Tek II 4 chamber slide (Nalge Nunc International) in complete DMEM containing sterile DMSO (control) or 2 μg/mL tunicamycin in DMSO. Approximately 24 h after the slide chamber was seeded, the cells were processed for immunocytochemistry as described below.

Immunocytochemistry. The HB6 ecto-apyrase was localized by indirect immunofluorescence by the following protocol. HB6-expressing COS cells were washed twice in PBS, fixed for 6 min in −20 °C methanol, washed again, and blocked in blocking buffer [1% (w/v) bovine serum albumin and 0.25% (w/v) saponin in PBS) for 1 h at room temperature. Anti-peptide polyclonal antiserum was then added to a final dilution of 1:500 in blocking buffer and incubated for 2 h at room temperature. HB6 control cells received an identical dilution of preimmune antiserum. After being washed with blocking buffer, the cells were exposed for 1 h to a 1:250 dilution of secondary antibody (goat anti-rabbit conjugated to CY3, Jackson Immunoresearch) prepared in blocking buffer. After being washed in blocking buffer followed by PBS alone, the slides were coverslipped in Dabco−glycerol mounting medium, and examined with a Bio-Rad MRC 600 laser-scanning confocal microscope and CoMOS image analysis software.

Size-Exclusion Chromatography. Ecto-apyrase-expressing COS cell membranes were detergent-solubilized with 1% NP-40 at a protein concentration of 0.1 mg/mL in 20 mM MOPS and 5 mM $MgCl_2$, pH 7.4, for 10 min at room temperature. Solubilization was also attempted with digitonin and saponin, but these detergents proved inefficient at solubilizing the ecto-apyrase from the COS cell membranes, consistent with a previous study (*24*). The solubilized membrane proteins were centrifuged at 13 000*g* for 2 min and then filtered through a 0.45 μm filter to remove any remaining insoluble proteins. The solubilized sample was loaded onto a Sephacryl S-300 column (1 cm × 45 cm) preequilibrated in 20 mM MOPS, 5 mM $MgCl_2$, and 0.1% saponin, pH 7.4, and allowed to separate with a flow rate of 0.4 mL/min. Fractions were collected every 2 min and analyzed for nucleotidase activity and western blot immunoreactivity. To calibrate the column, bovine serum albumin and gel-filtration standards (Bio-Rad) were run and fractions were analyzed by SDS−PAGE and protein assay to determine where the component standard proteins eluted.

Cross-Linking Experiments. Membrane preparations were obtained from COS cells previously transfected with the ecto-apyrase cDNA. Membranes (0.1 mg/mL) were incubated for 10 min at 23 °C in 20 mM MOPS and 5 mM $MgCl_2$, pH 7.4, in the presence (0−100 mM) of the hydrophobic, lysine-specific, primary amine cross-linker disuccinimido suberate (DSS), freshly prepared in dry DMSO. The lysine cross-linking reaction was stopped by dilution of samples into ice-cold 30 mM MOPS and 2 mM EDTA, pH 7.4, containing 12.5 mM lysine. The samples were immediately put on ice and precipitated overnight with 4 volumes of acetone at −20 °C. The precipitates were pelleted, solubilized with SDS buffer containing 8 M urea and 30 mM DTT reductant, vortexed, sonicated, and boiled for 5 min. The samples were then resolved by SDS−PAGE and western blotted as described above.

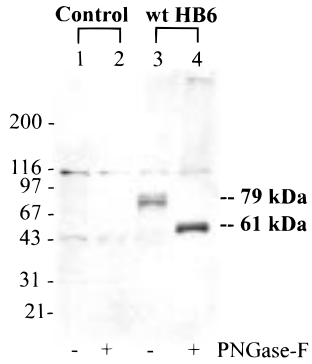

FIGURE 1: Western blot analysis of expressed ecto-apyrase with a polyclonal anti-peptide antibody generated against the C-terminal 15 amino acids. Control and HB6 ecto-apyrase-transfected COS cell membranes were electrophoresed on a reducing 4−15% linear gradient gel, transferred to PVDF membrane, and analyzed by western blotting. The symbol below each sample refers to whether the sample was (+, lanes 2 and 4) or was not (−, lanes 1 and 3) enzymatically deglycosylated with peptide *N*-glycosidase F (PNGase-F) prior to electrophoresis. The ecto-apyrase is shown as an approximately 79 kDa broad protein that shifts to a sharp 61 kDa protein band upon deglycosylation. HB6 apparent molecular masses were calculated by nonlinear curve-fitting of the molecular mass standards indicated at the left.

RESULTS

Expression and Core Molecular Weight of the Human Brain Ecto-apyrase. The isolation and characterization of the 2809 base human brain ecto-apyrase cDNA (HB6) was described in a previous publication (*15*). 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, as well as the seven potential *N*-glycosylation sites.

Identification of the expressed ecto-apyrase protein product was performed with polyclonal antiserum generated against the cytosolic C-terminal sequence. Western blot analyses of mock-transfected and HB6-transfected COS cells detected an approximately 79 kDa broad protein band only in the transfected cells (Figure 1, lane 3). No immunoreactive proteins were detected at this molecular weight in control COS cells (lane 1). The unidentified faint protein band at approximately 110 kDa present in both the control and HB6 transfected cells was observed upon probing with the preimmune sera as well (data not shown).

Seven putative glycosylation sites exist in the HB6 protein, suggesting that the enzyme is a glycoprotein. To determine the core molecular weight of the HB6 ecto-apyrase, control and HB6-transfected COS cells were subjected to enzymatic deglycosylation by peptide *N*-glycosidase F. Deglycosylation of HB6-transfected cell membranes caused a shift in the molecular weight of the immunoreactive 79 kDa ecto-apyrase

FIGURE 2: Time course of deglycosylation of native ecto-apyrase by peptide N-glycosidase F. HB6 ecto-apyrase-transfected COS cell membranes (35 μg) were incubated in the presence of 1.2 units of boiled, inactivated PNGase F (−) or active PNGase F (+), and aliquots were taken at the indicated times for analysis by SDS−PAGE. Aliquots (3 μg) of control and deglycosylated ecto-apyrase were electrophoresed under reducing conditions on a 5−15% linear gradient gel and analyzed by immunoblotting. The ecto-apyrase molecular mass is observed to decrease over time, consistent with the removal of glycans. A small amount of the core protein at 61 kDa is observed by 21 h (the faint narrow band in the rightmost lane). No obvious shift in molecular mass is observed for the control ecto-apyrase exposed to previously denatured PNGase F. Molecular size markers are indicated.

to approximately 61 kDa (Figure 1, lane 4), indicating that the protein is glycosylated. This is consistent with the calculated core molecular weight of 59 190 for this enzyme (*15*). No such immunoreactivity was observed in the control COS cells (Figure 1, lane 2).

Effect of Deglycosylation on Human Brain Ecto-apyrase Activity. To examine the effect of the removal of N-linked glycans on the enzymatic activity of the expressed ecto-apyrase, transfected COS cell membranes were exposed to peptide N-glycosidase F under nondenaturing conditions and aliquots were taken for SDS−PAGE and nucleotidase assays. As shown in Figure 2, exposure to the deglycosylating enzyme (indicated by a + in the figure) caused a gradual shift in the apparent molecular mass of the ecto-apyrase from 0 to 21 h, consistent with the removal of glycan chains. In contrast, no obvious change in molecular mass was observed for the control ecto-apyrase exposed to heat-inactivated PNGase F (indicated by a − in the figure) over the time course.

Enzymatic analyses of samples treated in a manner similar to those shown in Figure 2 were done to examine the effect of deglycosylation on the ATP and ADP hydrolyzing activity of the ecto-apyrase. As shown in Figure 3, enzymatic deglycosylation caused a time-dependent decrease in both the ATPase and ADPase activity of the enzyme. Both activities were inactivated in a similar manner. It was not determined which of the seven putative N-glycosylation sites were hydrolyzed by the PNGase F treatment, resulting in loss of enzymatic activity.

Effect of Tunicamycin on E-Type ATPase Activity and Molecular Mass. As shown in Figure 4 (lane 1), the ecto-apyrase protein is detected as an approximately 79 kDa, broad glycosylated protein band, with a core molecular weight of approximately 61 kDa evident after removal of all N-linked glycans by PNGase F deglycosylation (lane 5). To examine the effects of preventing carbohydrate addition on the size and enzymatic activity of the expressed protein, HB6 cDNA was transiently transfected in mammalian COS cells with tunicamycin added to the cellular medium at final

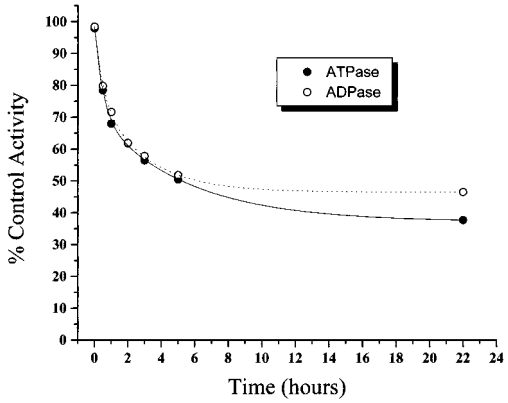

FIGURE 3: Loss of ATPase and ADPase activity upon PNGase F treatment. HB6 ecto-apyrase-transfected COS cell membranes were incubated in the presence of boiled, inactivated PNGase F or active PNGase F, and aliquots were taken at the indicated times for analysis of nucleotidase activity. Deglycosylation results in a time-dependent inactivation of both ATP and ADP hydrolyzing activity (corrected for slight decreases in control ecto-apyrase activity treated with heat-inactivated PNGase F). The specific activity of the control ecto-apyrase at the start of the reaction was 72 units/mg for ATP and 22.5 units/mg for ADP. The curves represent fits of the data to a double-exponential decay.

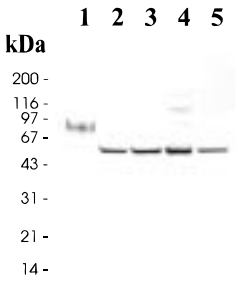

FIGURE 4: Effect of tunicamycin on the expression of the HB6 ecto-apyrase. HB6-transfected COS cells were incubated immediately after transfection in the absence (lane 1) or presence of 2, 5, and 10 μg/mL tunicamycin (lanes 2, 3, and 4, respectively). The following day, the medium was removed and replaced with medium containing fresh tunicamycin. On the next day, cell membranes were prepared and analyzed by western blotting. All concentrations of tunicamycin tested resulted in the synthesis of a 61 kDa ecto-apyrase core protein (lanes 2−4). The tunicamycin-expressed enzyme was the same molecular mass as the wild-type expressed enzyme after total deglycosylation with peptide N-glycosidase F (lane 5). No E-type ATPase activity was detectable from any of these enzymes synthesized in the presence of tunicamycin. Molecular size markers are indicated.

concentrations of 2, 5, and 10 μg/mL. Tunicamycin at all concentrations tested resulted in the production of an immunoreactive protein equal in mass to the core protein of 61 kDa (Figure 4, lanes 2−4). While the specific activity of the HB6 control membrane preparation (from transfection done in parallel to the tunicamycin-treated samples) was 149 units/mg for ATPase activity and 36.9 units/mg for ADPase activity, no ecto-apyrase (ATPase or ADPase) activity was detected in any of the three tunicamycin-treated membrane preparations shown in Figure 4.

Effect of Tunicamycin on the Cellular Localization of the HB6 Ecto-apyrase. To examine the cellular localization of the expressed enzyme in transfected cells, the HB6 cDNA was transiently transfected into mammalian COS cells in the presence and absence of 2 μg/mL tunicamycin. As shown in the confocal immunofluorescence images in Figure 5 (panels A and B), the glycosylated ecto-apyrase was detected

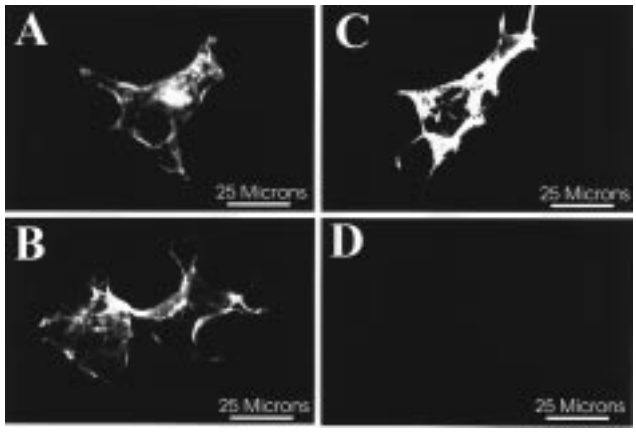

FIGURE 5: Immunolocalization of the ecto-apyrase in mammalian COS cells. COS cells were transfected with the HB6 ecto-apyrase cDNA and analyzed by indirect immunofluorescence as described under Experimental Procedures. In the control transfected cells not exposed to tunicamycin (panels A and B), the ecto-apyrase is seen distributed over the cell surface and clearly visible in the cell membrane border and cell processes. Transfected cells exposed to 2 μg/mL tunicamycin show a similar enzyme distribution, clearly staining along the cell edges and extensions (panel C). No immunoreactivity was observed when control and tunicamycin-treated cells were tested with the preimmune serum (panel D).

by diffuse staining over the cell and along the borders of the cells. Immunoreactivity can be seen along the ruffles and thin processes of the cells (panel B), consistent with localization mostly in the cell membrane. Similarly, cells exposed to tunicamycin during expression displayed immunoreactive protein also localized to the borders and extensions of the cell membrane (panel C). Some cells exposed to tunicamycin appeared somewhat rounded in shape but with a similar expression pattern (data not shown). No immunoreactivity was evident in mock-transfected cells or when transfected cells were probed with preimmune serum (panel D).

Size-Exclusion Chromatography of Detergent-Solubilized Ecto-apyrase. The solubilized size of the expressed, glycosylated ecto-apyrase was determined by size-exclusion chromatography calibrated with protein standards of known molecular weight. As shown in Figure 6, the glycosylated HB6 ecto-apyrase elutes primarily in two fractions (28 and 29; see inset), similar to where bovine IgG (MW 158 000) elutes. This indicates that the solubilized enzyme runs with a calculated molecular mass of 147 kDa and is consistent with the active, solubilized enzyme existing as a dimer of 79 kDa monomers. In contrast, the tunicamycin-treated (nonglycosylated) ecto-apyrase elutes in several fractions (centered around fraction 31; see Figure 6 inset), indicating a molecular mass of 84 kDa, indicative of the monomeric form of the enzyme.

Effect of Glycans on the Quaternary Structure of the Ecto-apyrase. The quaternary structure of the ecto-apyrase was investigated by nonreducing SDS−PAGE (Figure 7). When the HB6 control cell membranes were analyzed on the gel in the absence of reductant, nearly equal intensities of 79 kDa (monomer) and 158 kDa (dimer) bands are present (lane 2). Only the monomer form is evident when the sample is reduced by dithiothreitol prior to electrophoresis (lane 1). Tunicamycin-treated E-type ATPase migrates only as a 61 kDa core monomer, regardless of the presence or absence of reductant (lanes 3 and 4). Ecto-apyrase with all of the

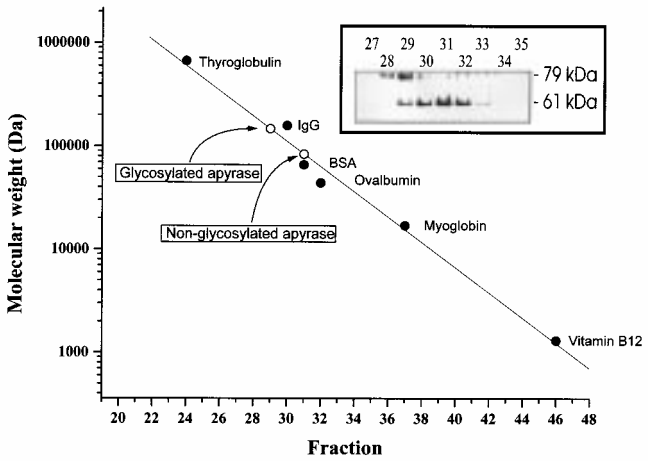

FIGURE 6: Size-exclusion chromatography of detergent-solubilized ecto-apyrase. Control and tunicamycin treated ecto-apyrase cell membranes were solubilized with NP-40 and pooled, and the proteins were separated on a Sephacryl S-300 column in the presence of 0.1% saponin. Fractions were taken and analyzed by western blotting for the presence of the ecto-apyrase after reducing SDS−PAGE (see inset). The column was standardized with protein standards of known molecular weight (thyroglobulin, 670 000; bovine γ-globulin, 158 000; bovine serum albumin, 66 000; ovalbumin, 44 000; myoglobin, 17 000; and vitamin B12, 1350). HB6 apparent molecular weights were calculated by curve-fitting of the molecular weight standards indicated. The glycosylated ecto-apyrase elutes with a calculated molecular weight of 147 000 and the nonglycosylated ecto-apyrase (which eluted in the same fraction as the 66 000 BSA protein) with a calculated molecular weight of 84 000.

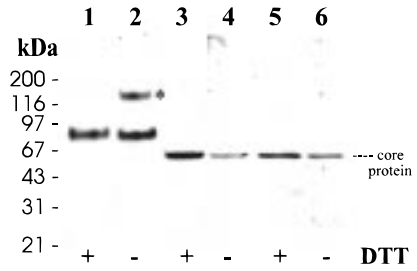

FIGURE 7: Effect of reduction conditions on the oligomerization of the ecto-apyrase. Control, tunicamycin-treated, and PNGase F-treated cell membranes were subjected to SDS−PAGE in the presence (+) and absence (−) of the reductant dithiothreitol and then western-blotted. Under reducing conditions, the ecto-apyrase runs as a single 79 kDa broad glycoprotein monomer (lane 1). Under nonreducing conditions, however (lane 2), the ecto-apyrase is observed as two bands—a monomer at 79 kDa and a dimer at approximately 158 kDa (indicated by an asterisk). Membranes from cells exposed to 2 μg/mL tunicamycin show the presence only of the core protein monomer (61 kDa) under both reducing and nonreducing conditions (lanes 3 and 4). Similarly, in cell membranes completely deglycosylated with PNGase F, the ecto-apyrase is detected only as a 61 kDa monomer (lanes 5 and 6). No evidence of dimer formation is seen in any ecto-apyrase preparation devoid of glycans. Molecular size markers are indicated.

N-linked glycosylation removed by peptide N-glycosidase F also migrates only as a 61 kDa monomer under reducing and nonreducing SDS−PAGE (lanes 5 and 6).

Effect of Cross-Linking on the Ecto-apyrase Oligomeric Structure. Transfected COS cell membranes containing expressed ecto-apyrase were treated in situ with increasing concentrations of the lysine-specific cross-linker disuccinimido suberate (DSS). A decrease in the immunoreactivity of the monomeric 79 kDa ecto-apyrase and the appearance

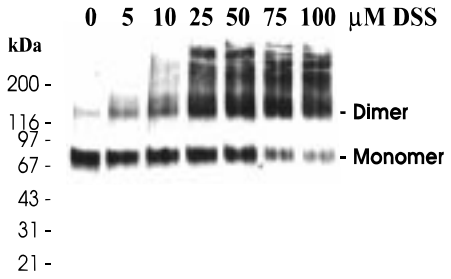

FIGURE 8: Immunoblot of DSS cross-linked COS cell membranes. HB6 ecto-apyrase-transfected COS cell membranes were cross-linked with varying concentrations of DSS, acetone-precipitated, and subjected to reducing SDS−PAGE. As analyzed by western blotting, the 79 kDa ecto-apyrase (monomer) was shown to shift to an approximately 158 kDa (dimer) as a function of DSS concentration, with a concomitant decrease in the amount of monomer present. At the higher concentrations of DSS (25−100 μM), higher order oligomers become evident. Molecular size markers are indicated.

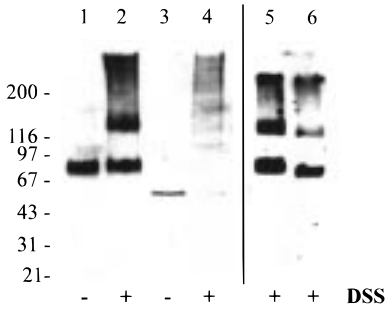

FIGURE 9: Chemical cross-linking of ecto-apyrase COS cell membranes treated with tunicamycin and PNGase F. Ecto-apyrase COS cell membranes (1.5 μg) were subjected to DSS cross-linking (+), subjected to reducing SDS−PAGE, and immunoblotted. With 100 μM DSS, some of the 79 kDa ecto-apyrase monomer (lane 1) was shown to shift to an approximately 158 kDa dimer (lane 2) in the fully glycosylated enzyme. Tunicamycin-treated ecto-apyrase, which runs as a deglycosylated core protein of 61 kDa (lane 3), was not cross-linked into a dimeric structure but rather a smear of high molecular weight aggregates (lane 4). In a separate experiment, ecto-apyrase (1.5 μg) first treated with boiled (lane 5) or active (lane 6) PNGase F deglycosylation enzyme for 3.75 h and subsequently cross-linked with DSS displayed evidence of dimer formation in the fully glycosylated ecto-apyrase (lane 5), but a greatly reduced amount of cross-linked dimer was formed from the partially deglycosylated ecto-apyrase (lane 6). This is consistent with the observed partial loss of glycans (compare lane 6 to lane 5) and activity (52% and 46% of the control ATPase and ADPase activity, respectively) of the E-type ATPase.

of a dimeric 158 kDa band was seen with increasing concentrations of DSS (Figure 8), consistent with the enzyme existing as a dimer in the membrane. At high concentrations of DSS, higher oligomeric structures could be seen. Glycan-deficient ecto-apyrase was also examined for the ability to be cross-linked into dimeric structures by DSS. As shown in Figure 9, tunicamycin-treated ecto-apyrase was not cross-linked into a dimer to any significant extent (lane 4). Rather, high molecular weight aggregates appeared. Similarly, native ecto-apyrase exposed to peptide N-glycosidase F was significantly reduced in its ability to be cross-linked to a dimer (lane 6), consistent with a partial loss of glycans after this limited digestion (compare shift in molecular weight of this monomer to control monomer, lane 5). These results are in contrast to the fully glycosylated 79 kDa ecto-apyrase (lane 1), which is shown to be cross-linked into a dimer of 158 kDa (lane 2). No recovery of enzymatic activity was evident after attempting to cross-link either the tunicamycin- or PNGase F-treated ecto-apyrase.

DISCUSSION

The class of enzymes known as the E-type ATPases are glycosylated proteins (*5*). These include the chicken oviduct ATP diphosphohydrolase (*25*), chicken gizzard ecto-ATPase (*26*), human ATP diphosphohydrolase (*15*), and human CD39 ecto-apyrase (*27*), containing 12, 4, 7, and 6 potential N-glycosylation sites, respectively. However, the role of the glycans in the functional activity of these enzymes has not been examined in great detail. In this work, we show the crucial role that N-linked glycans play in the enzymatic activity of a human ecto-apyrase.

The human brain HB6 ecto-apyrase studied in this work runs as a broad glycoprotein band at approximately 79 kDa that shifts to a tight, well-resolved protein band at 61 kDa after deglycosylation (Figure 1). Peptide N-glycosidase F completely deglycosylated the ecto-apyrase in an overnight incubation if the apyrase was first denatured by boiling in the presence of SDS. Under native (nondenaturing) conditions, however, the enzyme was more resistant to deglycosylation, with only partial deglycosylation after 21 h (see Figure 2). Only a slight amount of the core protein is evident after 21 h of incubation with the peptide N-glycosidase F. However, the enzymatic activity of the ecto-apyrase is gradually lost over time by treatment with the glycosidase (Figure 3). This is in agreement with results presented by Martina et al. (*28*), who showed that timed deglycosylation of GD3 synthase by peptide N-glycosidase F caused a gradual loss of enzymatic activity. Similarly, Nosjean et al. (*29*) showed, using various endoglycosidases, that N-glycosylation is absolutely essential for the catalytic activity of bone, kidney, and liver alkaline phosphatase. We have also examined COS cell membranes transfected with human brain CD39 ecto-apyrase cDNA, and we found that PNGase F treatment results in a time-dependent inactivation of the nucleotidase activity, very similar to that observed for the HB6 ecto-apyrase (i.e., 38% ADPase activity remaining for CD39 after 5 h of PNGase F treatment, as compared to 45% ADPase activity remaining for HB6 ecto-apyrase in an experiment run in parallel). However, these studies are in contrast to results reported by Christoforidis et al. (*14*), who showed that a placental ATP diphosphohydrolase, presumably CD39, that was partially deglycosylated with peptide N-glycosidase F retained its enzymatic activity. In addition, our group previously found that another E-type ATPase, the rabbit skeletal muscle T-tubule ecto-ATPase, was insensitive to the effects of PNGase F under nondenaturing conditions (*30*). It is not obvious why these discrepancies exist, except that both enzymes used in the previous studies were solubilized with detergents prior to PNGase F treatment and were different E-type ATPases isolated from diverse sources (i.e., human placenta and rabbit skeletal muscle).

To examine the effects of the prevention of carbohydrate additions on the E-type ATPase activity, transfected cells were cultured in the presence and absence of tunicamycin, an inhibitor of N-glycosylation formation. As shown in Figure 4, tunicamycin treatment resulted in expressed HB6 ecto-apyrase devoid of N-glycosylation. ATP and ADP hydrolyzing activity of the enzyme was completely lost after

this treatment. In addition, a parallel experiment utilizing COS cell expressed human CD39 ecto-apyrase demonstrated that this E-type ATPase was also inactivated by this treatment, since the CD39 ATP and ADP hydrolyzing activity was completely abolished after tunicamycin treatment (COS cells expressing glycosylated CD39 had an ATPase activity of 147 units/mg and an ADPase activity of 123 units/mg, while tunicamycin-treated COS cells expressing CD39 had no detectable ATPase or ADPase activity). This suggests that N-glycans might be important for the maintenance of enzymatic activity of all or most of the family of E-type ATPases. This finding is similar to the results observed for H^+, K^+-ATPase (*31*), GD3 synthase (*28*), and *N*-acetylglucosaminyltransferase III (*32*).

N-Glycosylation does not appear to be necessary for the proper trafficking and localization of the HB6 ecto-apyrase. In both the presence and absence of tunicamycin, ecto-apyrase-expressing COS cells showed a similar localization around the cellular periphery (Figure 5). Examples exist in the literature in which N-glycosylation is necessary for the proper cellular localization of some Golgi-specific enzymes such as *N*-acetylglucosaminyltransferase III (*32*) and GD3 synthase (*28*). However, in another report examining a plasma membrane enzyme, neither prevention of O-glycosylation nor defects in N-glycosylation prevented the proper protein trafficking of CD13 (aminopeptidase N) to the plasma membrane surface (*33*).

E-type ATPases are known to be capable of forming oligomeric structures under certain conditions, and it has been suggested that the proteins may exist as dimers in vivo (*34*). Enzymatic activity may also be regulated by the quaternary structure of the protein (*35*). Detergent-solubilized glycosylated HB6 ecto-apyrase analyzed by size-exclusion chromatography elutes with a weight indicative of a dimeric structure (Figure 6), consistent with previous results reported for the rabbit skeletal muscle E-type ATPase (*17*). Supporting this observation is the fact that SDS−PAGE analysis of the ecto-apyrase under nonreducing conditions reveals an apparent dimer in addition to the 79 kDa monomer seen under reducing conditions (Figure 7), consistent with the results obtained with the chicken oviduct and liver ecto-apyrase (*36*). It is interesting, but not unprecedented, that the ecto-apyrase maintains oligomeric structures after nonreducing SDS−PAGE. Alkaline phosphatases, which are also ecto-enzymes capable of forming dimers or tetramers (*37*), have been shown to run as oligomers on nonreducing SDS−PAGE (*29*).

Unlike the control ecto-apyrase protein, however, glycan-deficient ecto-apyrases (both tunicamycin- and PNGase F-treated) display only a single monomeric protein at 61 kDa under both reducing and nonreducing SDS−PAGE conditions (Figure 7). In addition, size-exclusion chromatography of tunicamycin-treated ecto-apyrase shows that the protein elutes with a size indicative of a monomer (Figure 6). It is possible that the carbohydrates are involved in helping to maintain the quaternary structure of the enzyme (and also the enzymatic activity). Kern et al. (*38*), using invertase as a model, found that the oligomerization of invertase into tetramers and octamers was dependent on glycosylation of the core enzyme. Invertase synthesized in the absence of glycans was unable to form higher order structures. In addition, studies on deglycosylated acid phosphatase showed that the modified protein lost enzymatic activity and the ability to form dimers as assessed by electrophoresis (*39*). However, it is also possible that the tunicamycin- and PNGase F-treated ecto-apyrase tertiary structure is disrupted due to the absence of glycan chains. Nosjean et al. (*29*) found that the enzymatic activity but not the oligomeric structure of nonspecific alkaline phosphatases was affected by deglycosylation with several glycosidases. The researchers proposed that the loss of enzymatic activity observed in the alkaline phosphatase was more likely due to local modulations in tertiary structures, i.e., changes in charge−charge interactions or in steric hindrance (*29*). Other studies suggest that the removal or absence of glycosylations can disrupt the proper folding of polypeptides and decrease the stabilization of the mature glycoprotein conformation (*40*).

The oligomeric nature of the E-type ATPase and the role of glycosylation on the quaternary structure were also studied by cross-linking the E-type ATPase enzyme directly in the COS cell membranes in which they were expressed. These in situ cross-linking results (Figure 8) also suggest that the ecto-apyrase is a homooligomer, consistent with cross-linking results obtained for other membrane-bound E-type ATPases (*35*, *41*, *42*). In addition, the present results (Figures 7 and 9) demonstrate that the ability to form the active dimer structure is adversely affected by lack of (or removal of) glycosylation.

We have previously hypothesized that many modulators of E-type ATPase activity act via effects on the quaternary structure (monomer versus dimer) (*35*). Now, we propose that the deglycosylation effects reported here may also be mediated by decreasing or abolishing the monomer interactions involved in homodimer formation or maintenance, thereby leading to inactivation of the E-type ATPase. However, it is also possible that the effect of deglycosylation on quaternary structure may also be mediated indirectly, e.g., by generally decreasing the stability of the protein tertiary structure, as is also known to occur with deglycosylation of other proteins (*40*). The physiological importance of this modulation of E-type ATPase activity by quaternary structure remains to be determined, but it is possible that it serves as a switch to turn on or off extracellular nucleotidase activity at points of interaction between cells or between cells and the extracellular matrix.

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