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THE *N*-GLYCOSYLATION STATE OF CD39 CORRELATES WITH SURFACE ACTIVITY AND LOCALIZATION*

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CD39 is a member of the membrane-bound ecto-nucleoside triphosphate diphosphohydrolase family. The active site for native CD39 is located on the outer surface of the cellular plasma membrane; however, it is not yet known at what stage this enzyme becomes active along the secretory pathway to the plasma membrane. In this study, sucrose density fractionations performed on CD39-transfected COS-7 cell membranes suggest that CD39 activity resides primarily in the plasma membrane. Furthermore, we have created recombinant, soluble versions of CD39, one that is secreted and others that are retained in the endoplasmic reticulum, to demonstrate that CD39 is not active until it reaches the plasma membrane both in yeast and COS-7 cells. Moreover, the secreted active soluble CD39 in COS-7 cells is found to receive a higher degree of *N*-glycan addition than the inactive form retained intracellularly. When COS-7 cells were treated with tunicamycin to prevent *N*-glycosylation, soluble CD39 was not detected in the extracellular medium and remained inactive intracellularly. Surface biotinylation analysis also revealed that surface-expressed wild type CD39 receives a higher degree of *N*-glycosylation than intracellular forms and that inhibition of *N*-glycosylation prevents its plasma membrane localization. In addition, both intact and digitonin-permeablized COS-7 cells transfected with CD39 possess similar ecto-ATPase activities, further supporting the conclusion that only surface-expressed CD39 is enzymatically active. All of these data suggest that intracellular CD39 is inactive and that only a fully glycosylated CD39 has apyrase activity and is localized at the cell surface.

Extracellular nucleotides such as ATP can act as signaling transmitters in nerve, muscle, and blood tissues through their interactions with specific plasma membrane purinergic receptors (1–3). The ability of ecto-nucleoside triphosphate diphosphohydrolases (E-NTPDases)[1] to hydrolyze nucleoside tri- and diphosphates in the presence of divalent cations (usually Ca^{2+} or Mg^{2+}) suggests that they may regulate the actions of purinergic receptors by controlling the extracellular conversion rate from ATP to adenosine (1–4). The molecular identity of the E-NTPDase family was initially discovered by the cloning of a soluble apyrase from potato tubers (*Solanum tuberosum*) (5). CD39, a human and mouse lymphoid cell antigen (6), E-NTPDase1, localizes to the plasma membrane (7) and is responsible for the inhibition of ADP-induced platelet aggregation (8, 9). Recent data from CD39-null mice indicate that CD39 indeed plays an important role in regulating the function of the ADP-dependent purinoreceptor P2Y1 (10).

CD39 is an integral membrane protein with two transmembrane domains and a large extracellular region (6) with NTPDase activity (7). This extracellular domain contains apyrase conserved regions (5, 7), some of which are similar to those of actin-hsp70-hexokinase β and γ-phosphate binding motifs, suggesting a possible role in nucleotide binding (5). The transmembrane domains of CD39 affect its enzymatic activity by forming a detergent-sensitive oligomeric structure (11). CD39 has six putative *N*-glycosylation sites within its sequence (6) and is heavily glycosylated (12). This ectoapyrase undergoes several other post-translational modifications as well, including proteolysis (13, 14) and constitutive palmitoylation within its NH_2-terminal intracytoplasmic region (15).

It has been well established that CD39 functions on the outer face of the plasma membrane (6, 7, 9, 11, 12, 15). However, it is not known whether CD39 is active in the compartments of the secretory pathway. One distinguishing hallmark of E-NTPDases is their ability to hydrolyze nucleotide substrates at extremely high turnover rates. The specific ecto-ATPase activity of the digitonin-solublized membrane-bound CD39 has been estimated to be ~2000 μmol/min/mg (11). If such high nucleotidase activity were present in the lumen of intracellular compartments such as the endoplasmic reticulum (ER) or the Golgi, it would deplete the lumenal ATP and potentially inhibit other ATP-dependent lumenal components and processes (16–19). It thus becomes of interest to determine whether cells can tolerate the presence of high nucleotidase activity within intracellular compartments and to identify the stage at which CD39 becomes active. In this study, we provide evidence that CD39 is not active until it reaches the plasma membrane, indicating that cells restrict CD39 activity to its target location. Our results also demonstrate that complete *N*-glycosylation of CD39 correlates with its enzymatic activity and that *N*-glycan addition is essential for its surface localization.

MATERIALS AND METHODS

Strains, Media, and Reagents—All DNA manipulations were performed using the *Escherichia coli* strain DH5α (*supE44D lacU169 (f80lacZDM15) hsdr17 recA1 endA1 gyrA96 thi-1 relA1*). The *Saccharomyces cerevisiae* strains used were BCY123 (*MATa pep4::HIS3 prb1::LEU2 bar1::HISG lys2::GAL1/10-GAL4 can1 ade2 trp1 ura3 his3 leu2-*

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[1] The abbreviations used are: E-NTPDase, ecto-nucleoside triphosphate diphosphohydrolase; ER, endoplasmic reticulum; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis.

3,112) and YPH500 (*MATα leu2 ura3 trp1 lys2 his3 ade2*). Standard rich (YPD) and complete minimal tryptophan or uracil drop-out media were used (20). Standard rich medium for *E. coli* was used (21). Nucleoside phosphates were purchased from Sigma.

Preparation of Crude Membrane from COS-7 Cells and Concentration of Secreted Proteins from the Medium of Transfected Cells—COS-7 cells were grown in a humidified incubator with 5% CO_2 at 37 °C in Dulbecco's modified Eagle's medium supplemented with penicillin, streptomycin, glutamine, and 10% fetal bovine serum. 100-mm plates of COS-7 cells at 50–70% confluency were transfected with 6 μg of supercoiled plasmid DNA by the LipofectAMINE method (Life Technologies, Inc.). For studies utilizing tunicamycin-treated cells, tunicamycin prepared in sterile Me_2SO was added immediately following transfection to the final concentration of 5 μg/ml. The control cells received Me_2SO alone. Crude membranes of transfected COS-7 cells were prepared as described (22) and resuspended in 50 μl of 50 mM Tris-HCl, pH 8.0/100-mm plate. To concentrate the secreted glycoproteins from the media as shown in Fig. 5, culture media from three plates of transfected COS-7 cells were collected by centrifugation at 3,000 rpm for 10 min in a clinical centrifuge and passed through a 1-ml ConA Sepharose column. The column was then washed with 20 ml of Tris-buffered saline solution containing 1 mM $CaCl_2$. Secreted glycoproteins were eluted with 2 ml of 1 M α-methyl-D-mannoside in the same solution. To concentrate the secreted proteins from the media as shown in Fig. 6, culture media were applied to a 1.5-ml nickel resin column (Invitrogen), washed with 6 ml of the Tris-buffered saline solution containing 1 mM $CaCl_2$ and eluted with 3 ml of Tris-buffered saline solution containing 0.5 M imidazole and 1 mM $CaCl_2$. All of the elutions were centrifuged in a Centricon-30 tube for 90 min at 5,000 rpm. The concentrated proteins were diluted 1:200 for nucleotidase assays.

Nucleotide Phosphatase Assay, Protein Deglycosylation, Yeast Crude Membrane Preparation, and Immunoblotting—Nucleotide phosphatase activity was assayed as described by Wang *et al.* (11). Briefly, the assays were done for 20 min in buffer A (20 mM Tris-HCl, pH 7.4, 120 mM NaCl, 5 mM KCl, 1 mM EGTA, 1 mM NaN_3, 1 mM Na_3VO_4, 0.2 mg/ml alamethicin, 2 mM ATP) with or without 5 mM $CaCl_2$ at room temperature with yeast cells and at 37 °C with COS-7 cells. The Ca^{2+}-stimulated ATPase activity was determined by measuring the inorganic phosphate released and by subtracting values obtained without $CaCl_2$ from those with 5 mM $CaCl_2$. Yeast crude membranes were prepared as described previously (23). *Flavobacterium menigosepticium* glycopeptidase F (New England Biolabs, Beverly MA) was used to deglycosylate asparagine-linked glycans as described (23). Immunoblotting was done as described previously (6). Rabbit anti-CD39 polyclonal antibody (12) was used at a dilution of 1:1,000. The α1-specific polyclonal rabbit antibody 620 (1:1,000) was raised against the rat kidney Na,K-ATPase by Dr. Jonathan Lytton in this lab (22). Rabbit anti-calnexin carboxyl terminus polyclonal antibody (1:2,000) was purchased from StressGen (Canada). Peroxidase-conjugated goat anti-rabbit antibody (1:2,000) and peroxidase-conjugated goat anti-mouse antibody (1:2,000) were purchased from Sigma.

Subcellular Fractionation of Crude Membranes of COS-7 Cells—The cell fractionation procedure of COS-7 crude membranes was essentially as described by Coppi and Guidotti (22). Briefly, the crude membranes obtained from three 100-mm plates of COS-7 cells and placed in 0.7 ml of 10 mM HEPES (pH 7.4) were combined with 2.3 ml of 65% sucrose (w/w in 10 mM HEPES-Tris, pH 7.5, 10 μg/ml aprotinin, 100 μM $CaCl_2$) and placed at the bottom of an SW40 centrifuge tube. Subsequently, 1 ml each of the following sucrose solutions (w/w in the same solution) was overlaid: 45, 40, 35, 30, 25, 20, 15, and 10%. Following centrifugation at 4 °C at 25,500 rpm for 18 h in a Beckman SW40 Ti rotor, 0.75-ml fractions were collected from the bottom of the tube. The sucrose concentration was measured using a refractometer. A 90-μl aliquot of each sucrose fraction sample was used for immunoblotting with anti-CD39, anti-calnexin, and anti-α1 subunit antibodies.

DNA Constructs—DNA manipulations were carried out according to standard protocols (24). To express CD39 in yeast, a *Bam*HI polymerase chain reaction (PCR) product containing the entire CD39 gene was cloned into the pG1 vector (pGZ131). Primer 112 (5′-CGCGGATCCGTAACCATGGAAGATATAAAGGATTCTAAG-3′, containing a *Bam*HI site, a Kozak sequence, and a sense sequence of the CD39 open reading frame (nucleotides 1–24)), and primer 113 (5′-CGCGGATCCTTATTCCATTGGCATGTATTCTACTGCCTC-3′, containing a *Bam*HI site and an antisense sequence of the CD39 open reading frame (nucleotides 1507–1536)) were used with pCI-neo-CD39 as a template (12).

To express soluble CD39 with an ER retention sequence (sCD39-HDEL) in yeast, two different *Bam*HI/*Xho*I PCR products encoding a fusion of the NH_2-terminal 85-amino acid leader sequence of yeast

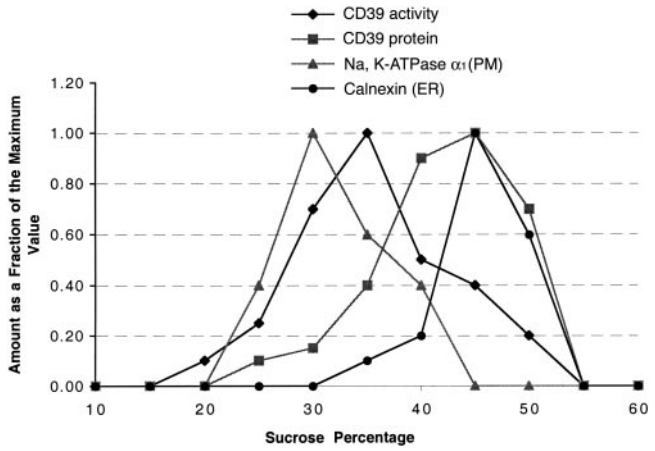

FIG. 1. **Subcellular fractionation of COS-7 cells transfected with CD39 cDNA.** Crude membranes were prepared from COS-7 cells transfected with CD39 cDNA and fractionated on a discontinuous sucrose gradient as described under "Materials and Methods." The data for Ca^{2+}-stimulated nucleotidase activity of CD39 are plotted as fractions of the maximal enzymatic activity observed. The data for calnexin, endogenous Na,K-ATPase α_1 subunit, and CD39 are plotted as percentages of the maximal immunostaining observed. The values are the means of three independent experiments; the standard deviations were ~10%.

α-factor (25) and residues 38–470 of CD39 were cloned into the pG1 vector (pGZ155). One PCR product was generated with primer 146 (5′-GCGGGATCCTTATAATTCGTCGTGGGATAATGGCTGTTCAGCTGGGAT-3′, containing a *Bam*HI site, an antisense codon for the amino acids HDEL, and an antisense sequence of the CD39 open reading frame (nucleotides 1387–1410)) and primer 149 (5′-CCGCTCGAGACCCACAACAAACCATTGCCAGAA-3′, containing a *Xho*I site and a sense sequence of the CD39 open reading frame (nucleotides 112–135)) and with the template pCI-neo-CD39. Another PCR product was generated with primer 147 (5′-GCGGGATCCGTAACCATGAGATTTCCTTCAATTTTTACT-3′, containing a *Bam*HI site, a Kozak sequence, and the sense sequence of the yeast α-factor open reading frame (nucleotides 1–24)) and primer 148 (5′- CCGCTCGAGTCTTTTATCCAAAGATACCCCTTC-3′, containing a *Xho*I site and an antisense sequence of the yeast α-factor open reading frame (nucleotides 232–255)) and with chromosomal DNA isolated from YPH500 cells.

To express sCD39-KDEL in COS-7 cells, an *Eco*RI/*Eco*RI PCR product encoding residues 39–470 of CD39 and a COOH-terminal KDEL motif replaced a corresponding DNA fragment in pcDNA3-CD4-sCD39 (26) (pGZ167). The PCR product was generated with primer TM12E (5′-GGAATTCCCACAACAAACCATTGCCA-3′, containing an *Eco*RI site and a sense sequence of the CD39 open reading frame (nucleotides 115–132)) and primer 170 (5′-GGAATTCTTATAATTCGTCTTT-GGATAATGGCTGTTCAGCTGGGAT-3′, containing an *Eco*RI site, antisense codons for KDEL, and an antisense sequence of the CD39 open reading frame (nucleotides 1507–1536)) and with the template pcDNA3-CD4-sCD39.

Cell Surface Biotinylation and Streptavidin Precipitation—The procedure was modified as described by Feraille *et al.* (27). Briefly, two 100-mm plates of COS-7 cells transfected with pCI-neo-CD39 were washed three times with ice-cold PBS buffer. The cells were then incubated at 4 °C for 1 h with biotinylation buffer (0.25 mg/ml EZ-link sulfo-NHS-LC-biotin (Pierce) in PBS buffer). After removal of the biotinylation buffer, the cells were incubated for 20 min at 4 °C in quenching buffer (100 mM glycine in PBS buffer), washed once with ice-cold PBS buffer, and lysed in 1 ml of lysis buffer (1% (w/v) sodium deoxycholate, 20 mM HEPES/KOH, pH 7.4, 100 mM NaCl, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 10 μg/ml leupeptin). 200 μl of protein extract were added to 100 μl of streptavidin-agarose beads (Immunopure immobilized streptavidin; Pierce) diluted in precipitation buffer (0.1% digitonin, 20 mM HEPES/KOH, pH 7.4, 100 mM NaCl, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 10 μg/ml leupeptin) and were incubated overnight at 4 °C with end-to-end rotation. The incubation mixture was centrifuged for 4,000 rpm for 1 min. Although the supernatant fraction was saved, the beads were washed twice with 1 ml of the precipitation buffer and extracted in 100 μl of sample buffer with boiling (designated as the pellet fraction). The quantitative results are the means of two to four experiments, as is indicated in the legends to Figs. 1 and 4–7.

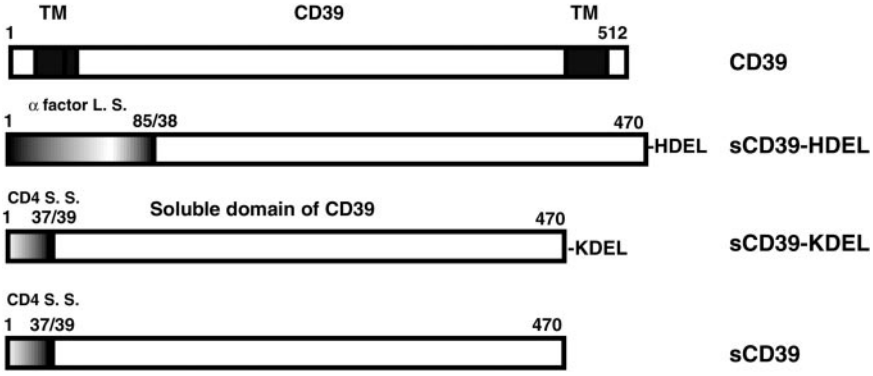

FIG. 2. **Schematic representation of the constructs for wild type CD39 and secretable soluble CD39 with or without an ER retention signal for yeast and COS-7 expression.** The transmembrane domains of CD39, the CD4 signal peptide, and cleavage sequence (*CD4 S.S.*), the α-factor leader peptide sequence (α factor *L.S.*), and the (K/H)DEL tags are indicated. The α-factor leader sequence is comprised of an initial signal sequence of 22 amino acids and a subsequent pro-sequence from amino acids 23–85.

RESULTS

Subcellular Distribution of CD39 Activity—As a first step in determining the stage at which CD39 becomes active, the activity of CD39 in different subcellular fractions was assessed. Crude membranes were isolated from COS-7 cells transfected with CD39 cDNA and were fractionated across a discontinuous sucrose gradient. As shown in Fig. 1, the ER and plasma membrane of COS-7 cells can be identified by their distinct sedimentation patterns in the discontinuous sucrose gradient. The plasma membrane marker, the endogenous α_1 subunit of the Na,K-ATPase (22), peaks at roughly 30% sucrose. The ER marker, endogenous calnexin (28), has a distribution at a higher sucrose density that peaks at roughly 45% sucrose.

Although CD39 is known to be a plasma membrane protein (Fig. 1), immunoblotting with anti-CD39 showed that the ectoapyrase was present in a range of fractions (35–55% sucrose, peak at 40–45%) that more closely resembled the ER-localized calnexin. This might be a result of the overexpression of CD39 in COS-7 cells because of the transfection. On the other hand, the activity of CD39 was principally distributed in the 25–40% sucrose fractions, which is more consistent with a functional role for CD39 primarily limited to the plasma membrane. The results of the sucrose density gradient indicate that CD39 activity does not directly correlate with CD39 concentration within the plasma membrane and intracellular organelles such as the ER.

Construction of Various Versions of CD39—As a second way of determining whether CD39 is active intracellularly, various constructs of CD39 were made. As illustrated in Fig. 2, we constructed yeast vectors capable of expressing intact CD39 as well as a soluble version using CD39 (sCD39) with an ER retention sequence (29, 30). We also created a mammalian vector capable of expressing a soluble form of CD39 with an ER retention sequence. With these constructs, we could test whether CD39 is active only at the cell surface or also when confined within intracellular organelles.

Expression of CD39 in Yeast Does Not Cause a Cell Growth Defect—To assess whether overexpression of CD39 and sCD39-HDEL in yeast affects cell growth, plasmids pGZ131 (wild type CD39), pGZ155 (sCD39-HDEL), and vector pG1 were transformed into yeast strain BCY123. As shown in Fig. 3*A*, the transformants of these three plasmids generated yeast colonies of similar size after 3 days of incubation on tryptophan dropout plates at 30 °C. When these transformants were transferred to liquid medium, vegetative growth rates were also observed to be similar (data not shown). These results indicate that the overexpression of CD39 and sCD39-HDEL in yeast does not cause any observable cell growth defects.

To find out whether the products of these constructs had actually been expressed in the yeast cells, crude membranes from the three yeast strains, BCY123/pG1, BCY123/pGZ131, and BCY123/pGZ155, were isolated. Fig. 3*B* illustrates that

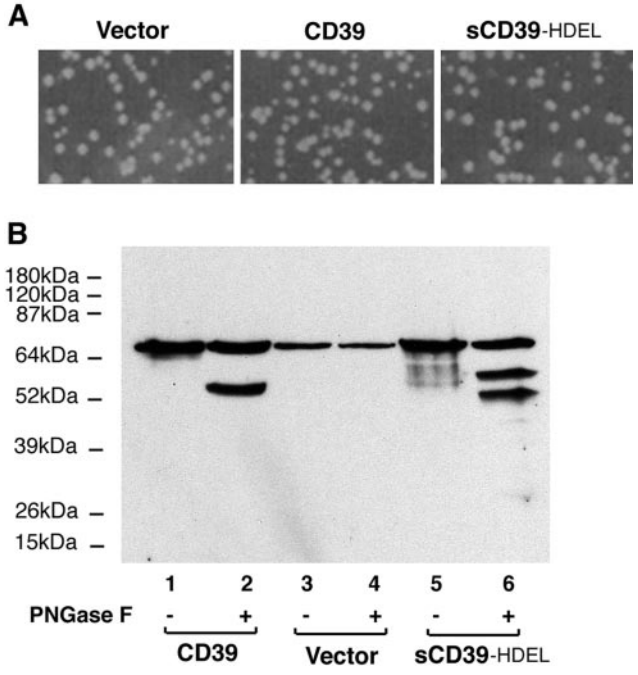

FIG. 3. **Expression of CD39 and sCD39-HDEL in yeast.** *A*, expression of CD39 and sCD39-HDEL in yeast does not cause a cell growth defect. The yeast strain BCY123 was transformed with equal amounts of plasmid pG1 (control vector), plasmid pGZ131 (expressing CD39 cDNA), or plasmid pGZ155 (expressing sCD39-HDEL cDNA) using the lithium acetate method. Identical aliquots of the transformation reactions were plated on SD plates lacking tryptophan to select for Trp^+ transformants and allowed to grow at 30 °C for 3 days. *B*, expression of CD39 and sCD39-HDEL in yeast. 50 μg of crude membrane proteins from BCY123/pGZ131 (*lanes 1* and *2*), BCY123/pG1 (*lanes 3* and *4*), and BCY123/pGZ155 (*lanes 5* and *6*) were treated or not treated with glycopeptidase F (*PNGase F*). The samples were subjected to 10% SDS-PAGE and examined by immunoblotting with anti-CD39 antibody.

anti-CD39 serum recognized a nonspecific 72-kDa protein band within all of the samples. However, additional smeared bands close to 70 kDa were recognized by the antibody in the sample of yeast cells transformed with the CD39 plasmid (compare *lane 1* with *lanes 2–4*). When this sample was deglycosylated with glycopeptidase F, a 56-kDa band was observed (*lane 2*), corresponding roughly to the correct molecular mass of unglycosylated CD39. No such band was detected in the sample of the control vector treated with glycopeptidase F (*lane 4*). These results indicate that CD39 was not only expressed in yeast cells but also post-translationally modified. Anti-CD39 antibodies recognized some smeared bands ranging from 58 to 72 kDa in the sample of yeast cells transformed with the sCD39-HDEL plasmid (*lane 5*). One 58-kDa band and one 52-kDa band appeared in the same sample treated with glycopeptidase F (*lane 6*). The 58-kDa band is probably the deglycosylated form of

sCD39-HDEL, whereas the 52-kDa band is most likely a protease degradation product. Both protein bands were not detectable in the extracellular medium (data not shown). We recognize that sCD39-HDEL at 58 kDa migrates slower than native CD39 at 56 kDa despite having a calculated smaller molecular mass (Figs. 2 and 3*B*). This observation is attributed to the high density of negatively charged residues in the pro-sequence of α-factor attached to this soluble CD39, which tends to slow migration in SDS-PAGE. These results confirm that sCD39-HDEL was also expressed in yeast cells and retained intracellularly.

Wild Type CD39 Is Active in Yeast Cells; sCD39-HDEL Is Not—To verify that CD39 is active and reaches the plasma membrane when it is expressed in yeast, intact yeast cells were used to measure the Ca^{2+}-stimulated ecto-ATPase activity on the cell surface. In addition to BCY123/pG1, yeast strain BCY123/pVT-mdr3 (31) was also used as a negative control. In yeast, the mouse mdr3 P-glycoprotein, which contains two cytoplasmic ATPase domains, was demonstrated to pump drugs out of cells (31). Fig. 4*A* shows that yeast strain BCY123/pGZ131 (expressing wild type CD39) possessed constitutive surface Ca^{2+}-dependent ecto-ATPase activity. This activity was not inhibited by P- or V-type ATPase inhibitors, because 1 mM Na_3VO_4 and 1 mM NaN_3 were included in the reaction buffer. As expected, no such activity was detectable on the surface of BCY123/pG1, BCY123/pGZ155 (sCD39-HDEL), and BCY123/pVT-MDR3. These results suggest that wild type CD39 is functionally expressed at the plasma membrane in yeast cells upon transfection.

To determine whether sCD39-HDEL is active in yeast cells, the Ca^{2+}-stimulated ecto-ATPase activities of crude membranes isolated from yeast strains BCY123/pGZ155, BCY123/pGZ131, and BCY123/pG1 were measured and compared. Crude membranes with wild type CD39 possessed four times higher activity than that of control crude membranes from cells transfected with the pG1 vector alone. However, the activity of crude membranes containing sCD39-HDEL was almost the same as the activity found in the control (Fig. 4*B*). No sCD39-HDEL protein was detected in the supernatant with immunoblotting (data not shown) after the membrane purification was completed, suggesting that the majority of the sCD39-HDEL remained associated within intracellular organelles. This indicates that sCD39-HDEL is not active in yeast and hence that lumenal ATP should not be depleted, which is consistent with the data that overexpression of this construct in yeast does not result in a cell growth defect.

Extracellular sCD39 Expressed in COS-7 Cells Is Active, but Neither Intracellular sCD39 nor sCD39-KDEL Is Active—To further investigate whether CD39 is active before it reaches the plasma membrane, plasmids containing sCD39 cDNA and sCD39-KDEL cDNA were transfected into mammalian COS-7 cells. Secreted glycoproteins including sCD39 were partially purified from the extracellular medium of the transfected cells using a ConA-Sepharose column. Crude membranes were also isolated from transfected COS-7 cells. The immunoblot depicted in Fig. 5*A* confirmed that both sCD39 and sCD39-KDEL were expressed and associated with the crude membranes of corresponding transfected cells (*lanes 1–4*). sCD39 was also found to be secreted into the extracellular medium (*lanes 5* and *6*), whereas sCD39-KDEL was not found in the medium (*lanes 7* and *8*), indicating that this protein was retained within the cells because of the ER retention signal.

The activities of these samples were measured, and as shown in Fig. 5*B*, the activities of both crude membranes containing lumenal sCD39 and crude membranes with sCD39-KDEL were similar to the background activity of the crude membranes

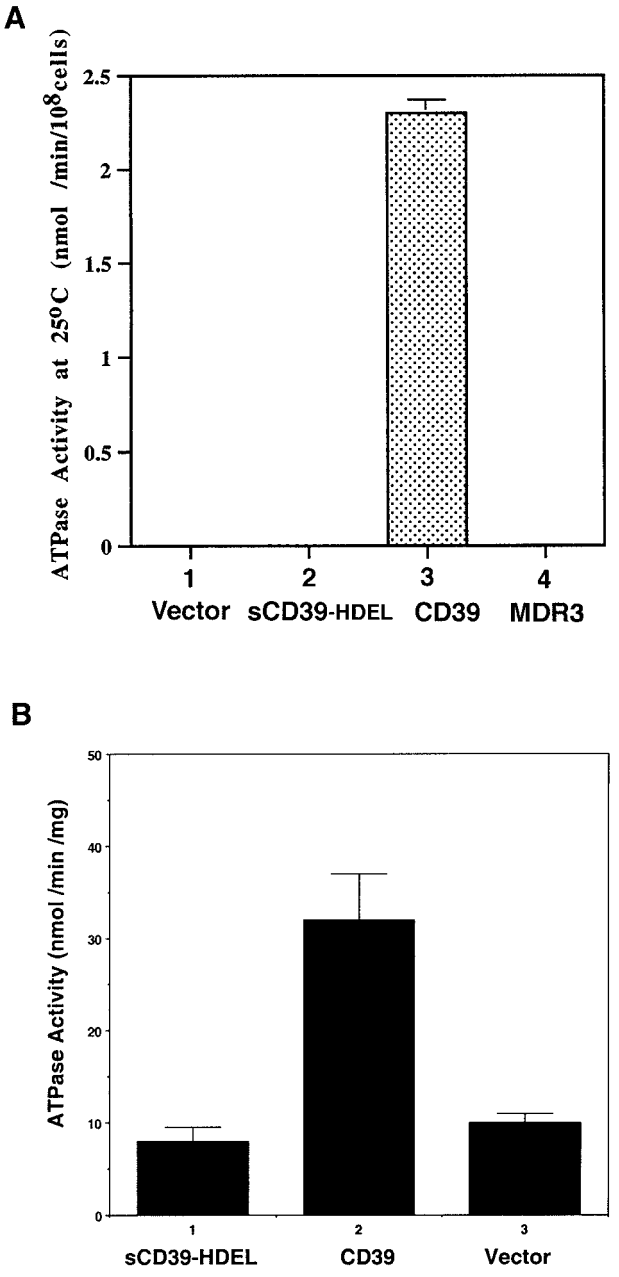

FIG. 4. **Wild type CD39, but not sCD39-HDEL, has enzymatic activity when expressed in yeast.** *A*, Wild type CD39 expressed in yeast is active and reaches the cell surface. Overnight cultures (at 30 °C in tryptophan or uracil drop-out medium) of BCY123/pG1, BCY123/pGZ131, BCY123/pGZ155, and BCY123/pVT-mdr3 yeast strains were washed with the assay buffer (20 mM Tris-HCl, pH 7.4, 120 mM NaCl, 5 mM KCl, 1 mM EGTA, 1 mM NaN_3, 1 mM Na_3VO_4) and resuspended in the same buffer with 2 mM ATP and ± 5 mM $CaCl_2$. The surface activity assay was done at room temperature for 30 min. *B*, sCD39-HDEL expressed in yeast is not active. The Ca^{2+}-stimulated ecto-ATPase activity of yeast crude membranes was measured at room temperature as described under "Materials and Methods" with 25 μg of membrane proteins from BCY123/pG1, BCY123/pGZ131, and BCY123/pGZ155. All of the values are the means ± S.D. (n = 4).

from cells with control vector, indicating that both sCD39 and sCD39-KDEL were not significantly active in the crude membranes. On the contrary, the activity of sCD39 secreted from cells was more than 20 times higher than that of sCD39 in the crude membranes, even though both samples contained similar amounts of sCD39 (Fig. 5, compare *A* with *B*). No such activity was observed in the sample collected from the extracellular medium of the cells transfected with sCD39-KDEL. These data

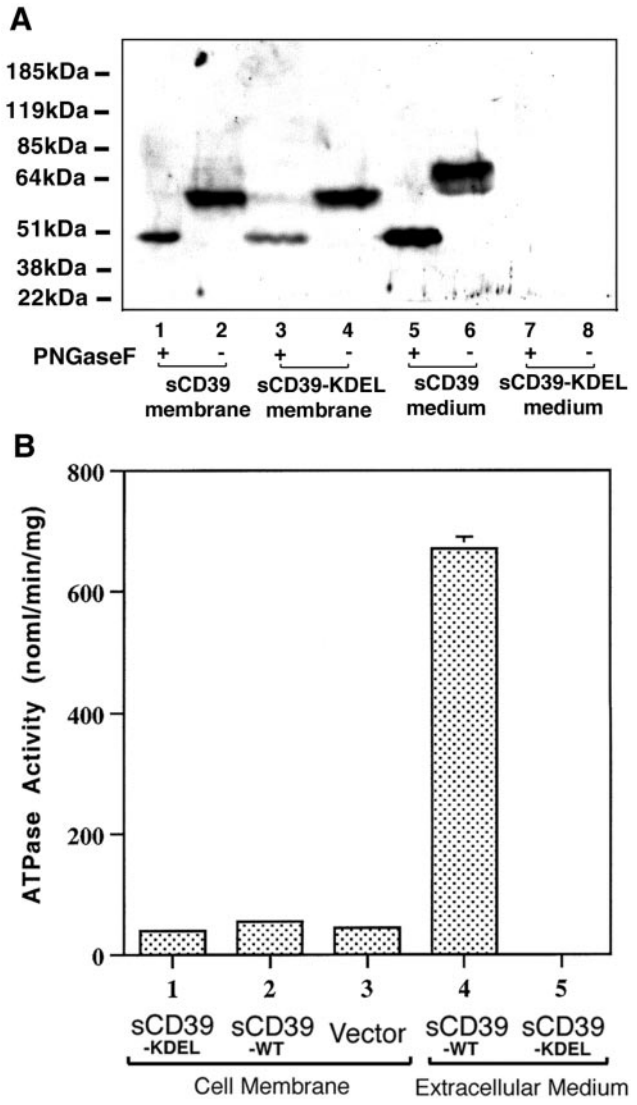

FIG. 5. **Only secreted sCD39 exhibits activity when expressed in COS-7 cells.** *A*, expression of sCD39 and sCD39-KDEL in COS-7 cells. 25 μg of membrane proteins and 10 μg of secreted glycoproteins not treated or treated with glycopeptidase F (*PNGaseF*) were separated by 7.5% SDS-PAGE and examined by immunoblotting with anti-CD39 antibody. *B*, Ca^{2+}-stimulated ecto-ATPase activity of COS-7 crude membranes and secreted glycoproteins isolated from the extracellular medium. 2.5 μg of proteins were used for the Ca^{2+}-stimulated ecto-ATPase activity assay described under "Materials and Methods." The assays were done at 37 °C for 25 min. All of the values are the means ± S.D. (n = 4).

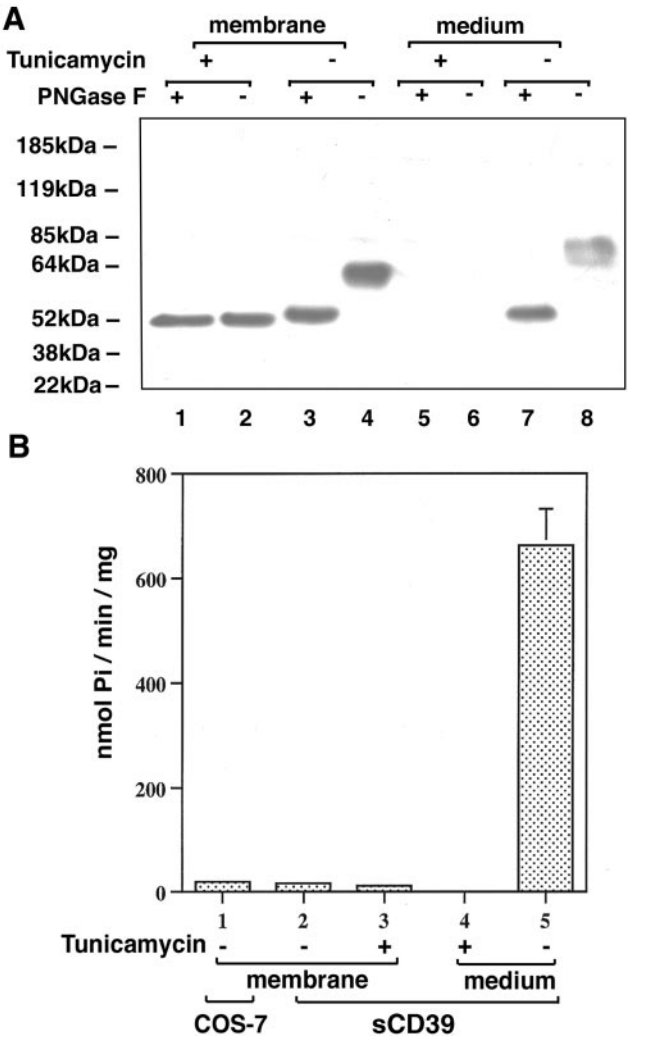

FIG. 6. **Without glycosylation, soluble CD39 expressed in COS-7 cells is retained intracellularly and is inactive.** *A*, expression of sCD39 in COS-7 cells treated with or without tunicamycin. As described under "Materials and Methods," membrane fractions and secreted proteins in the medium were isolated from COS-7 cells transfected with sCD39 cDNA, which were incubated with tunicamycin (5 μg/ml) or Me_2SO. 20 μg of membrane proteins and 30 μg of secreted proteins, not treated or treated with glycopeptidase F (*PNGase F*), were separated by 7.5% SDS-PAGE, and examined by immunoblotting with anti-CD39 antibody. *B*, Ca^{2+}-stimulated ecto-ATPase activity of COS-7 crude membranes and secreted glycoproteins isolated from the extracellular medium. 5 μg of proteins were used for the Ca^{2+}-stimulated ecto-ATPase activity assay described under "Materials and Methods." The assays were done at 37 °C for 10 min. All of the values are the means ± S.D. (n = 4).

illustrate that sCD39 is active only when it is secreted from the cells. Because sCD39 cannot be retained within the plasma membrane because of the lack of transmembrane domains, sCD39 present in the crude membranes should presumably be trapped inside the intracellular compartments. Based on the results that sCD39 as well as sCD39-KDEL in the crude membranes were not active while the secreted sCD39 in the medium was active, these experiments further demonstrate that CD39 is not active intracellularly.

Without Glycosylation, Soluble CD39 Expressed in COS-7 Cells Is Retained Intracellularly and Is Inactive—In Fig. 5*A*, we noticed that secreted soluble CD39 migrates slower than intracellular sCD39 in SDS-PAGE (compare *lanes 2* and *4* with *lane 6*). When we treated these samples with *N*-glycopeptidase F, they all migrate to the same position (*lanes 1*, *3*, and *5*), indicating that the migration difference between secreted sCD39 and intracellular sCD39 is due to terminal modification of *N*-glycans. To determine whether *N*-glycosylation affects both enzymatic activity and cellular localization of sCD39, COS-7 cells transfected with sCD39 cDNA were treated with tunicamycin to prevent *N*-glycosylation. As shown in Fig. 6*A*, sCD39 in control cells is glycosylated as demonstrated by the treatment of these cells with *N*-glycopeptidase F (*lanes 3*, *4*, *7*, and *8*), and is secreted into the extracellular medium (*lanes 7* and *8*). On the contrary, sCD39 in cells treated with tunicamycin is not glycosylated (compare *lanes 1* and *2*), and is not found in the extracellular medium (*lanes 5* and *6*), indicating that sCD39 devoid of *N*-glycosylation cannot be properly targeted to the extracellular space. Consistently, both partially glycosylated and unglycosylated forms of intracellular sCD39 are inactive when ecto-ATPase activity is measured (Fig. 6*B*). Only secreted sCD39 in a fully glycosylated state is active. These data indicate that complete *N*-glycan modification is essential

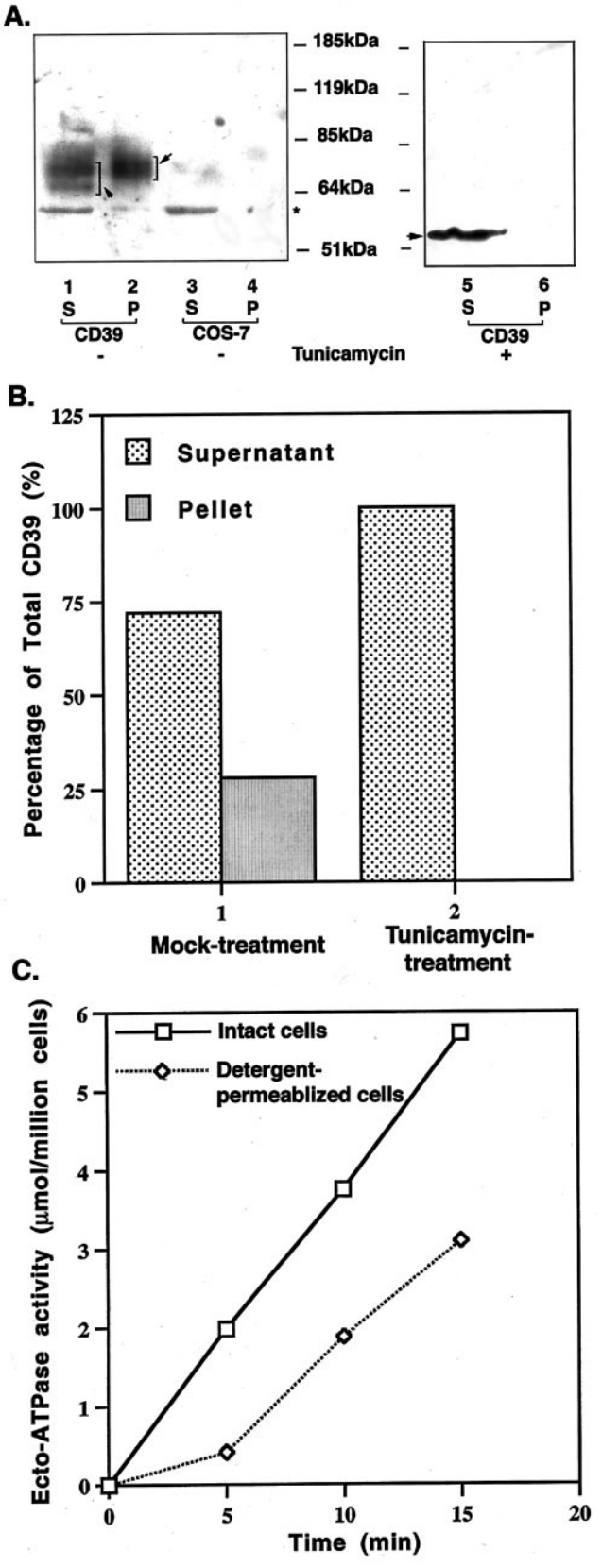

FIG. 7. **Only surface-expressed intact CD39 with full glycosylation is active in COS-7 cells.** *A*, surface biotinylation analysis of COS-7 cells transfected with CD39 cDNA. Intact cells treated with or without tunicamycin were biotinylated and precipitated with streptavidin-agarose as described under "Materials and Methods." Precipitates (100% of total) and supernatant after streptavidin precipitation (25% of total) were separated by 10% SDS-PAGE and examined by immunoblotting with anti-CD39 antibody. A control is also shown with COS-7 cells not transfected with CD39 cDNA (*lanes 3* and *4*). The *arrows* point to CD39-specific bands, whereas a *star* indicates a nonspecific band that was found in both transfected and control cells. *B*, Quantitative analysis of *A*. *C*, time course of Ca^{2+}-stimulated ecto-ATPase activity of intact or digitonin-permeablized COS-7 cells transfected with CD39 cDNA. The cells cultured and transfected in a six-well plate were washed gently with assay buffer containing 20 mM HEPES-Tris, pH 7.4, 120 mM NaCl, 5 mM KCl, 1 mM EGTA, 0.5 mM Na_3VO_4, and 1 mM NaN_3; then 500 μl of the same buffer containing 2 mM ATP with or without 5 mM $CaCl_2$ were added to start the reactions. The mixture was incubated at room temperature for the indicated time. 50 μl of the mixture solution was assayed for inorganic phosphate. Subsequently, the same cells were washed three times with the assay buffer, and 500 μl of the assay buffer containing 1% digitonin, 2 mM ATP with or without 5 mM $CaCl_2$ were added to start the reaction. The mixture was incubated at room temperature for the indicated time. 50 μl of the mixture solution was assayed for inorganic phosphate. All of the values are the means ± S.D. (n = 2). The standard deviations are the sizes of the symbols.

for both enzymatic activity and cellular localization of soluble CD39.

Only Surface-expressed Wild Type CD39 with Full Glycosylation Is Active in COS-7 Cells—To further examine whether intact CD39 is also affected by *N*-glycosylation with regard to both enzymatic activity and cellular localization, we performed surface biotinylation analysis of COS-7 cells transfected with CD39 cDNA, both with and without tunicamycin. The intact cells were biotinylated on the cell surface with a membrane-impermeable biotin conjugated cross-linker. Cell lysates were precipitated with streptavidin-agarose so that biotinylated surface-expressed CD39 (Fig. 7*A*, *lanes 2* and *6*) could be separated from intracellular CD39 remaining in the supernatant (*lanes 1* and *5*). As shown in Fig. 7 (*A* and *B*), ~28% of total CD39 in normal cells was precipitated by the streptavidin-agarose, whereas 72% of total CD39 was found in the supernatant (*lanes 1* and *2*). In contrast, all of the CD39 in tunicamycin-treated cells was found in the supernatant (*lane 5*), and no CD39 could be precipitated by the streptavidin-agarose (*lane 6*). No ecto-ATPase activity was observed from a total membrane preparation isolated from tunicamycin-treated cells (data not shown). These results are consistent with the data shown in Fig. 6, indicating that *N*-glycosylation is essential for both surface expression and enzymatic activity of intact CD39. Fig. 7*A* also shows that surface-expressed CD39 migrates slower than intracellular CD39 (compare *lane 2* with *lane 1*). This migration difference is due to *N*-glycosylation but not biotin labeling, as indicated by *N*-glycopeptidase F treatment (data not shown). To further determine whether only surface-expressed CD39 is active, we measured the ecto-ATPase activity of CD39 in both intact and digitonin-permeablized COS-7 cells transfected with CD39 cDNA (Fig. 7*C*). If intracellular CD39 were active, digitonin-permeablized cells should have a much higher activity than that of intact cells, given that the majority of CD39 is localized intracellularly (Fig. 7*B*). As shown in Fig. 7*C*, the ecto-ATPase activities of both intact cells and digitonin-permeablized cells are similar. In fact, the ecto-ATPase activity of the digitonin-permeablized cells was found to be 70% of that of intact cells. Control experiments showed that digitonin treatment of isolated membranse from cells expressing CD39 also reduced the ecto-ATPase activity to ~70% of that of untreated membranes (data not shown). This evidence further supports the conclusion that CD39 activity is found only at the cell surface.

DISCUSSION

In this study, we have provided multiple lines of evidence that the mammalian E-NTPDase CD39 is not active until it reaches the plasma membrane. First, the results of sucrose density fractionation of COS-7 membranes illustrated that although transfected CD39 primarily localized within the ER

fraction, CD39 activity localized to the plasma membrane fraction. Secondly, soluble CD39 is active when it is secreted into the extracellular medium, but it is not active when it is within the COS-7 cells. Thirdly, secretable soluble CD39 with an ER retention signal is not active in both COS-7 and yeast cells. Finally, overexpression of either CD39 or sCD39-HDEL in yeast does not cause a slow growth phenotype, even though CD39 is active in the yeast plasma membrane.

Furthermore, both wild type CD39 and soluble CD39 devoid of *N*-glycosylation are retained intracellularly and remain inactive. In addition, similar ecto-ATPase activities are observed for both intact and digitonin-permeablized cells transfected with CD39, suggesting that CD39 is normally only active at the cell surface. Thus, it is concluded that full *N*-glycosylation correlates with the activation of CD39 and is required for its surface localization.

Our findings are consistent with the notion that maintaining lumenal ATP concentrations within intracellular compartments is essential. Many steps in ER protein processing are catalyzed by lumenal ATP-dependent factors. The post-translational transfer of secretory protein precursors in yeast can only occur if a sufficient level of ATP is present inside the ER lumen (32, 33). Mammalian lumenal chaperone protein BiP and its yeast homolog Kar2p are crucial for ATP-dependent protein translocation (34–37). In addition, Kar2p and other ATP-dependent chaperones are critical for the proper folding of translocated protein inside the ER lumen and ensure that only correctly folded proteins proceed along the secretory pathway toward their final destination (38, 39). Golgi lumenal ATP is vital for the phosphorylation of secreted proteins and Golgi integral membrane proteins (40). Futile hydrolysis of this lumenal ATP by intracellular CD39 would presumably impair these biological processes. It seems necessary for biological systems to minimize CD39 activity during its processing and shipping in the ER and Golgi.

CD39 is a prototypical member of the rapidly expanding E-NTPDase family. Not only do some members localize to the plasma membrane or to the extracellular space (41–45), but there are also members that localize within the Golgi (23, 46–48) and the ER (49). Interestingly, most of these intracellular E-NTPDases, such as mammalian Golgi-UDPase (48), mammalian ER-UDPase (49), and yeast Golgi GDPase (46), are ecto-NDPases and possess little ecto-ATPase activity. The only reported Golgi E-NTPDase possessing high ecto-ATPase activity is the yeast protein Ynd1p (23, 47). However, the activity of this enzyme is normally down-regulated by Vma13p, a peripheral protein associated with the vacuolar H^+-ATPase (50). When this repressor is missing, Ynd1p activity increases drastically. Overexpression of Ynd1p causes a slow growth phenotype in such cells (50). These observations, together with the data in this paper, support the notion that ecto-ATPase activity present in the secretory pathway is toxic to cells and therefore is tightly regulated.

What are the potential mechanisms that ensure the inactive state of CD39 before its arrival to the plasma membrane? CD39 does not contain a cleavable propeptide sequence, although a limited extent of tryptic cleavage of intact CD39 on the cell surface has been reported that augments NTPDase activity by roughly 2-fold (13). The surrounding pH does not appear to be a major factor that affects CD39 activity in the intracellular compartments. CD39 retains ~50% of its activity at pH 6.5 (51), whereas the pH in the ER lumen is close to neutral (52), and the pH in the Golgi lumen was measured to be 6.45 (53). Palmitoylation within the NH_2 terminus of CD39 has been reported to be important both in its plasma membrane association and in its targeting to caveolae (15). However, this modification is not essential for the activation of CD39 because sCD39, which lacks the NH_2-terminal modification site, is active when it is secreted into the extracellular medium (Fig. 5*B* and Refs. 11 and 51).

Although the oligomeric state of CD39 in the intracellular compartments may have an effect on enzymatic activity, it can only be a partial effect because monomeric CD39 retains at least 10% of its oligomeric activity (11). Thus, even if CD39 were monomeric in the intracellular compartment, it would still have sufficient activity that would require further silencing.

The addition and processing of *N*-linked glycans in the ER and Golgi play a critical role in the biogenesis and quality control of many membrane proteins and secretory proteins (54). Whereas glycosylation can promote the proper folding and assembly of glycoproteins to increase their stability and trafficking, glycosylation is not required for the cell surface expression of many glycoproteins and secretory proteins (55–57). Previous work has shown that *N*-glycosylation is important for maintaining the enzymatic activities of HB6, another member of the E-NTPDase family (58). The data presented here demonstrate that *N*-glycosylation is essential for the surface localization of CD39 and correlates with the activation of the protein. Furthermore, intracellular sCD39 and sCD39-(H/K)DEL are partially glycosylated (Figs. 3*B* and 5*A*) within the ER and probably within the early compartment of the Golgi, because soluble ER-resident proteins with the (K/H)DEL signal are retrieved back to the ER from early Golgi compartments via (K/H)DEL receptors (29, 30). But these proteins are still not active. This suggests that terminal glycosylation of CD39 in the mid-Golgi or late Golgi compartments is critical for CD39 activity. It is possible that terminal glycosylation of CD39 is required for proper folding of the active enzyme.

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Mammalian Plasma Membrane Ecto-nucleoside Triphosphate Diphosphohydrolase 1, CD39, Is Not Active Intracellularly: THE N-GLYCOSYLATION STATE OF CD39 CORRELATES WITH SURFACE ACTIVITY AND LOCALIZATION

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