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

The Harvard community has made this article openly available. Please share how this access benefits you. Your story matters

Citation
Zhong, Xiaotian, Rajeev Malhotra, Rachel Woodruff, and Guido Guidotti. 2001. “Mammalian Plasma Membrane Ecto-Nucleoside Triphosphate Diphosphohydrolase 1, CD39, Is Not Active Intracellularly.” Journal of Biological Chemistry 276 (44): 41518–25. https://doi.org/10.1074/jbc.m104415200.

Citable link
http://nrs.harvard.edu/urn-3:HUL.InstRepos:41467410

Terms of Use
This article was downloaded from Harvard University’s DASH repository, and is made available under the terms and conditions applicable to Other Posted Material, as set forth at http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#LAA
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

Received for publication, May 15, 2001, and in revised form, September 6, 2001
Published, JBC Papers in Press, September 6, 2001, DOI 10.1074/jbc.M104415200

Xiaotian Zhong, Rajeev Malhotra, Rachel Woodruff, and Guido Guidotti‡

From the Department of Molecular and Cellular Biology, Harvard University, Cambridge, Massachusetts 02138

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-glycosylation 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-permeabilized 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)† to hydrolyze nucleoside tri- and diphosphates in the presence of divalent cations (usually Ca²⁺ or Mg²⁺) 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 NH₂-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-solubilized 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 luminal ATP and potentially inhibit other ATP-dependent luminal 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(supE44 dcmG lacU169 [80lacZDM15] hisd17 recA1 endA1 gyrA96 thi-1 relA1). The Saccharomyces cerevisiae strains used were BGY123 (MATa pep4::HIS3 prb1:: LEU2 bar1::HISG lys2::GAL1/10–GAL4 can1 ade2 trpl1 ura3 his3 leu2-
3,112) and YPH500 (MATa 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% CO₂ 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 Me₃SO was added immediately following transfection to the final concentration of 5 μg/ml. The control cells received Me₃SO alone. Crude membranes from 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 CaCl₂. Secreted glycoproteins were eluted with 2 ml of 10 mM HEPES (pH 7.4) were combined with 2.3 ml of 65% sucrose obtained from three 100-mm plates of COS-7 cells and placed in 0.7 ml of 10 mM HEPES buffered saline solution containing 10 mM CaCl₂ and eluted with 3 ml of Tris-buffered saline solution containing 0.5 x imidazole and 1 mM CaCl₂. 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 nucleotidease assays.

Nucleotide Phosphatase Assay, Protein Deglycosylation, Yeast Crude Membrane Preparation, and Immunoblotting—Nucleotide phosphatase activity was assayed as described by Wang et al. (111). 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 EDTA, 1 mM NaN₃, 1 mM Na₂VO₄, 0.2 mg/ml amethicin, 2 mM ATP) with or without 5 mM CaCl₂ at room temperature with yeast cells and at 37 °C with COS-7 cells. The Ca⁺⁺-stimulated ATPase activity was determined by measuring the inorganic phosphate released and by subtracting values obtained without CaCl₂ from those with 5 mM CaCl₂. Yeast crude membranes were prepared as described previously (23). "Flavobacterium menegesicpticum" 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 column was then washed with 20 ml of Tris-buffered saline solution containing 1 mM CaCl₂ and eluted with 3 ml of Tris-buffered saline solution containing 0.5 x imidazole and 1 mM CaCl₂. 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 nucleotidease assays.

Cell surface biotinylation and streptavidin precipitation—The procedure was modified as described by Feraillle 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 biotinylination buffer (0.25 mg/ml EZ-link sulfo-NHS-LC-biotin (Pierce) in PBS buffer). After removal of the biotinylatin 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 EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 200 μl of protein extract were added to 100 μl of streptavidin-garose beads (Immunopure immobilized streptavidin; Pierce) diluted in the precipitation buffer (0.1% digitonin, 20 mM HEPES/KOH, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml antinstead, 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.
CD39 Is Not Active Intracellularly

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 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 ecto-5’-nucleotidase 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. 3A, the transformants of these three plasmids generated yeast colonies of similar size after 3 days of incubation on tryptophan drop-out 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. 3B illustrates that 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
CD39 is functionally expressed at the plasma membrane in BCY123/pVT-MDR3. These results suggest that wild type surface of BCY123/pG1, BCY123/pGZ155 (sCD39-HDEL), and buffer. As expected, no such activity was detectable on the same as the activity found in the control (Fig. 4B). Crude membranes containing sCD39-HDEL was almost the 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. 4B). 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 luminal 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. Immunoblotting (data not shown) after the membrane purification was 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. 5B, the activities of both crude membranes containing luminal sCD39 and crude membranes with sCD39-KDEL were similar to the background activity of the crude membranes 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 much 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...
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 and secreted glycoproteins isolated from the extracellular medium. 2.5 μg of proteins were used for the Ca<sup>2+</sup>-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).

Without Glycosylation, Soluble CD39 Expressed in COS-7 Cells Is Retained Intracellularly and Is Inactive—In Fig. 5A, 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, which were incubated with tunicamycin (5 μg/ml) or Me<sub>2</sub>SO, 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<sup>2+</sup>-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<sup>2+</sup>-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).
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. 7A, 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. 7A 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-permeabilized COS-7 cells transfected with CD39 cDNA (Fig. 7C). If intracellular CD39 were active, digitonin-permeabilized cells should have a much higher activity than that of intact cells, given that the majority of CD39 is localized intracellularly (Fig. 7B). As shown in Fig. 7C, the ecto-ATPase activities of both intact cells and digitonin-permeabilized cells are similar. In fact, the ecto-ATPase activity of the digitonin-permeabilized cells was found to be 70% of that of intact cells. Control experiments showed that digitonin treatment of isolated membrane 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 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-permeabilized 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_{3}VO_{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.

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-permeabilized 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_{3}VO_{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.
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-permeabilized 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 luminal ATP concentrations within intracellular compartments is essential. Many steps in ER protein processing are catalyzed by luminal 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 luminal 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 luminal ATP is vital for the phosphorylation of secreted proteins and Golgi integral membrane proteins (40). Futile hydrolysis of this luminal 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 prepeptide 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 NH2 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 NH2-terminal modification site, is active when it is secreted into the extracellular medium (Fig. 5B 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. 3B and 5A) 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.

REFERENCES

1. Dubyk, G. R., and el-Mostassim, C. (1993) Am. J. Physiol. 265, C577–C606
2. Zimmermann, H. (1994) Trends Neurosci. 17, 420–426
3. Brake, A. J., and Julius, D. (1996) Annu. Rev. Cell Dev. Biol. 12, 519–541
4. Plesner, L. (1995) Int. Rev. Cytol. 158, 141–214
5. Handa, M., and Guidotti, G. (1996) Biochem. Biophys. Res. Commun. 218, 916–923
6. Maliszewski, C. R., Dellespesse, G. J., Schoenborn, M. A., Armitage, R. J., Fanold, W. C., Nakajima, T., Baker, E., Sutherland, G. R., Poidexter, K., Birks, C., et al. (1994) J. Immunol. 153, 3574–3583
7. Wang, T. F., and Guidotti, G. (1996) J. Biol. Chem. 271, 9898–9901
8. Kasznarek, E., Kozak, K., Sevigny, J., Siegel, J. B., Anrather, J., Beaudoin, A. R., Bach, F. H., and Robson, S. C. (1996) J. Biol. Chem. 271, 33116–33122
9. Marcus, A. J., Brockman, M. J., Drossopoulos, J. H., Islam, N., Alynynycheva, T. N., Saifer, L. B., Harmata, K., Pomsett, D. N., Schoenborn, M. A., Schookey, K. A., Gayle, R. B., and Maliszewski, C. R. (1997) J. Clin. Invest. 99, 1341–1360
10. Enjyoji, K., Sevigny, J., Lin, Y., Fenrette, P. S., Christie, P. D., Esch, J. S., Sh packin, J., Safi, M., Beaudoin, K., Imai, M., Edelberg, J. M., Rayburn, H., Lech, M., Beeler, D. L., Caizmadia, E., Wagner, D. D., Robson, S. C., and Rosenberg, R. D. (1999) Nat. Med. 5, 1010–1017
11. Wang, T. F., Ou, Y., and Guidotti, G. (1998) J. Biol. Chem. 273, 24814–24821
12. Wang, T. F., Rosenberg, P. A., and Guidotti, G. (1997) Mol. Brain Res. 47, 295–302
13. Schulte am Esch, J., Sevigny, J., Kasznarek, E., Siegel, J. B., Imai, M., Kozak, K., Beaudoin, A. R., and Robson, S. C. (1999) Biochemistry 38, 2248–2258
14. Robson, S. C., Daoud, S., Begin, M., Cote, Y. P., Siegel, J. B., Bach, F. H., and Beaudoin, A. R. (1997) Blood Coagul. Fibrinolysis 8, 21–27
15. Kozak, K., Kasznarek, E., Kittel, A., Sevigny, J., Bluzstajn, J. K., Schulte am Esch, J., Imai, M., Guckelberger, O., Goepfert, C., Qawi, I., and Robson, S. C. (2000) J. Biol. Chem. 275, 2057–2062
16. Quemeneur, G., Guthapfel, R., and Guesguen, P. (1994) J. Biol. Chem. 269, 5455–5458
17. Pfeffer, S. R., and Rothman, J. E. (1987) Annu. Rev. Biochem. 56, 829–852
18. Munro, S., and Pelham, H. R. (1986) J. Cell Biol. 104, C606–C611
19. Braakman, I., Helenius, J., and Helenius, A. (1992) Nature 356, 260–262
20. Sherman, F., and Hicks, J. B. (1986) Methods in Enzymology, pp. 160–175, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
21. Miller, J. H. (1992) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
22. Ceppi, M. V., and Guidotti, G. (1997) Arch. Biochem. Biophys. 346, 312–321
23. Fauci, D. R., Gabbay, H., and Marini, P. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
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
Xiaotian Zhong, Rajeev Malhotra, Rachel Woodruff and Guido Guidotti

J. Biol. Chem. 2001, 276:41518-41525.
doi: 10.1074/jbc.M104415200 originally published online September 6, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M104415200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 55 references, 25 of which can be accessed free at
http://www.jbc.org/content/276/44/41518.full.html#ref-list-1