Palmitoylation Targets CD39/Endothelial ATP Diphosphohydrolase to Caveolae*

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Ectonucleotidases influence purinergic receptor function by the hydrolysis of extracellular nucleotides. CD39 is an integral membrane protein that is a prototype member of the nucleoside 5’-triphosphate diphosphohydrolase family. The native CD39 protein has two intracytoplasmic and two transmembrane domains. There is a large extracellular domain that undergoes extensive glycosylation and can be post-translationally modified by limited proteolysis. We have identified a potential thioester linkage site for S-acylation within the N-terminal region of CD39 and demonstrate that this region undergoes palmitoylation in a constitutive manner. The covalent lipid modification of this region of the protein appears to be important both in plasma membrane association and in targeting CD39 to caveolae. These specialized plasmalemmal domains are enriched in G protein-coupled receptors and appear to integrate cellular activation events. We suggest that palmitoylation could modulate the function of CD39 in regulating cellular signal transduction pathways.

The vascular ATP or NTP diphosphohydrolase (ATPDase or NTPDase; EC 3.6.1.5), now known to be CD39, is a plasma membrane-bound enzyme that plays the dominant role in the hydrolysis of extracellular tri- and/or diphosphate nucleotides in blood (1, 2). Our recent data from cd39-null mice indicate that this specific ectonucleotidase also plays a pivotal role in the regulation of an ADP-purinoreceptor P2Y1 function; absence of ATPDase activity results in desensitization of this G protein-coupled receptor with profound effects on hemostasis and thromboregulation (3).

Established topological models of CD39 suggest the presence of two transmembrane domains at both termini of the molecule and an extracellular loop containing a central hydrophobic region (1, 4, 5). The transmembrane domains of ATPDases appear to influence the formation of detergent-sensitive mul- timers (6). Examination of CD39 amino acid sequences reveals a total of 11 cysteine (Cys) residues, with an unpaired Cys13 contained within the intracellular N terminus of the protein (4). Analysis of the CD39 sequence, by a computer algorithm PROSITE, further indicates six putative N-glycosylation sites with several potential casein kinase, cAMP/cGMP-dependent protein kinase or protein kinase C phosphorylation sites (7, 8).

We have described several post-translational modifications of CD39. There are differences in the extent of glycosylation of human CD39 in endothelial cells, platelets, and leukocytes (9). Effects of limited serine proteolysis of native CD39 on ATPDase activity have been documented (5), and there is also a propensity for CD39 to undergo autophosphorylation reactions (data not shown). In addition, we have shown that cellular interactions with free fatty acids modulate ATPDase enzymatic activity in vitro and have postulated that acylation could also influence CD39 structure (10). Here we study this possibility and specifically examine palmitoylation, a reversible and potentially regulated post-translational modification, in which the 16-carbon saturated fatty acid is attached to intracytoplasmic cysteine residues via high energy thioester linkages (11).

Generalized functions for palmitoylation have not been established but many palmitoylated proteins serve as signaling molecules (G protein subunits, β-adrenergic receptors, nonreceptor tyrosine kinases, and others) (12, 13). The function of lipid modification of at least some proteins is believed to facilitate membrane association and therefore regulate distribution between plasma membrane and cytoplasm (13–16). There is additional evidence indicating that palmitoylation might direct proteins to caveolae; this may be of relevance as these plasmalemmal microdomains have been implicated in the compartmentalization of signaling molecules (17, 18).

We demonstrate here that CD39 undergoes constitutive palmitoylation within the N-terminal intracytoplasmic region that contains only one potential site for thioester linkage, Cys13. This process is associated with strengthened membrane interaction and preferential targeting of the native protein to caveolae. This observation may be pertinent to differential functions, membrane expression and regulation of the increasing number of cell-associated or soluble CD39 family members (8, 19–21).

This paper is available online at http://www.jbc.org

* This work was supported in part by National Institutes of Health Grants R01 HL57307 (to S. C. R.) and P01 AG099525 (to J. K. B.) and American Heart Association Grant-in-aid 9650490N (to S. C. R.). A poster demonstrating the caveolar localization of CD39 was presented by Agnes Kittel et al at the 2nd International Workshop on Ecto-ATPases and Ectonucleotidases, Diepenbeek, Belgium in June 1999. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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§ Recipient of studentships from the Heart and Stroke Foundation of Canada and “Fonds pour la Formation de Chercheurs et l’Aide à la Recherche du Québec.”

** Funded by a postdoctoral fellowship from the Deutsche Forschungsgemeinschaft.

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1 The abbreviations used are: ACR, apyrase conserved regions; ATP-Dase (or NTPDase), ATP (or NTP) diphosphohydrolase (now known to be CD39); EC, endothelial cell; mAb, monoclonal antibody.
EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Anti-human CD39 (BU61) monoclonal antibody (mAb) was purchased from AnCell (Bayport, MN). Anti-mouse and anti-rabbit IgG-fluorescein isothiocyanate conjugates, as well as monoclonal mouse M2 anti-FLAG, were purchased from Sigma; peroxidase-conjugated anti-rabbit antibodies were purchased from Pierce. The rabbit polyclonal antibodies used included KY102/130 directed at the carboxyl terminus of the apyrase conserved region 2 and RO2092217 to apyrase conserved region 4; both have been found to react with human CD39/ATPDase expressed by COS-7 transfectants (5). All biochemical reagents were from Sigma unless otherwise specified and of highest grade available.

Cell Culture—Human umbilical vein endothelial cells (HUVECs) from fresh umbilical veins were cultured in M199 with 20% fetal calf serum, 200 U/ml penicillin, 0.2 KIU/ml aprotinin, 100 mg/ml streptomycin, 50 mg/ml gentamicin, 0.5% tryptamine (50 mg/ml/ml), and penicillin (100 mg/ml/ml) and endothelial cell growth factor (50 mg/ml/ml) (BioWhittaker, Walkersville, MD), and COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum. Both media were supplemented with l-glutamine (2 mM), penicillin G (100 units/ml), and streptomycin (100 mg/ml). All cells were grown in culture dishes at 37 °C in a humidified incubator with a 5% CO2 atmosphere. Cell lysates were clarified by centrifugation at 10,000 g for 10 min at room temperature. COS-7 cells, at 80% confluence, were transfected using LipofectAMINE (Life Technologies, Inc; 9 μl/g/cm2) for 30 min at room temperature. COS-7 cells, at 80% confluence, were transfected with pcDNA3 vector containing native, N- or C-truncated CD39 cDNA inserts at 50 ng/cm2 of the culture plate area) was incubated with LipofectAmine (Life Technologies, Inc; 3 μg/cm2) for 30 min at room temperature. COS-7 cells, at 80% confluence, were transfected with the DNA/lipofectamine mixture for 5 h at 37 °C in serum-free medium. The transfection was terminated by adding 6 ml of Dulbecco’s modified Eagle’s medium containing 20% fetal calf serum. Medium was changed after 48 h and cells were harvested for assays after 72 h. Both control COS-7 cells used for transfection and empty vector transfected cells were negative for CD39 as analyzed by Western blotting and flow cytometry with anti-CD39 mAb; in both controls ATPDase activity was negligible.

Native and Mutant CD39 Expression Vectors—For expression of native recombinant CD39 in COS-7 cells, an identical approach to that described previously was utilized (1). Each truncated mutation was generated by polymerase chain reaction using 0.25 μg of a CD39-pcDNA3 vector, described previously (5). Polymerase chain reaction products were gel-cleaned, digested, and ligated into poly linker of the vector utilizing a BamHI site at the 5′-end and a XhoI site at the 3′-end. C-terminal truncated CD39 as well as N- and C-terminal truncations that removed the transmembrane and intracytoplasmic domains at either site. The pcDNA3 vectors were utilized for the transmembrane domain truncations and the FLAG-tagged native protein. To assure attachment of a FLAG tag in C-terminal truncated forms and FLAG-tagged CD39, the antisense primer used for polymerase chain reaction included the nucleotide sequence coding for FLAG. Constructs in expression vectors were sequenced using an Applied Biosystems 373 Fluorescent DNA Sequencer (Perkin-Elmer, Foster City, CA) using SP6 and T7 primers as well as custom sequencing primers (5).

Cell Labeling and Immunoprecipitation—Forty-eight h post-transfection, COS-7 cells were washed with serum-free Dulbecco’s modified Eagle’s medium and then incubated with 0.4 μCi/ml of [9,10-3H]myristic acid and [1-14C]palmitic acid. Cell lysates were preincubated with 1-b-h incubation with 10 μl of protein A/G-agarose at 4 °C. Labeled CD39 was immunoprecipitated with either site. The pcDNA3 vectors were utilized for the transmembrane domain truncations and the FLAG-tagged native protein. To assure attachment of a FLAG tag in C-terminal truncated forms and FLAG-tagged CD39, the antisense primer used for polymerase chain reaction included the nucleotide sequence coding for FLAG. Constructs in expression vectors were sequenced using an Applied Biosystems 373 Fluorescent DNA Sequencer (Perkin-Elmer, Foster City, CA) using SP6 and T7 primers as well as custom sequencing primers (5).

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ersham Pharmacia Biotech, Buckinghamshire, UK), 1 mM ouabain (Na+/K+ -ATPase inhibitor; Merck, Darmstadt, Germany), 50 μM α,β-methylene-ADP (5′-nucleotidase inhibitor) and KCl (5 mM), in Tris-maleate buffer (70 mM, pH 7.4) for 30 min at 37 °C. This incubation was post-fixed for 30 min in 1% OsO4 (Taab, Aldermaston, Berkshire, UK) followed by three rinses in Tris-maleate buffer, and samples were then dissolved in cacodylate buffer, dehydrated, and embedded in Epon (Fluka, Buchs, Switzerland). Ultra-thin sections were cut and examined in a Hitachi 2001 transmission electron microscope (Hitachi Corp., Tokyo, Japan). To demonstrate the specificity of the reaction product, in control experiments, the relevant substrate (ATP or ADP) was omitted from the incubation medium.

Immunostaining with Anti-CD39—Fixation was carried out as above and after rinsing with PBS, HUVECs or transfected cells were blocked with 5% control human serum in PBS for 30 min and incubated overnight with the antibodies (CD39 mAb in 1:500 dilution) on a shaking plate at 4 °C. The Vectastain ABC system was used according to the manufacturer’s instruction (Vector Lab, Burlinghame, CA). Post-fixation, embedding, and electron microscopic investigations were performed as for enzymatic histochemical staining.

Membrane Association of CD39 and Mutants—Aliquots of conditioned supernatant fluids were collected from all transfected COS-7 cells, ultracentrifuged for 60 min at 100,000 × g and 4 °C. Supernatants were then freeze-dried and concentrated specimens dissolved by boiling in 2 × nonreducing Laemmli sample buffers. Samples (equivalents of 100 μl of conditioned medium) were analyzed by Western blotting with mAb to CD39, and bands were visualized with ECL as described above.

RESULTS

Palmitoylation of CD39 and Mutant Forms—Following incubation of transfected COS-7 cells with [3H]palmitic acid, anti-CD39 (or anti-FLAG; not shown) mAb was used to immunoprecipitate radiolabeled proteins from cell lysates. The subsequent analysis of the immunoprecipitated protein by Western blotting with BU61 and other anti-CD39 established that the transfected COS-7 cells expressed the tagged CD39, N- (Δ1–37) and C-truncated (Δ477–510) proteins at near equivalent levels (Fig. 1A).

In parallel, we also observed, by autoradiography, radioactive signals indicating incorporation of the [3H]palmitic acid label into native CD39 and the C-truncated mutant, but not the N-truncated protein (Fig. 1B). There were no radiolabeled bands detected for empty vector transfected cells (data not shown). Palmitoylation of CD39 expressed in HUVECs was also demonstrated (data not shown). These results suggested that the Cys13 present within the intracytoplasmic domain at the N terminus of CD39 underwent palmitoylation.

However, palmitic acid can be metabolized into myristate by cells in culture (15). Therefore, to confirm the nature of the protein acylation, CD39-transfected cell lysates were treated with either 1 mM hydroxylamine, pH 7.5, to cleave thioester bonds linking fatty acid (palmitic acid or its putative metabolite, myristic acid) to the protein backbone or with 1 mM Tris-HCl, pH 7.5, as the parallel control. Hydrolyzed fatty acids were then extracted into chloroform:methanol:water (2:1:1), dried under nitrogen, and analyzed by C-18 reverse phase TLC (Fig. 2). The positions of standards ([3H]myristic acid (MA) and [3H]palmitic acids (PA)) and test samples were determined after development in acetic acid:acetoniitrile (1:1). Both standard fatty acids and other labeled membrane lipids were detected and readily separated by TLC; evidence of conversion of the labeled palmitic acid to myristate in vitro was demonstrated by analysis of the total transfected cell lysate fractions hydrolyzed with either hydroxylamine or incubated with Tris-HCl (Fig. 2A and B, respectively).

We further evaluated the specific nature of modification of CD39 by performing first the immunoprecipitation with anti-CD39 followed by incubation of the entire product with either 1 mM hydroxylamine or 1 mM Tris-HCl, as described above. Examination of radiolabeled released fatty acids by TLC clearly identified that palmitic acid was incorporated into CD39, confirming that the endonucleotidease was only subject to palmitoylation. This acylation appeared to be via thioester linkages to the Cys13 within the N-terminal domain (Fig. 2C). In the parallel control experiment, no palmitic acid was released (Fig. 2D).

Membrane Association of CD39 and Truncated Mutants—We then investigated the membrane association of CD39 and mutants to evaluate potential release of the truncated proteins.
CD39 Palmitoylation

Fig. 3. Western blot analysis of conditioned medium. COS-7 cells were transiently transfected with pcDNA3-CD39, N- or C-truncated CD39 mutants. Only the N-terminal truncated mutant protein was detected in a soluble state in the concentrated conditioned medium of transfected cells.

from the cell membrane. Conditioned media free of cellular debris were collected from COS-7 cultures transfected with native, N and C terminus truncated mutants of CD39. The deletion of the N-terminal region of CD39 and consequent lack of acylation resulted in some release of this mutant to the medium, unlike the native protein or C-terminal truncated mutant that were strictly cell-associated (Fig. 3). We estimated that less than 10% of the immunoreactive N-terminal mutant antigen was solubilized (not shown).

However, this observation suggested that the N terminus might play some regulatory role in the attachment of CD39 to the cell membrane. We further examined the effects of mutagenesis and deletion of the intracytoplasmic domains on CD39 membrane distribution patterns by additional immunohistochemistry and cytochemical studies.

Electron Microscopy and Cytochemistry—Functional CD39 expression was determined by visualization of CD39 and mutant ectonucleotidase ATPase activity in transiently transfected COS-7 cells. Empty vector-transfected cells had no specific ATPase activity (Fig. 4A). The pattern of membrane expression of native recombinant CD39 was remarkable in that ATPase activity was largely concentrated in plasmalemmal microdomains, highly suggestive of caveolar structures (Fig. 4B), and in keeping with our prior morphological observations (26). The C-terminal truncated mutant also appeared to be enriched in the caveolae, whereas the N-terminal mutant did not localize in these domains and was only observed in decreased amounts within the plasma membrane (Fig. 4, C and D, respectively).

HUVECs were also shown to express high levels of immunoreactive and enzymatically active CD39 in caveolae-like structures (Fig. 5, A and B). We have previously demonstrated that antibodies to CD39 and caveolin both react with these vesicles, confirming specific localization of CD39 within caveolae (26). A similar distribution of CD39-antigen and associated specific ATPase activity was shown in stable transfectants expressing CD39 (Fig. 5, C and D, respectively).

DISCUSSION

In this manuscript, we demonstrate that CD39 could undergo a process of S-acylation and show that labeled free palmitic acid could associate with the native protein by a thioester bond, as observed for other membrane proteins (12, 13, 15). Truncated forms of CD39 lacking the N-terminal intracytoplasmic region, and the associated Cys<sup>13</sup> residue (5), were not subject to palmitoylation. This process of palmitoylation in the native protein appeared to be constitutive and to contribute to the integral membrane association of this ectonucleotidase. Thus, a small proportion of this N-terminal truncated CD39 protein was also found in conditioned medium of transfected cells in a soluble form (Fig. 3). The apparent targeting of CD39 to caveolae also appeared linked to this post-translational modification as the mutant lacking this potential palmitoylation site was not detected in these plasmalemmal microdomains (Fig. 4D).

Because the hydrolysis of palmitic acid from CD39 could occur in an alkaline environment, it was presumed that the linkage was a thioester bond (15, 27). We found no evidence for myristoylation of CD39 (Fig. 2C), and there are no putative N-terminal glycine residues that would be accessible for amide N-acylation linkages (4). Hence, unlike the majority of acylated membrane proteins, palmitic acid substitution of CD39 did not require prior modification by myristoyl groups. Nor was there any potential for prenylation at the C-terminal region, given the absence of strict consensus sequences in this region (13). Other proteins that are only palmitoylated are generally targeted to the inner surface of the plasma membrane and include G<sub>i</sub>, family members (12, 14) and neuronal proteins, e.g. neuromodulin and SNAP-25 (11).

In general, the functional significance of palmitoylation remains unclear. In part, this relates to technical limitations with respect to the generation of mutants, that preclude palmitoylation by conversion of Cys residues or by deletion mutagenesis. These changes may also influence protein structure <i>ab initio</i> (13). However, alternative approaches are limited in that proposed inhibitors of palmitoylation, such as tunicamycin (28), would be coconitent inhibitors of CD39 glycosylation (5). Given this caveat, it remains feasible that palmitoylation could influence the association of CD39 with the cell membrane (29).
The biological properties of caveolae appear to bolster these speculations. These plasma-membrane microdomains have the potential to compartmentalize signaling responses and are enriched in proteins intimately associated with the regulation of calcium flux and signaling pathways (38). Our data imply that both CD39 antigen and the associated ATPase activity are associated with caveolae (Figs. 4 and 5). The targeting of palmitoylated CD39 to caveolae could influence defined G protein-coupled receptors within this plasma-membrane microenvironment (17).

We show that the prototype CD39 family member undergoes palmitoylation at the N-terminal intracytoplasmic domain and is also targeted to caveolae. This post-translational modification would not be anticipated in CD39L1 but may also occur in other CD39-like proteins, e.g. CD39L3 has putative palmitoylation sites within both the N- and C-intracytoplasmic regions (20, 39). Other members of the CD39 family have only a single transmembrane sequence at the N terminus that, in the case of the macrophage-associated CD39L4, undergoes cleavage to result in a soluble enzyme (21). How any of these modifications influence the pathophysiological function(s) of the CD39/E-NTPDase family members remains to be determined.

Acknowledgments—We thank Dr. Susan Hagen for the access to electron microscopy equipment at the Center for Advanced Microscopy, Beth Israel Deaconess Hospital and for her generous help and advice. We thank Eva Csizmadia for endothelial cell culture and Piotr Kaczmarek for preparation of illustrations.

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