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Cholesterol-dependent Lipid Assemblies Regulate the Activity of the Ecto-nucleotidase CD39*

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CD39 (ecto-nucleoside triphosphate diphosphohydrolase-1; E-NTPDase1) is a plasma membrane ecto-enzyme that regulates purinergic receptor signaling by controlling the levels of extracellular nucleotides. In blood vessels this enzyme exhibits a thromboregulatory role through the control of platelet aggregation. CD39 is localized in caveolae, which are plasma membrane invaginations with distinct lipid composition, similar to dynamic lipid microdomains, called rafts. Cholesterol is enriched together with sphingolipids in both rafts and caveolae, as well as in other specialized domains of the membrane, and plays a key role in their function. Here, we examine the potential role of cholesterol-enriched domains in CD39 function. Using polarized Madin-Darby canine kidney (MDCK) cells and caveolin-1 gene-disrupted mice, we show that caveolae are not essential either for the enzymatic activity of CD39 or for its targeting to plasma membrane. On the other hand, flotation experiments using detergent-free or detergent-based approaches indicate that CD39 associates, at least in part, with distinct lipid assemblies. In the apical membrane of MDCK cells, which lacks caveolae, CD39 is localized in microvilli, which are also cholesterol and raft-dependent membrane domains. Interfering with cholesterol levels using drugs that either deplete or sequester membrane cholesterol results in a strong inhibition of the enzymatic and anti-platelet activity of CD39. The effects of cholesterol depletion are completely reversed by replenishment of membranes with pure cholesterol, but not by cholestenone. These data suggest a functional link between the localization of CD39 in cholesterol-rich domains of the membrane and its role in thromboregulation.

CD39 (also known as ecto-nucleoside triphosphate diphosphohydrolase-1, ATP diphosphohydrolase, E-NTPDase1, EC 3.6.1.5) is a plasma membrane ecto-nucleotidase that hydrolyzes tri- and diphosphonucleosides (including ATP and ADP) but not monophosphonucleosides. It plays an important role in regulating the extracellular levels of nucleotides, thereby controlling purinergic receptor signaling (1–3). CD39 is mainly expressed in endothelial cells and has been shown to play a crucial role in thromboregulation through inhibition of platelet aggregation by hydrolyzing ADP, a platelet aggregator (for a review see Ref. 4). Detailed localization studies based on CD39 enzymatic assays (5–9) and immunochemistry (6, 8, 10) have shown that this enzyme is localized in characteristic flask-shaped invaginations of the plasma membrane, called caveolae.

Although caveolae have been implicated in many cellular functions such as endocytosis, transcytosis, and cholesterol transport, most attention has been focused on their role in signal transduction (11, 12). Caveolar pits are formed by polymerization of caveolin-1 with lipid-assemblies, called rafts, which are cholesterol- and glycosphingolipid-enriched microdomains (12, 13). Both caveolae and rafts depend on the presence of cholesterol, because removal of this component from membranes disrupts their structure and function (11, 12). Therefore, caveolae are considered as a specialized type of raft responsible for spatial and temporal compartmentalization of signaling complexes at the plasma membrane and for regulation of signal transduction initiation. This is achieved by allowing only a subset of molecules of the plasma membrane to be partitioned into caveolae and by excluding others (11–13). Association of a protein with lipid assemblies is believed to be the driving mechanism for sorting into caveolae (14, 15). Transmembrane domains, together with palmitoylation, are among the structural features that are required to target proteins into rafts (12, 13, 16). Additionally, protein oligomerization can increase significantly the affinity for association with these lipid assemblies (12, 15, 17). Interestingly, all the above structural characteristics exist in CD39 (8, 18, 19).

Despite the importance of caveolae for various cellular functions, there are cells that lack these organelles. In this case, it is believed that rafts take over their role in signaling. In support of this hypothesis, loss of caveolae in mice, as a result of disruption of the caveolin-1 gene, was not lethal (20, 21), suggesting that rafts may substitute in part for these organelles (20). On the other hand, rafts can also exist outside caveolae, at the plasma membrane and intracellularly, and have been shown to be involved in sorting and transport from Golgi to the apical surface of polarized cells (12, 14, 17, 22). In MDCK cells, rafts exist both in the apical and basolateral surface. However, caveolae are exclusively found basolaterally (23, 24), whereas apical rafts are responsible for formation of microvilli (25, 26)

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and targeting of membrane proteins into these membrane extensions (22). Therefore, rafts play a fundamental role in signaling, protein sorting, and formation of specific membrane structures such as caveolae and microvilli (12, 13, 17, 25, 26).

Although it is established that CD39 is localized in caveolae, it is not known whether these plasmalemma invaginations are involved in the regulation of CD39 function. Furthermore, it remains unclear whether CD39 associates with other cholesterol-dependent structures of the membrane and the possible role of such association in the biological activity of this ecto-nucleotidase. Our study provides insights into these questions and suggests a functional link between cholesterol-rich domains of the membrane and the anti-platelet activity of CD39.

MATERIALS AND METHODS

Antibodies—Monoclonal antibody against CD39 (Clone BU61) was from Ancell Corp. (Bayport, MN), polyclonal antibodies against caveolin-1 and 5′-ecto-nucleotidase were from Santa Cruz Biotechnology, and monoclonal antibody against CD31 was from DAKO (Glostrup, Denmark). Rabbit polyclonal antibody against rat NTPDase1 was a gift from Jean Sevigny (Centre de Recherche en Rhumatologie et Immunologie, Universite Laval, Sainte-Foy, Quebec), monoclonal antibody against transferrin receptor, clone H68.4, was kindly donated by Marino Zerial (MPI-CBG, Dresden) and monoclonal gp58 antibody was a gift from Kai Simons (MPI-CBG, Dresden). Polyclonal anti-CD39 antibodies were produced against full-length human placental CD39. The enzyme, 600 μg, was isolated from human term placenta (27) and further purified by preparative SDS-PAGE to avoid any contaminants. The final preparation was used for the immunization of two rabbits.

Plasmid Constructions—Given that several experiments in this study were carried out using isolated human placental membranes, expression of CD39 in tissue culture cells was also performed using the variant of CD39 that is expressed in human placenta. Human placental CD39 differs in two amino acids and has an insertion of seven amino acids at its N-terminal cytoplasmic tail in comparison to the gene product cloned from human B cells and endothelial cells (28). These changes were introduced by PCR using as template a plasmid containing human endothelial CD39 (pcDNA3-CD39, kindly provided by Simon Robson) and appropriate forward and reverse primers. The product of the PCR reaction was cloned into the pCDNA3 expression vector (Invitrogen), purified using a DNA purification kit from Qiagen, and verified by sequencing.

Cell Culture—MDCK cells, type II were grown in Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen), penicillin (100 units/ml) and streptomycin (100 μg/ml) in 10% CO_2. Primary cultures of human umbilical vein endothelial cells were obtained from human umbilical veins by collagenase digestion as previously described (29) and were used 24 h after plating. They were maintained in M199 medium (with Earle's and L-glutamine, Invitrogen) supplemented with 20% fetal bovine serum, 50 μg/ml EC growth supplement (Sigma), 10 units/μl heparin (Sigma), penicillin (100 units/ml), and streptomycin (100 μg/ml), in 5% CO_2. 24-well plates and glass coverslips were coated with 50 μg/ml type I rat tail collagen (BD Biosciences). Baby hamster kidney-21 (BHK-21) cells were cultured in Glasgow minimal essential medium supplemented with 5% heat-inactivated fetal calf serum and 10% tryptose phosphate.

Transient Transfection and Generation of MDCK Stable Cell Lines—Cells were transfected using Lipofectamine 2000 according to the supplier's protocol (Invitrogen). Selection of MDCK cells stably expressing CD39 was accomplished in the presence of 1 mg/ml G418 (Sigma). Positive clones were identified by immunofluorescence and kept in culture in the continuous presence of the selective antibiotic.

Polarized MDCK Cells Grown on Filters—MDCK cells (2.5×10^5) stably expressing CD39 were seeded and grown on 24-mm diameter Transwell polycarbonate filter units with a pore size of 0.4 μm (Costar Corp.) until they were fully polarized (4–5 days). Formation of tight monolayers accompanied by full polarization of the cells was tested by measuring transepithelial electrical resistance with an epithelial volt-ohmmeter (World Precision Instruments).

Indirect Immunofluorescence and Confocal Microscopy—MDCK cells grown on coverslips or filters or human umbilical vein endothelial cells grown on coverslips were fixed in 3.8% paraformaldehyde and quenched with 50 mM ammonium chloride for 30 min, and nonspecific sites were blocked with 10% fetal calf serum in PBS. Primary antibodies were incubated with the cells for 45 min at room temperature in 10% fetal calf serum in PBS. Then the cells were washed three times with PBS, and incubated with donkey anti-mouse and anti-rabbit IgG secondary antibodies (diluted in 10% fetal calf serum in PBS) conjugated with fluorescein isothiocyanate or TRITC, purchased from Dianova (Hamburg, Germany) and used at 1:200 dilution. Actin localization was assessed by rhodamine-phalloidin staining. Then, coverslips were washed three times with PBS and mounted on glass slides using Mowiol containing 100 mg/ml diazabicyclo[2.2.2]octane (Sigma), while filters were placed in 50% glycerol solution between two glass slides. Excitation of fluorescein isothiocyanate and TRITC was achieved using the 488 and 568 nm wavelengths, respectively. Images were collected on a Leica TCS-SP scanning confocal microscope, equipped with an argon/krypton laser and Leica TCS software.

Immunohistochemistry—Unfixed frozen sections (4 μm) of mouse liver (n = 2 of each, wild type (wt),[1] knock-out animals) were air-dried. Indirect immunofluorescence staining of the tissues was performed by overlaying each section with 50 μl of rabbit polyclonal anti-NTPdase1 antibody (dilution 1:50) for 45 min at room temperature. A secondary fluorescein isothiocyanate-coupled goat anti-rabbit Ig (dilution 1:80, Dianova, Hamburg, Germany) was applied for 45 min at room temperature. All washings were done with PBS.

Detergent Lysis and Sucrose Flotation Gradients—Membranes isolated from human placenta (27) were mixed with TNE buffer (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA), 1 mM dithiothreitol, 2% Triton X-100, or Lubrol WX (ICN Biomedicals) or Brij 96 (Fluka), protease inhibitors, and passed 25 times through a 27-gauge needle. Then the mixture (0.58 ml) was incubated for 30 min at 4 °C with rotation, adjusted to 40% w/w sucrose by addition of 0.82 ml of 62% w/w sucrose, loaded into the bottom of MLS-50 centrifuge tubes (Beckman), and overlaid sequentially with 35%, 30%, 25%, 20%, 15%, and 10% (w/w) sucrose solutions (0.6 ml each). Tubes were then centrifuged at 45,000 rpm for 18 h at 4 °C. After centrifugation, 600-μl fractions were collected from the top to the bottom and were subjected to Western blot analysis.

Detergent-free Flotation Gradients—This protocol has been described previously (30, 31). Briefly, human placental membranes were resuspended in 0.58 ml of 500 mM Na_2CO_3, pH 11.0. Homogenization was carried out using the following devices sequentially: Dounce homogenizer (10 strokes), Polytron tissue grinder (three 10-s bursts with 20-s intervals, Kinematica), and a sonicator (three 20-s bursts). The homogenate was then adjusted to 40% w/w sucrose by addition of 0.82 ml of 62% w/w sucrose prepared in MBS (25 mM Mes, pH 6.5, 0.15 M NaCl) and loaded into the bottom of MLS-50 centrifuge tubes (Beckman). A 30 to 5% w/w discontinuous sucrose gradient was formed above (3.3 ml of 30% w/w sucrose and 0.3 ml of 5% w/w sucrose) and centrifuged at 45,000 rpm for 20 h at 4 °C. After centrifugation, 300-μl fractions were collected from the top to the bottom and were subjected to Western blot analysis.

Cholesterol Depletion and Replenishment—Human placenta or mouse lung membranes isolated as described before (27) or BHK cells expressing CD39 were incubated in TNE buffer containing the indicated concentration (0–20 mM) of methyl-β-cyclodextrin (MβCD) and incubated for different time periods (0–60 min) at room temperature or 37 °C with rotation (32). The membranes were then used directly for subsequent assays, or after spinning them down to remove excess MβCD and resuspending them in the appropriate buffer. To replenish depleted membranes with cholesterol, the sample containing cholesterol-depleted membranes (after removal of the excess of MβCD) was incubated for 30 min at room temperature in the presence of MβCD-cholesterol complex in TNE buffer with agitation, as described previously (32). Preparation of the complex between MβCD and cholesterol for replenishment experiments has been reported before (32). MβCD-cholestenone complex was prepared in the same way.

Depletion of cholesterol from MDCK cells prior to immunofluorescence experiments was performed by incubating the cells with 50 mM MβCD, which is the concentration required to deplete efficiently cholesterol from this cellular system according to previous reports (22, 25), at 37 °C, for 1 h.

Treatment of Membranes with Filipin and Nystatin—Human placental membranes in TNE buffer containing the indicated concentration of filipin (0–10 μg/ml) or 100 μg/ml nystatin were incubated for 1 h with rotation at room temperature or at 37 °C respectively. After treatment,

[1] The abbreviations used are: wt, wild type; KO, knockout; BHK, baby hamster kidney cells; MDCK, Madin-Darby canine kidney cells; TRITC, tetramethylrhodamine isothiocyanate; PBS, phosphate-buffered saline; Mes, 4-morpholineethanesulfonic acid; MβCD, methyl-β-cyclodextrin.

the enzymatic activity of CD39 was determined in the membranes.

Platelet Aggregation—ADP-induced platelet aggregation studies were performed in a platelet Lumi-aggregometer (Chronolog, model 560) using human citrated platelet-rich plasma at a final platelet count of 2.5×10^8/ml at 37 °C, with continuous stirring at 1200 rpm. Platelet aggregation was followed by the increase of light transmission. Platelet sample was mixed with human placental membranes properly pretreated to alter cholesterol-sensitive domains by manipulation of their cholesterol content (see above), and aggregation was initiated by the addition of 5 μM ADP.

Enzymatic Assays—Polarized MDCK cells stably expressing CD39 and grown on polycarbonate filters were washed with HNMC buffer (20 mM Hepes, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$) and incubated with 0.5 ml of HNMC buffer on the apical side and 1.5 ml of the same buffer on the basolateral side, both containing 1 mM ATP or ADP as substrate for 20 min at 37 °C with occasional gentle agitation. The enzymatic reaction was stopped with addition of EDTA and EGTA at 10 mM final concentration each.

To measure CD39 or 5′-ecto-nucleotidase enzymatic activity in human placental membranes, the enzymatic reaction was initiated by addition of assay buffer (67 mM Tris-HCl, pH 8, 1 mM $MgCl_2$, 40 mM L-Phe, and 1 mM levamisole, and 1 mM ADP or AMP, respectively) to crude membranes followed by incubation for 10 min at 37 °C. Placental alkaline phosphatase activity was assayed by incubating membranes with buffer containing 67 mM Tris-HCl, pH 8, 1 mM $MgCl_2$, and 1 mM *p*-nitrophenylphosphate, for 10 min at 37 °C. All enzymatic reactions were terminated with addition of the malachite green reagent. The released inorganic phosphate was determined by the malachite green colorimetric assay (33).

Measurement of Cholesterol Content—Placental membranes were treated with MβCD and MβCD-cholesterol complex as described above. Then the samples were ultracentrifuged at $100{,}000 \times g$ for 1 h at 4 °C, the membrane pellets were washed with TNE buffer, recentrifuged as above, and resuspended in TNE buffer. Cholesterol content of each sample was measured by using the Amplex red kit (Molecular Probes), according to the manufacturer's instructions.

Other Methods—Normalization of CD39 protein content in different samples was achieved by quantitating the corresponding immunoreactive zones of the immunoblots using the Gel Analyzer software (Biosure). SDS-PAGE and immunoblotting analysis were carried out using established protocols.

RESULTS

Do Caveolae Play a Role in CD39 Activity?—Given the localization of CD39 in caveolae, we tested whether the enzymatic activity of CD39 depends on this localization. To address this question, we used the *in vitro* model system of polarized MDCK cells. When these cells are grown on polycarbonate filters they become fully polarized and contain caveolae exclusively on the basolateral side (23, 24). This system allowed us to test whether CD39 can be targeted to a membrane that lacks caveolae (*e.g.* the apical membrane of MDCK cells), and whether this enzyme remains active in the absence of these plasmalemma invaginations. We generated MDCK cells stably expressing CD39, allowed them to fully polarize on filters, and performed immunofluorescence confocal microscopy using antibodies that recognize the ecto-domain of CD39. Cells were not permeabilized to detect signals exclusively arising from the apical and basolateral plasma membrane avoiding interference from intracellular CD39. As can be seen in Fig. 1*A*, confocal scanning of the sample from the top of the cell to its bottom revealed a strong signal at the apical membrane in contrast to the low staining of the lateral or basal side. The basolateral marker gp58 exhibited an opposite staining pattern (Fig. 1*A*), whereas caveolin-1 stained both the apical and basolateral surface (Fig. 1*B*), consistent with previous reports (23, 34). Heterogeneous appearance of the CD39 staining on the apical membrane is due to different expression levels of CD39 in individual cells within the monolayer, combined with varying heights of the cells. Identical results were obtained with two different antibodies (polyclonal and monoclonal) against CD39, whereas control MDCK cells (stably transformed by empty vector) showed no staining (data not shown). We therefore conclude that CD39

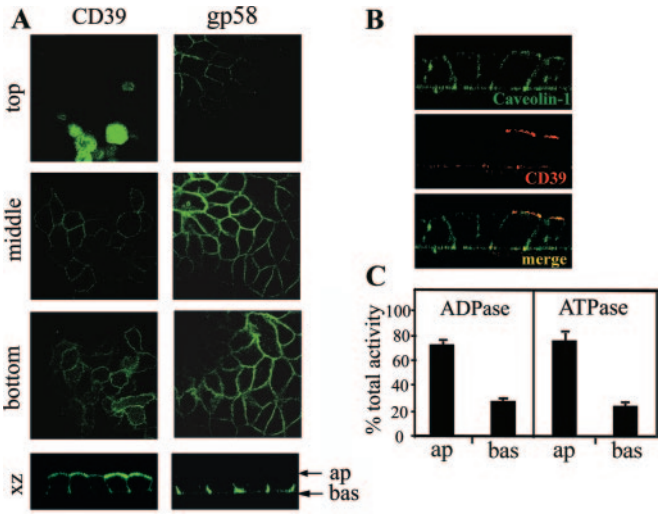

FIG. 1. **CD39 is targeted apically in polarized MDCK cells and is enzymatically active in the apical membrane.** *A*, polarized MDCK cells stably expressing CD39 were grown on filters, fixed, and stained with polyclonal anti-CD39 or monoclonal anti-gp58 (basolateral marker) antibodies and analyzed by confocal fluorescence microscopy. Optical sections from the *top*, *middle*, and *bottom* of the cell layers and *xz* sections are shown to reveal apical (*ap*) and basolateral (*bas*) staining. *B*, optical *xz* section of polarized MDCK cells showing the localization of CD39 (*green*), caveolin-1 (*red*), and the two together (*merge*). *C*, ADPase and ATPase activities of intact filter-grown MDCK cells, stably expressing CD39, were measured in the presence of ADP or ATP, respectively. Apical and basolateral hydrolytic activities are expressed as percentage of the total activity. Activity assays were performed in duplicate in at least two independent experiments.

is preferentially targeted to the apical side of the plasma membrane in MDCK cells. Next we tested whether CD39 is enzymatically active in the absence of caveolae. To address this question, we measured the apical and basolateral ecto-ATPase and ecto-ADPase activities of the CD39-expressing cell line. After removal of the medium and washing, we added assay buffer containing ATP or ADP to the apical and basolateral sides of the filter chambers containing fully polarized intact cells. Both ecto-ATPase and ecto-ADPase activities corresponding to exogenously expressed CD39 (after subtracting the activity of control cells from CD39-expressing cells) were preferably detected on the apical side (Fig. 1*C*), consistent with the immunofluorescence data. These data indicate that caveolae are not necessary for the enzymatic activity of CD39 and that delivery of CD39 to the membrane occurs independently of the presence of caveolae.

To further support the above results, we took advantage of the caveolin-1 knock-out mice and the fact that disruption of caveolin-1 gene in these animals leads to complete disappearance of caveolae from the cell surface (20, 21). Membrane fractions were isolated from control and knock-out mice from lungs and spleen, which are highly vascularized tissues and contain high amounts of CD39. The absence of caveolin-1 in the knock-out sample was confirmed by immunoblotting (Fig. 2*A*). To test whether disruption of caveolin-1 gene has any effect on the surface localization of CD39, we performed immunocytochemistry experiments using tissues from wild type and gene-disrupted mice. Fig. 2*B* shows that CD39 is expressed in the apical surface of endothelial cells of large vessels of mouse liver sections consistent with previous reports (35, 36). A similar staining pattern is observed in caveolin-1 gene-disrupted mice. Therefore, caveolin-1 has no effect on polarized targeting of CD39 to plasma membrane. Furthermore, the ADPase activity remains largely unaffected by disruption of the caveolin-1 gene (Fig. 2*C*). Because >90% of the ADPase activity in these membrane fractions is attrib-

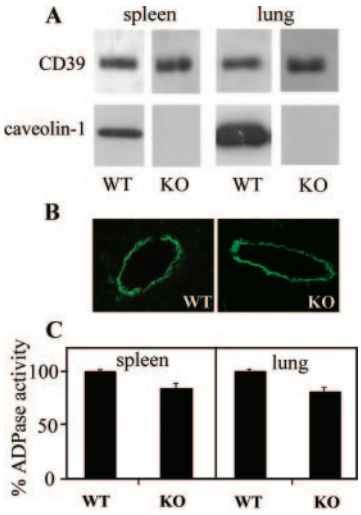

FIG. 2. **The activity of CD39 is not substantially affected in caveolin-1 gene-disrupted mice.** *A*, spleen and lung particulate membrane fractions were isolated from wild type (*WT*) and caveolin-1 gene-disrupted (*KO*) mice and were analyzed by Western blotting using a polyclonal anti-CD39 antibody. Polyclonal anti-caveolin-1 antibodies were used to show lack of caveolin-1 expression in samples obtained from caveolin-1 gene-disrupted mice. *B*, immunofluorescence demonstration of CD39 in blood vessels of liver sections obtained from wild type mice (*WT*) and caveolin-1 gene-disrupted mice (*KO*), at 300 times magnification. *C*, the ADPase activity of spleen and lung particulate membrane fractions from caveolin-1 gene-disrupted (*KO*) mice is expressed as percentage of the activity of the wild type tissues. The *bars* indicate normalized ADPase activity by densitometric quantitation of CD39 immunoreactive zones shown in *A*. Activity assays were performed in duplicate in at least three independent experiments.

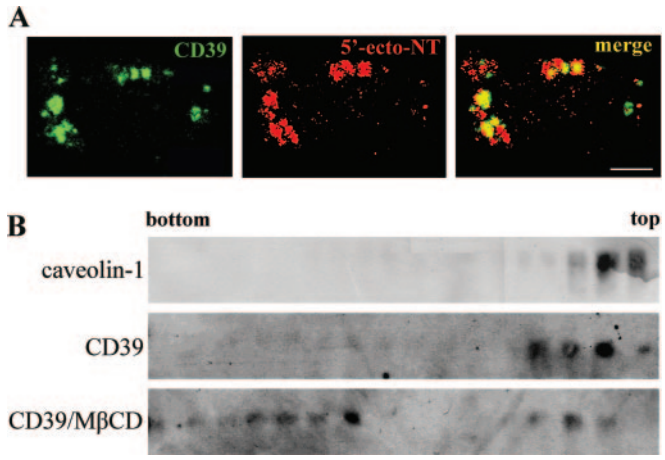

FIG. 3. **CD39 colocalizes with the raft marker ecto-5′-NT and floats to low-density fractions under detergent-free conditions.** *A*, human umbilical vein endothelial cells were subjected to double cell-surface indirect immunofluorescence using polyclonal anti-CD39 and monoclonal anti-ecto-5′-NT antibodies, and were analyzed by confocal microscopy. Single optical sections are shown. Colocalization between CD39 and ecto-5′-NT appears as *yellow color* in the *merged image*. *Scale bar*, 2 μm. *B*, human placental membranes were treated with 0.5 M carbonate buffer, pH 11.0, sonicated at 4 °C, adjusted to 40% sucrose, overlaid with 30 and 5% sucrose layers, and subjected to ultracentrifugation. The distribution of proteins was analyzed by immunoblotting using monoclonal anti-CD39, and polyclonal anti-caveolin-1 antibodies. In the *bottom panel*, membranes were first incubated with MβCD prior to the above treatment. Low density fractions containing caveolae/rafts exist on the *top* of the gradients, whereas heavy density fractions containing the non-raft fraction are at the *bottom*.

uted to the CD39 gene product (1), we conclude that CD39 activity is not substantially influenced when caveolin-1 and caveolae are removed from the plasma membrane.

CD39 Associates Partially with Lipid Microdomains—Recent studies have proposed that caveolae arise from the coalescence of small rafts, and that association of a protein with these microdomains may cause its translocation into caveolar pits (14, 15). Moreover, rafts exist also independently of caveolae and may take over the function of these organelles in caveolin-1 gene-disrupted mice (20). Therefore, an association of CD39 with lipid rafts could explain both the localization of this enzyme in caveolae and the lack of a significant influence on the enzymatic activity when these organelles are eliminated from the membrane. A first indication that supported the hypothesis that CD39 is present in lipid rafts was obtained by indirect immunofluorescence experiments in primary endothelial cells. Fig. 3*A* shows that, in a subset of cell population, large aggregates of endogenous CD39 exhibit a substantial degree of colocalization with ecto-5′-nucleotidase (ecto-5′-NT), a glycosylphosphatidylinositol-anchored enzyme that associates with lipid rafts (37).

To investigate whether CD39 is present in lipid rafts, we used a detergent-free approach that is based on sonication of the membranes under alkaline pH (30, 31). Under such conditions, a significant amount of CD39 was found to float to low density fractions, which contain the rafts/caveolae domains of the membrane, in parallel with caveolin-1 (Fig. 3*B*). To test whether the flotation profile of CD39 is cholesterol-dependent, we treated the membranes with methyl-β-cyclodextrin (MβCD). This drug has been widely used to remove cholesterol from membranes (32, 38) and causes disruption of lipid rafts and caveolae (11, 12). MβCD treatment caused a shift of a substantial amount of CD39 from the low to the high density fractions (Fig. 3*B*), which implies that cholesterol is an important component in the lipid microenvironment of CD39.

In a complementary approach, we analyzed detergent-solubilized membranes in sucrose flotation gradients, a commonly used procedure to detect lipid-protein association in rafts. For this experiment we took into consideration recent studies which showed that multiple and distinct types of raft-like assemblies, with diverse lipid-protein composition, coexist within membranes, and different detergents are required to reveal them (22, 38–42). In practice, this means that a given protein may remain lipid-raft associated in one detergent, but its lipid association may be disrupted by a different one (22, 38–42). Therefore, we have used three different detergents (Triton X-100, Lubrol WX, and Brij-96) (22, 38–42) to resolve multiple raft subpopulations. When Triton was used for solubilization, hardly any CD39 was found to float at low density fractions (Fig. 4*A*). On the other hand, when the same experiment was carried out using Lubrol WX (Fig. 4*B*), although most CD39 remained at the bottom of the tube, a significant amount was found to float toward the top of the gradient at the fractions where caveolin-1, a known raft-associated protein, was also detected. This is identical to the distribution pattern of other proteins that are associated with lipid-rafts such as prominin (22), CDC42 (39), and human neurotrophin receptor (43), after solubilization with Lubrol WX. Transferrin receptor used as a control of non-raft proteins (38), remained at the bottom fractions of the gradient. Similarly, when Brij-96 was used, part of CD39 was found to float at light density fractions where caveolin-1 rafts are located (Fig. 4*C*). It is noteworthy that CD39 was detected even at the very low density fraction of 10–15% sucrose in both Lubrol WX and Brij 96 (Fig. 4, *B* and C). An endothelium-specific raft-associated molecule, CD31 (44), overlapped with CD39 in the low density fractions obtained in Lubrol gradients, but not in Brij 96 (Fig. 4*C*), underscoring the diversity of lipid rafts in cell membranes (22, 38–42). All the above data suggest that CD39 exists, at least partially, in lipid assemblies.

CD39 Is Localized in Microvilli of the Apical Membrane of MDCK Cells—The differential association of CD39 with lipid

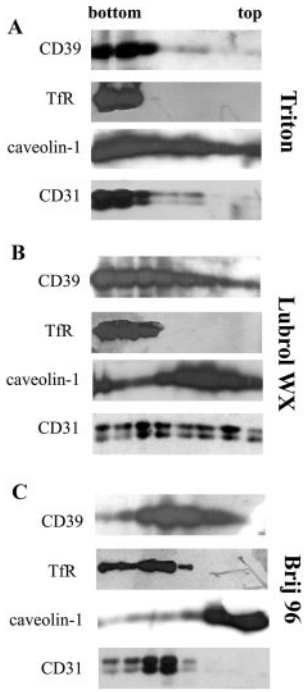

FIG. 4. **CD39 associates partially with detergent-resistant lipid microdomains.** Human placental membranes were extracted with 2% Triton (*A*), or 2% Lubrol WX (*B*), or 2% Brij 96 (*C*) at 4 °C, adjusted to 40% sucrose, and floated on sucrose step gradients (35 to 10% w/w). Low density fractions exist on the *top* of the gradients, while heavy density fractions are at the *bottom*. The distribution of proteins was analyzed by immunoblotting using monoclonal anti-CD39, anti-CD31, and anti-transferrin receptor and polyclonal anti-caveolin-1 antibodies.

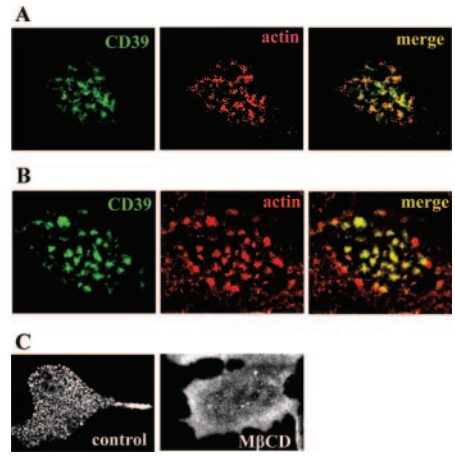

FIG. 5. **CD39 is colocalized with actin filaments in microvilli.** MDCK cells stably expressing CD39 were incubated with the appropriate probes and analyzed by laser scanning confocal microscopy. Single optical sections are shown from the apical surface of the cells. *A*, cells were stained with anti-CD39 polyclonal antibodies (*green*) and rhodamine-phalloidin (*red*) for actin filament staining. Colocalization between CD39 and actin filaments is shown in the *merged image*. *B*, cells were incubated with 2 μM cytochalasin D for 1 h at 37 °C to disrupt the actin cytoskeleton and then stained as in *A*. *C*, control cells (*left panel*) or incubated with MβCD (*right panel*) were stained with anti-CD39 polyclonal antibodies.

assemblies might play a role in the localization of CD39 in specialized domains of the membrane. This assumption is based on recent findings showing that the association of prominin with Lubrol rafts is required to target this protein to microvilli (22). Furthermore, in cellular membranes that lack caveolae, *e.g.* the apical membrane of MDCK cells, microvilli are good candidate domains for the localization of raft-associated molecules, because they are also cholesterol- and raft-dependent structures (25, 26). It is well known that microvilli in the apical surface of MDCK are visualized by the characteristic punctate staining of filamentous actin (26, 45, 46), which constitutes the core of microvilli (45). Based on the above studies, to test whether CD39 is localized in microvillar structures, we performed colocalization studies between CD39 and actin filaments in MDCK cells by immunofluorescence and scanning laser confocal microscopy (Fig. 5*A*). Filamentous actin showed a punctate staining at the apex of the cells, which is characteristic of the actin-based core of the microvilli (26, 45, 46). Interestingly, a substantial degree of colocalization between these actin-decorated microvilli and CD39 was observed.

It is well established that cytochalasin D treatment disrupts actin cytoskeleton by creating short filaments and actin aggregates. This treatment does not lead to disappearance of microvilli, but instead causes their aggregation (22, 45, 46). To further support the localization of CD39 in microvilli, we tested the influence of cytochalasin D treatment on CD39 localization. Interestingly, CD39 followed the reorganization of actin in the apical surface, and there was a substantial degree of colocalization between CD39 and actin at large patches of actin bundles (Fig. 5*B*), which suggests that CD39 is localized in the aggregated microvilli. Importantly, although basal actin filaments were also reorganized into focal aggregates (45, 46), there was no colocalization between these actin aggregates and the portion of CD39 found in the basal side of the cells (not shown). However, when cholesterol was removed from MDCK cells by MβCD, the speckled CD39 staining pattern in microvilli was converted into a diffuse surface distribution (Fig. 5*C*), which is also observed for the microvilli-localized prominin (22). Taken together, these data suggest that CD39 is localized in microvilli of MDCK cells and further imply that cholesterol-dependent microdomains could be responsible for such localization. Furthermore, the above results show that, when caveolae are absent from cellular membranes, CD39 may be localized in other cholesterol rich domains, such as the microvilli.

Cholesterol-dependent Lipid Assemblies Regulate the Enzymatic Activity of CD39—To test further the role of lipid assemblies on CD39 function, we next examined whether cholesterol depletion has a functional significance for its enzymatic activity. For this purpose we treated placental membranes with MβCD and tested the effect on the ADPase activity of CD39. Treatment of isolated human placental membranes with this reagent resulted in dramatic inhibition of ADPase activity in a concentration-dependent manner (Fig. 6*A*). Measurement of the concentration of cholesterol that remained in the membrane after treatment with the drug showed that the activity of CD39 declined in parallel with the amount of cholesterol remaining in the membrane (Fig. 6*A*), indicating that cholesterol is critical for CD39 activity. Moreover, inhibition of CD39 is not due to possible extraction of the protein from the membrane, because CD39 was found exclusively in the pellet fraction (unlike treatment with Triton) when MβCD-treated membranes were subjected to high speed centrifugation, similarly to caveolin-1 (Fig. 6*B*).

Two lines of evidence show that the above results are specifically due to inhibition of CD39 and not to other ADPases of this tissue. First, similar inhibition of ADPase activity was observed when tissue culture cells transfected with a CD39 expression plasmid were treated with MβCD (Fig. 6*C*). Second, the ADPase activity of this tissue is mainly due to CD39 (27–29, 47, 48). Remarkably, cholesterol depletion did not have a general effect on plasma membrane ecto-nucleotidases, because alkaline phosphatase (placental alkaline phosphatase) and 5′-nucleotidase remained unaffected (Fig. 6*D*), further supporting the specificity of MβCD treatment on CD39 function.

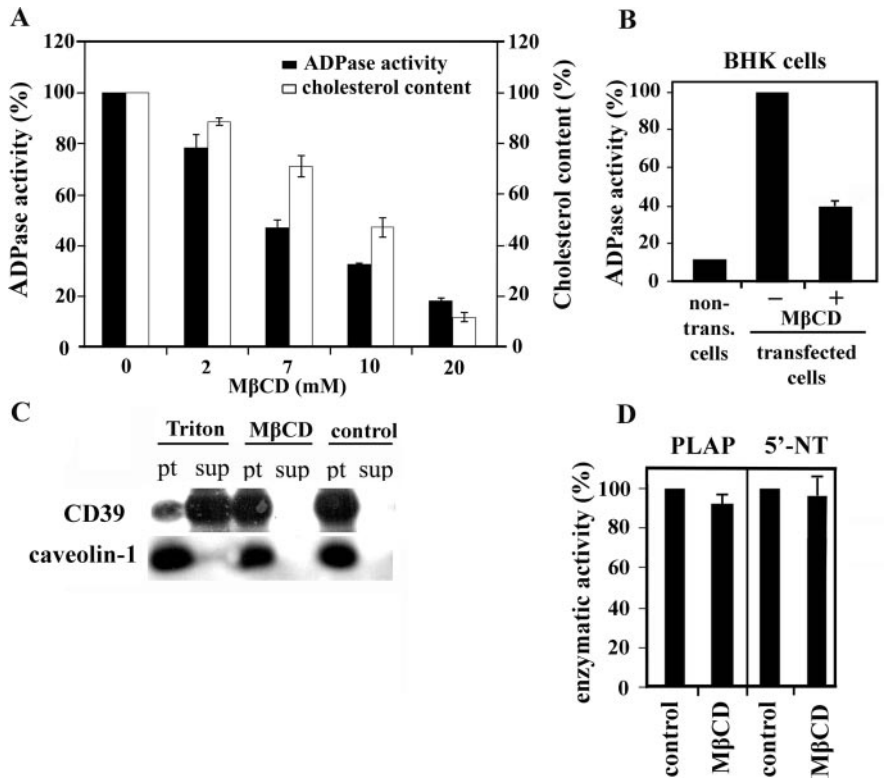

FIG. 6. **The enzymatic activity of CD39 is inhibited by cholesterol depletion of the membranes.** *A*, human placental membranes were treated with the indicated concentrations of MβCD at 37 °C for 30 min with agitation. The samples were centrifuged to remove the released cholesterol, membrane pellets were washed, resuspended in the appropriate buffer, and split in two aliquots for measurements of the ADPase activity of CD39 and the cholesterol content. The values are expressed as percentage of the control sample, which was treated as above in the absence of MβCD. *B*, human placental membranes were treated with 1.5% Triton at 4 °C (*Triton*), or 20 mM MβCD at 37 °C (*MβCD*), or left untreated (*control*) and centrifuged for 1 h at 100,000 × *g*. Supernatant (*sup*) and pellet (*pt*) fractions were analyzed by SDS-PAGE followed by immunoblotting using monoclonal anti-CD39 and polyclonal anti-caveolin-1 antibodies. *C*, BHK cells transfected with CD39 expression plasmid were treated with 10 mM MβCD for 1 h at room temperature with agitation. ADPase activity was measured and expressed as percentage of the activity of cells treated without the drug. *D*, placental alkaline phosphatase and ecto-5′-NT enzymatic activities of MβCD-treated (20 mM MβCD, 37 °C) and control placental membranes were assayed with the appropriate substrates.

MβCD treatment may have unspecific effects irrelevant of alteration of cholesterol levels. If the effect of MβCD treatment was solely due to cholesterol depletion one would expect that CD39 activity would be rescued by restoring the cholesterol levels of the membrane, as shown in other biological systems (32, 49). Cholesterol can be efficiently delivered to membranes when added as a preformed complex with MβCD (32, 49). Incubation of the MβCD-treated placental membranes with this complex completely restored the ADPase activity of CD39 in a concentration-dependent manner (Fig. 7*A*). Recovery of ADPase activity was accompanied by complete restoration of membrane cholesterol levels (101% compared with native membranes) when the concentration of the complex was 1 mM. Interestingly, when cholesterol was replaced by its oxidation product cholestenone, the activity of CD39 was not rescued (Fig. 7*A*), although cholestenone can be efficiently delivered to cholesterol-depleted membranes (50). These data suggest that the inhibitory effect by MβCD treatment was specifically due to cholesterol removal. Furthermore, when native membranes were loaded with 60% extra cholesterol the hydrolytic activity of CD39 was increased by 50% (Fig. 7*B*).

To further support the role of cholesterol on CD39 activity we used other drugs that interfere with cholesterol levels in different ways. For example, filipin is an invasive drug that, unlike MβCD, does not extract cholesterol from membranes, but is inserted into the membrane and sequesters cholesterol within the lipid bilayer (12). Treatment with filipin caused a dose-dependent inhibition of CD39 activity (Fig. 8). The concentration of filipin required to inhibit CD39 activity by 80% was 10 μg/ml (15.3 μM), whereas membrane cholesterol concentration was 12.5 μM. We conclude therefore that filipin exerts a very strong and specific effect at almost stoichiometric amounts. In addition, another cholesterol-binding drug, nystatin, when used at the concentration of 100 μg/ml resulted in 50% inhibition of the ADPase activity (data not shown). Collectively, all the above data suggest that cholesterol-dependent lipid assemblies play a critical role for CD39 activity.

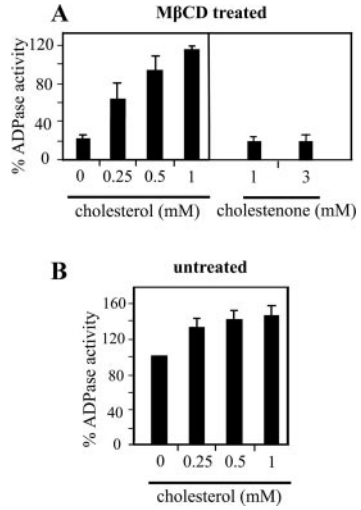

FIG. 7. **CD39 activity in cholesterol-depleted membranes is specifically rescued by cholesterol replenishment.** Cholesterol-depleted membranes (*A*) or untreated membranes (*B*) from human placenta were incubated with increasing amounts of pure cholesterol or cholestenone complexed to MβCD. Subsequently, ADPase activity was measured and expressed as percentage of the activity of control membranes. Activity assays were performed in duplicate in at least three independent experiments.

Cholesterol-dependent Lipid Assemblies Regulate the Anti-platelet Activity of CD39—A key function of CD39 is to protect blood vessels from thrombosis through inhibition of platelet aggregation (4). To address the role of cholesterol-based lipid assemblies on CD39 anti-platelet activity, we performed platelet aggregation assays in the presence of membranes that have been treated with MβCD. Membranes isolated from human placenta caused a significant inhibition of the primary ADP-induced platelet aggregation (Fig. 9*A*). Interestingly, MβCD treatment reduced substantially the anti-platelet activity of membranes, and this activity was completely rescued by re-

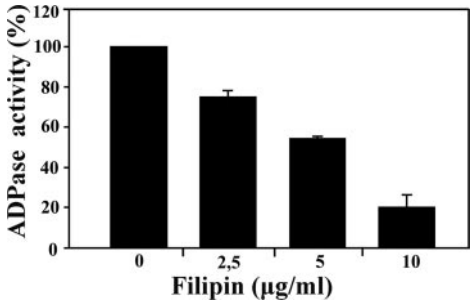

FIG. 8. **The enzymatic activity of CD39 is inhibited by treatment of membranes with filipin.** Human placental membranes were incubated with the indicated concentrations of filipin at room temperature for 30 min with agitation. The ADPase activity of the samples was measured and expressed as percentage of the activity of the control sample.

plenishment of cholesterol. More dramatic was the effect of cholesterol depletion on the activity of membranes to disaggregate platelets after their initial aggregation phase. Untreated membranes caused an immediate, almost complete (90%) disaggregation of the initially aggregated platelets (Fig. 9, *A* and *B*). Cholesterol depletion resulted in a significant delay (Fig. 9*A*) and a substantial decrease of this disaggregation activity to 35% (Fig. 9*B*), as measured at the time point when disaggregation by control membranes was almost complete (90%, see *dashed line* in Fig. 9*A*). Intriguingly, replenishment of the depleted membranes with pure cholesterol completely rescued both activities of the treated membranes (Fig. 9, *A* and *B*). These data are largely attributed to the ADPase activity of CD39 for three reasons. First, they are in agreement with the effects observed by enzymatic activity assays (Figs. 6 and 7). Second, platelet aggregation was induced by ADP, which is the substrate of CD39 and third, hydrolysis of ADP by its pretreatment with membranes abolished ADP-induced platelet aggregation (Fig. 9*A*). Therefore, from all the above results it is concluded that cholesterol-dependent lipid microdomains are functionally important for the anti-platelet activity of CD39.

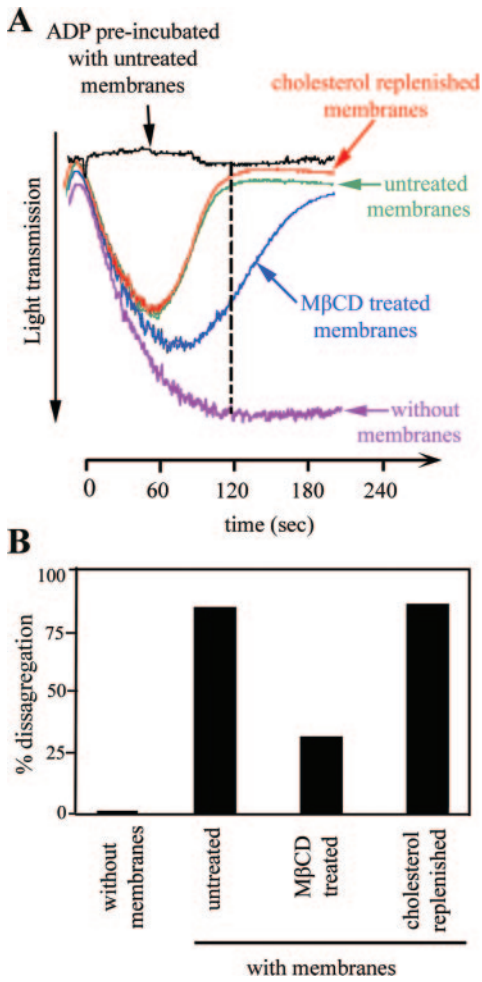

FIG. 9. **Cholesterol-dependent microdomains are important for the anti-platelet activity of CD39.** *A*, platelet aggregation assays were performed in the presence of untreated human placental membranes (*untreated membranes*), or treated with MβCD (*MβCD treated membranes*), or MβCD-treated membranes after replenishment of cholesterol (*cholesterol replenished membranes*), or in the absence of membranes (*without membranes*). Reactions were initiated with the addition of 5 μM ADP, and progression of platelet aggregation was monitored using a platelet aggregometer. The *top black line* refers to the condition where ADP was first preincubated for 1 min with untreated membranes, and then this mixture was added to platelets. The *dashed vertical line* is discussed in *B*. *B*, disaggregation (%) refers to the percentage of disaggregated platelets based on values obtained in *graph A* at the time point when disaggregation by untreated membranes was almost complete (90%, see *vertical dashed line* in *A* at $t = 2$ min). 0% disaggregation is defined as the maximum value of light transmission reached after platelets have been aggregated with ADP in the absence of membranes, and 100% as the value corresponding to light transmission of platelets in the absence of the agonist.

DISCUSSION

Membrane compartmentalization is an important feature of the cell surface, which warrants divergent cellular functions to take place at specific and distinct domains of the membrane. Caveolae and rafts are domains of the membrane from where many signal transduction pathways are initiated (12, 16, 42). They share a unique lipid composition, being rich in cholesterol and sphingolipids. Due to this similarity and the overlapping functions of these two domains, it has been difficult to functionally distinguish rafts and caveolae, the latter being considered as specialized rafts. For example, depletion of cholesterol interferes with the function of both rafts and caveolae, not allowing to decipher their individual role (11). Despite these similarities, there are regulatory mechanisms that are fulfilled specifically within the microenvironment of caveolae, and rafts are unable to substitute for these functions (16, 20, 21, 51).

In the present study we examined whether the activity of CD39, a caveolae-localized ecto-nucleotidase (5–10) involved in thromboregulation, depends on the caveolar environment. Using polarized MDCK cells and mice lacking caveolin-1, we have shown that the enzymatic activity of CD39 is not significantly affected when cells are devoid of caveolae in their plasma membrane. However, removal of caveolae has been proposed to be compensated by rafts (20). Therefore, we tested whether CD39 associates with these lipid assemblies. Several lines of evidence presented here indicate that CD39 associates, at least in part, with distinct raft-like lipid assemblies. First, CD39 forms patches on the cell surface of endothelial cells, which overlap with domains of ecto-5′-NT, a glycosylphosphatidylinositol-anchored and raft-associated 5′-nucleotidase. Second, a fraction of CD39 floats in sucrose gradients after solubilization of the membranes by detergents. Using a detergent-free approach, a significant amount of CD39 is detected in the caveolae/rafts fraction in a cholesterol-dependent manner. Third, interference with membrane cholesterol results in a substantial decrease of hydrolytic and anti-platelet activity of CD39. Fourth, CD39 is targeted to the apical side in polarized MDCK cells, a transport pathway that has been mainly attributed to raft function (12, 14, 17, 22). Finally, apical CD39 is localized in microvilli of MDCK cells, which are cholesterol/raft-dependent membrane structures (22, 25, 26).

The lipid assemblies where CD39 is present are distinct from the classic type of Triton-resistant rafts, because hardly

any CD39 floats after solubilization with this detergent. Instead, CD39 is detected in low sucrose density fractions upon solubilization with Lubrol WX, exactly as shown for other Lubrol-raft proteins (22, 39, 43). Lubrol defines a distinct class of lipid assemblies, which in several cases is strictly separated from Triton rafts (22, 39, 41, 42) and shows substantial differences in lipid composition (39). We also observed that CD39, caveolin-1, and CD31 are differently distributed along the gradients when different detergents are used. Therefore, our data support the coexistence of multiple, distinct types of lipid assemblies within membranes that exhibit differential sensitivity to various detergents (22, 38–42). These distinct subpopulations of rafts underlie the generation and maintenance of membrane subdomains.

Very little is known about the function of the newly identified, diverse microdomains. A Lubrol-raft-associated protein, prominin, has been shown to be localized in microvilli in a cholesterol and raft-dependent manner (22). Microvilli are membrane structures whose formation depends on cholesterol and on the integrity of rafts (25, 26). We show here that CD39 is also localized in microvilli of MDCK cells, in agreement with a recent report showing that this enzyme is detected in microvilli of placental syncytiotrophoblast cells (52). Therefore, we propose that association of CD39 with lipid microdomains could be involved in its targeting to these membrane protrusions. This result further implies that, in cells which lack caveolae, areas of the membrane that are rich in cholesterol, such as microvilli, might be the sites where CD39 is located. Either present in caveolae or in microvilli, targeting of CD39 to such cholesterol-rich domains warrants a high hydrolytic activity of this enzyme.

In our study we provide evidence for a functional link between cholesterol-based microdomains and the enzymatic activity of CD39. Cholesterol depletion from the membrane by MβCD or cholesterol sequestration within the membrane by filipin or nystatin strongly inhibit this enzyme. Replenishment of the cholesterol-depleted membranes with pure cholesterol completely restores CD39 function. Finally, substitution of cholesterol by the structurally related molecule cholestenone is not able to support CD39 activity, which provides mechanistic insights regarding the role of cholesterol on this enzyme. Cholesterol acts as the main lipid rigidifier in natural membranes (13, 53), whereas its depletion increases membrane fluidity (50), an effect which can be reversed by addition of cholestenone (50). Membrane fluidity is a biophysical property of the lipid bilayer that affects the activity of a number of plasma membrane proteins. For example, the α-secretase ADAM-10 and the cholecystokinin receptor, which are functionally dependent on cholesterol, retain their activity when cholesterol is replaced by cholestenone (50, 54). Therefore, because the same replacement cannot support CD39 activity, we conclude that the effect of cholesterol on CD39 is not due to the concomitant biophysical changes of membrane fluidity, but rather due to a structure-specific role of cholesterol that may affect the conformation of the catalytic ecto-domain. This hypothesis is supported by a recent study that showed that CD39 activity depends on interactions between the two transmembrane domains that undergo dynamic motions within the membrane (55). Therefore, cholesterol may affect the ability of this enzyme to undergo conformational changes required for nucleotide hydrolysis.

Under our experimental conditions, only part of CD39 was found to associate with detergent-resistant complexes. However, the amount of the protein that floats in sucrose gradients may not necessarily reflect the *in vivo* situation (38). Most likely, there are additional lipid-protein and protein-protein interactions *in vivo* that facilitate participation of proteins in rafts which may be disrupted by detergents (38). This suggestion is supported by the finding that a significantly higher proportion of CD39 floats in the low density fractions when a detergent-free method was employed. A similar distribution pattern has been observed for other proteins, such as epidermal growth factor receptor (30, 56, 57) and scavenger receptor class B type I (58). In addition, oligomerization can increase the affinity of a protein weakly associated with rafts to become a strong raft partner (12, 15, 17). Given that detergents disrupt CD39 oligomers (18), flotation of CD39 may be affected by membrane solubilization. Moreover, activation of a protein can affect dynamically the percentage of molecules participating in lipid assemblies (12, 14). Indeed, the caveolae-associated ecto-ATPase amount and/or activity were increased upon activation of endothelial and other cells (5). Post-translational modifications (*e.g.* palmitoylation (8) and/or phosphorylation), known to influence association of proteins with rafts (12, 13, 16, 42), may account for a dynamic association of CD39 with lipid assemblies. Therefore, it is likely that the relative amount of CD39 present in lipid assemblies is subjected to regulation. Regardless of the regulatory mechanism, our data show that this enzyme functionally depends on and physically associates with lipid assemblies.

Other members of the NTPdase family seem to be also regulated by cholesterol. For example, cholesterol oxidase treatment results in inhibition of brain synaptic plasma membrane Ca^{2+} plus Mg^{2+}-ATPase (59) and transverse tubule ecto-ATPase (60), whereas cholesterol increases the thermal stability of Ca^{2+}/Mg^{2+}-ATPase from bovine cardiac microsomes (61). Although in these cases cholesterol depletion/replenishment experiments were not performed to assign a direct role of this lipid on the activity of the enzymes, nevertheless they suggest that, like CD39, other members of the NTPdase family may be also regulated by cholesterol-sensitive domains.

Taking into account these findings, one might ask whether cells ever experience alterations in cholesterol levels *in vivo*. Indeed, cellular cholesterol levels can be changed under physiological or disease conditions (62–64), resulting in alteration of lipid-raft function (39, 65, 66). Therefore, it is possible that CD39 activity is regulated by changes of cholesterol levels at the microdomains of rafts/caveolae or other cholesterol-dependent structures, such as microvilli. In support to this hypothesis, administration of cholesterol to rabbits was accompanied by increased ecto-ATPase activity in blood vessels (67). Because high cholesterol levels in blood vessels (*e.g.* hypercholesterolemia) increase the risk of atherosclerosis and of thrombotic disorders, it can be postulated that under these conditions activation of CD39 will have a beneficial effect against thrombus formation.

Finally, our finding that CD39 activity depends on cholesterol-based lipid microdomains but not on caveolae may provide insights into the physiological role of CD39 localization in these plasmalemma invaginations. Given that ATP and adenosine signaling depend on caveolae (68, 69), a possible explanation would be that caveolae localization of CD39 serves to regulate the levels of ATP and adenosine (coordinately with ecto-5′-NT) inside caveolae, thereby controlling their signaling. In this case, it is tempting to speculate that a dynamic equilibrium between non-raft and raft-associated CD39 plays a dual role: it ensures a fine tuning of the enzymatic activity in a cholesterol-dependent manner, and in addition, it regulates CD39 localization in and out of caveolae in a dynamic way, as opposed to a more stable association of ecto-5′-NT. Therefore, CD39 could play a central regulatory role in determining spatially and temporally the balance between ATP *versus* adenosine levels in the caveolae pits, functioning as a switch that

leads from P2 to adenosine receptors activation. Such a mechanism can provide a protective role in inflammation and thrombosis and prompts further investigation.

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REFERENCES

1. Enjyoji, K., Sevigny, J., Lin, Y., Frenette, P. S., Christie, P. D., Esch, J. S., 2nd, Imai, M., Edelberg, J. M., Rayburn, H., Lech, M., Beeler, D. L., Csizmadia, E., Wagner, D. D., Robson, S. C., and Rosenberg, R. D. (1999) *Nat. Med.* **5,** 1010–1017
2. Goepfert, C., Imai, M., Brouard, S., Csizmadia, E., Kaczmarek, E., and Robson, S. C. (2000) *Mol. Med.* **6,** 591–603
3. Goepfert, C., Sundberg, C., Sevigny, J., Enjyoji, K., Hoshi, T., Csizmadia, E., and Robson, S. (2001) *Circulation* **104,** 3109–3115
4. Marcus, A. J., Broekman, M. J., Drosopoulos, J. H., Islam, N., Pinsky, D. J., Sesti, C., and Levi, R. (2003) *J. Thromb. Haemost.* **1,** 2497–2509
5. Kittel, A. (1999) *J. Histochem. Cytochem.* **47,** 393–400
6. Kittel, A., Kaczmarek, E., Sevigny, J., Lengyel, K., Csizmadia, E., and Robson, S. C. (1999) *Biochem. Biophys. Res. Commun.* **262,** 596–599
7. Zinchuk, V. S., Okada, T., and Kobayashi, T. (1999) *Cell Tissue Res.* **298,** 499–509
8. Koziak, K., Kaczmarek, E., Kittel, A., Sevigny, J., Blusztajn, J. K., Schulte Am Esch, J., 2nd, Imai, M., Guckelberger, O., Goepfert, C., Qawi, I., and Robson, S. C. (2000) *J. Biol. Chem.* **275,** 2057–2062
9. Zinchuk, V. S., Okada, T., and Seguchi, H. (2002) *Microsc. Res. Tech.* **58,** 427–431
10. Kittel, A., Pelletier, J., Bigonnesse, F., Guckelberger, O., Kordas, K., Braun, N., Robson, S. C., and Sevigny, J. (2004) *J. Histochem. Cytochem.* **52,** 861–871
11. Harder, T., and Simons, K. (1997) *Curr. Opin. Cell Biol.* **9,** 534–542
12. Simons, K., and Toomre, D. (2000) *Nat. Rev. Mol. Cell. Biol.* **1,** 31–39
13. Sprong, H., van der Sluijs, P., and van Meer, G. (2001) *Nat. Rev. Mol. Cell. Biol.* **2,** 504–513
14. Simons, K., and Ikonen, E. (1997) *Nature* **387,** 569–572
15. Helms, J. B., and Zurzolo, C. (2004) *Traffic* **5,** 247–254
16. Resh, M. D. (2004) *Subcell. Biochem.* **37,** 217–232
17. Fullekrug, J., and Simons, K. (2004) *Ann. N. Y. Acad. Sci.* **1014,** 164–169
18. Wang, T. F., Ou, Y., and Guidotti, G. (1998) *J. Biol. Chem.* **273,** 24814–24821
19. Schulte Am Esch, J., 2nd, Sevigny, J., Kaczmarek, E., Siegel, J. B., Imai, M., Koziak, K., Beaudoin, A. R., and Robson, S. C. (1999) *Biochemistry* **38,** 2248–2258
20. Drab, M., Verkade, P., Elger, M., Kasper, M., Lohn, M., Lauterbach, B., Menne, J., Lindschau, C., Mende, F., Luft, F. C., Schedl, A., Haller, H., and Kurzchalia, T. V. (2001) *Science* **293,** 2449–2452
21. Razani, B., Engelman, J. A., Wang, X. B., Schubert, W., Zhang, X. L., Marks, C. B., Macaluso, F., Russell, R. G., Li, M., Pestell, R. G., Di Vizio, D., Hou, H., Jr., Kneitz, B., Lagaud, G., Christ, G. J., Edelmann, W., and Lisanti, M. P. (2001) *J. Biol. Chem.* **276,** 38121–38138
22. Roper, K., Corbeil, D., and Huttner, W. B. (2000) *Nat. Cell Biol.* **2,** 582–592
23. Scheiffele, P., Verkade, P., Fra, A. M., Virta, H., Simons, K., and Ikonen, E. (1998) *J. Cell Biol.* **140,** 795–806
24. Vogel, U., Sandvig, K., and van Deurs, B. (1998) *J. Cell Sci.* **111,** 825–832
25. Francis, S. A., Kelly, J. M., McCormack, J., Rogers, R. A., Lai, J., Schneeberger, E. E., and Lynch, R. D. (1999) *Eur. J. Cell Biol.* **78,** 473–484
26. Poole, K., Meder, D., Simons, K., and Muller, D. (2004) *FEBS Lett.* **565,** 53–58
27. Christoforidis, S., Papamarcaki, T., Galaris, D., Kellner, R., and Tsolas, O. (1995) *Eur. J. Biochem.* **234,** 66–74
28. Matsumoto, M., Sakurai, Y., Kokubo, T., Yagi, H., Makita, K., Matsui, T., Titani, K., Fujimura, Y., and Narita, N. (1999) *FEBS Lett.* **453,** 335–340
29. Marcus, A. J., Broekman, M. J., Drosopoulos, J. H., Islam, N., Alyonycheva, T. N., Safier, L. B., Hajjar, K. A., Posnett, D. N., Schoenborn, M. A., Schooley, K. A., Gayle, R. B., and Maliszewski, C. R. (1997) *J. Clin. Invest.* **99,** 1351–1360
30. Smart, E. J., Ying, Y. S., Mineo, C., and Anderson, R. G. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 10104–10108
31. Song, K. S., Li, S., Okamoto, T., Quilliam, L. A., Sargiacomo, M., and Lisanti, M. P. (1996) *J. Biol. Chem.* **271,** 9690–9697
32. Klein, U., Gimpl, G., and Fahrenholz, F. (1995) *Biochemistry* **34,** 13784–13793
33. Lanzetta, P. A., Alvarez, L. J., Reinach, P. S., and Candia, O. A. (1979) *Anal. Biochem.* **100,** 95–97
34. Dupree, P., Parton, R. G., Raposo, G., Kurzchalia, T. V., and Simons, K. (1993) *EMBO J.* **12,** 1597–1605
35. Sevigny, J., Robson, S. C., Waelkens, E., Csizmadia, E., Smith, R. N., and Lemmens, R. (2000) *J. Biol. Chem.* **275,** 5640–5647
36. Dranoff, J. A., Kruglov, E. A., Robson, S. C., Braun, N., Zimmermann, H., and Sevigny, J. (2002) *Hepatology* **36,** 1135–1144
37. Strohmeier, G. R., Lencer, W. I., Patapoff, T. W., Thompson, L. F., Carlson, S. L., Moe, S. J., Carnes, D. K., Mrsny, R. J., and Madara, J. L. (1997) *J. Clin. Invest.* **99,** 2588–2601
38. Schuck, S., Honsho, M., Ekroos, K., Shevchenko, A., and Simons, K. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 5795–5800
39. Drobnik, W., Borsukova, H., Bottcher, A., Pfeiffer, A., Liebisch, G., Schutz, G. J., Schindler, H., and Schmitz, G. (2002) *Traffic* **3,** 268–278
40. Taylor, C. M., Coetzee, T., and Pfeiffer, S. E. (2002) *J. Neurochem.* **81,** 993–1004
41. Slimane, T. A., Trugnan, G., Van, I. S. C., and Hoekstra, D. (2003) *Mol. Biol. Cell* **14,** 611–624
42. Pike, L. J. (2004) *Biochem. J.* **378,** 281–292
43. Breuza, L., Garcia, M., Delgrossi, M. H., and Le Bivic, A. (2002) *Exp. Cell Res.* **273,** 178–186
44. Gratzinger, D., Canosa, S., Engelhardt, B., and Madri, J. A. (2003) *FASEB J.* **17,** 1458–1469
45. Gottlieb, T. A., Ivanov, I. E., Adesnik, M., and Sabatini, D. D. (1993) *J. Cell Biol.* **120,** 695–710
46. Maples, C. J., Ruiz, W. G., and Apodaca, G. (1997) *J. Biol. Chem.* **272,** 6741–6751
47. Wang, T. F., and Guidotti, G. (1996) *J. Biol. Chem.* **271,** 9898–9901
48. Kaczmarek, E., Koziak, 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
49. Simons, M., Keller, P., De Strooper, B., Beyreuther, K., Dotti, C. G., and Simons, K. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 6460–6464
50. Gimpl, G., Burger, K., and Fahrenholz, F. (1997) *Biochemistry* **36,** 10959–10974
51. Sowa, G., Pypaert, M., and Sessa, W. C. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 14072–14077
52. Kittel, A., Csapo, Z. S., Csizmadia, E., Jackson, S. W., and Robson, S. C. (2004) *Eur. J. Histochem.* **48,** 253–259
53. Bretscher, M. S., and Munro, S. (1993) *Science* **261,** 1280–1281
54. Kojro, E., Gimpl, G., Lammich, S., Marz, W., and Fahrenholz, F. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 5815–5820
55. Grinthal, A., and Guidotti, G. (2004) *Biochemistry* **43,** 13849–13858
56. Pike, L. J., and Casey, L. (1996) *J. Biol. Chem.* **271,** 26453–26456
57. Couet, J., Sargiacomo, M., and Lisanti, M. P. (1997) *J. Biol. Chem.* **272,** 30429–30438
58. Rhainds, D., Bourgeois, P., Bourret, G., Huard, K., Falstrault, L., and Brissette, L. (2004) *J. Cell Sci.* **117,** 3095–3105
59. Wood, W. G., Igbavboa, U., Rao, A. M., Schroeder, F., and Avdulov, N. A. (1995) *Brain Res.* **683,** 36–42
60. Megias, A., Martinez-Senac, M. M., Delgado, J., and Saborido, A. (2001) *Biochem. J.* **353,** 521–529
61. Ortega, A., Santiago-Garcia, J., Mas-Oliva, J., and Lepock, J. R. (1996) *Biochim. Biophys. Acta* **1283,** 45–50
62. Blair, A., Shaul, P. W., Yuhanna, I. S., Conrad, P. A., and Smart, E. J. (1999) *J. Biol. Chem.* **274,** 32512–32519
63. Millard, E. E., Srivastava, K., Traub, L. M., Schaffer, J. E., and Ory, D. S. (2000) *J. Biol. Chem.* **275,** 38445–38451
64. Mauch, D. H., Nagler, K., Schumacher, S., Goritz, C., Muller, E. C., Otto, A., and Pfrieger, F. W. (2001) *Science* **294,** 1354–1357
65. Simons, K., and Ehehalt, R. (2002) *J. Clin. Invest.* **110,** 597–603
66. Ledesma, M. D., Abad-Rodriguez, J., Galvan, C., Biondi, E., Navarro, P., Delacourte, A., Dingwall, C., and Dotti, C. G. (2003) *EMBO Rep.* **4,** 1190–1196
67. Ataman, O. V. (1991) *Fiziol. Zh.* **37,** 32–37
68. Lasley, R. D., and Smart, E. J. (2001) *Trends Cardiovasc. Med.* **11,** 259–263
69. Bhatnagar, A., Sheffler, D. J., Kroeze, W. K., Compton-Toth, B., and Roth, B. L. (2004) *J. Biol. Chem.* **279,** 34614–34623