Transmembrane Domains Confer Different Substrate Specificities and Adenosine Diphosphate Hydrolysis Mechanisms on CD39, CD39L1, and Chimeras†

Alison Grinthal* and Guido Guidotti

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

Received July 2, 2001; Revised Manuscript Received November 29, 2001

ABSTRACT: Members of the ecto-nucleoside triphosphate diphosphohydrolase (eNTPDase) family exhibit distinctive substrate specificities, but how such specificities are achieved by enzymes with identical putative catalytic domains is unknown. Previously we showed that H59G substitution changes CD39 from an apyrase to an adenosine diphosphatase (ADPase) in a manner that depends on intact associations of both transmembrane domains with the membrane. Here we show that the extracellular domain of CD39L1 ecto-adenosine triphosphatase (ecto-ATPase) has the same 3:1 ATP:ADP hydrolysis ratio as the extracellular domain of CD39, suggesting that the transmembrane domains are required to confer the native substrate specificities on each enzyme. As in CD39, H50G substitution has little effect on the activity of the CD39L1 extracellular domain or solubilized monomers. However, H50G substitution diminishes both ATPase and ADPase activities of native CD39L1, in contrast to its selective effect on ATPase activity in CD39, suggesting that the transmembrane domains confer different ADP hydrolysis mechanisms on CD39 and CD39L1. We then show that the transmembrane domains of CD39L1 can substitute for those of CD39 in conferring native CD39 substrate specificity and regulation of H59 but that the transmembrane domains of CD39 confer neither CD39 nor CD39L1 properties on the CD39L1 extracellular domain. These results suggest that non-apyrase conserved region residues in the extracellular domain contain the information specifying CD39 native properties but have a nonspecific requirement for two transmembrane domains to manifest the information.

Nucleotides are transported from the cytoplasm into the extracellular space and into the lumen of intracellular organelles (*1*−*5*), where they influence a variety of physiological functions including neuronal signaling, platelet clotting, vascular homeostasis, immune function, and protein and lipid modification (*6*−*10*). Nucleoside triphosphates (NTPs) and their derivatives nucleoside diphosphates (NDPs), nucleoside monophosphates (NMPs), and nucleosides act on overlapping groups of receptors and other regulatory systems with an array of additive and antagonistic effects (*11*−*14*). As a result, the overall state of each system is a function of both the total and relative local concentrations of NTPs and their derivatives. Since nucleotides cannot be transported back into the cytoplasm until all of their phosphates have been removed (*15*), nucleotide-hydrolyzing enzymes with extracytoplasmic active sites play a primary role in regulating the local concentrations of NTPs, NDPs, NMPs, and nucleosides.

Ecto-nucleoside triphosphate diphosphohydrolases (eNTPDases) are a ubiquitous family of ectoenzymes that specifically hydrolyze the terminal phosphoanhydride bonds of NTPs and NDPs (*16*, *17*). While all family members catalyze hydrolysis both of NTP to NDP and of NDP to NMP, the relative rate constants for these reactions vary significantly, resulting in enzymes that hydrolyze primarily NTPs, primarily NDPs, or both NTPs and NDPs. Initial efforts to understand the structural basis for differences in substrate specificity focused on five domains, called the apyrase conserved regions (ACRs), found in all eNTPDases (*18*, *19*). In particular, ACR1 and ACR4 are homologous to the phosphate binding loops of a family of cytoplasmic ATPases including actin and hsp70 and are therefore likely to contain catalytic residues (*18*, *20*−*24*). Previously we demonstrated that changing one amino acid in ACR1, H59, turns CD39 (now referred to as eNTPDase 1) from an apyrase to an ADPase (*25*). Smith et al. (*26*) also found that double mutation of D219 in ACR4 and W459 in ACR5 turns the related HB6 apyrase (eNTPDase 3) into an NTPase. Both studies point to amino acids in the ACRs as determinants of apyrase substrate specificity. However, related enzymes with different specificities have the same amino acids in their ACRs (*27*−*30*). The discovery that the effect of H59G substitution on specificity depends on the transmembrane domain and quaternary structure context (*25*) led to the idea that although these key residues are conserved, their contributions to specificity may not be; their roles in the ATPase and ADPase mechanisms may depend on the transmembrane domain context of the particular enzyme.

To begin to address the question of whether the interplay between ACR residues and transmembrane domains has anything to do with the specificity differences between different enzymes, we compared CD39 with the ATPase CD39L1 (eNTPDase 2). CD39L1 is also an ectoenzyme with

† This work was supported by Grant HL08893 from the National Institutes of Health.

* To whom correspondence should be addressed at 7 Divinity Ave., Cambridge, MA 02138. Phone 617-495-2399; fax 617-495-8308; e-mail agrinth@fas.harvard.edu.

10.1021/bi015563h CCC: $22.00
Published on Web 01/12/2002

two terminal transmembrane domains, has the same five ACRs as CD39 (*27*, *29*), and is thought to form oligomers (*31*), but it hydrolyzes primarily NTPs (*27*, *32*). NDPs are also substrates but with one-tenth to one-hundedth the hydrolysis rate of NTPs (*32*). We first sought to determine the contribution of the transmembrane domains and cytoplasmic tails to the total and relative ATPase and ADPase activities of CD39L1. In particular, we wondered whether CD39 monomers and CD39L1 monomers might have similar substrate specificities. We then looked at the effect of H50G substitution in CD39L1, the amino acid equivalent of H59G substitution in CD39, to determine whether H50 plays the same role in the ATPase and ADPase mechanisms of the two enzymes and how its role may depend on membrane context. The results of these experiments suggested that the transmembrane domains confer different substrate specificities and regulation of ACR1 on the two enzymes. We therefore created two sets of chimeric enzymes in which the extracellular domains of CD39 and CD39L1 with and without the HG mutation were attached to each other's transmembrane and cytoplasmic domains in order to investigate how the transmembrane domains confer specificity.

MATERIALS AND METHODS

Reagents. ATP and ADP were purchased from Sigma (St. Louis, MO). Triton X-100 was purchased from Calbiochem (La Jolla, CA) Fetal bovine serum, penicillin/streptomycin/L-glutamine, Dulbecco's modified Eagle medium (DMEM), and lipofectamine were purchased from Gibco−BRL (Gaithersburg, MD). Chemiluminescence reagents were purchased from Pierce (Rockford, IL).

DNA Construction. Cloning of human CD39L1 cDNA and insertion into the *Eco*RI site of pGW1 expression vector were performed by T.-F. Wang. Soluble CD39L1 was constructed by PCR amplification of the extracellular domain, including residues T29−S512, addition of a C-terminal hexaHis tag encoded by the 3′ end primer, and subcloning into a vector previously constructed by T.-F. Wang (*31*) containing the CD4 signal sequence. The H50G mutation was introduced into pGW1-CD39L1 by production of two overlapping PCR fragments each containing the sequence 5′-GGTTCTTCAG-GTACCTCCATG-3′, corresponding to ACR1 amino acids G47−M53 and including the H to G substitution as well as an introduced *Kpn*I site. These fragments were digested with *Kpn*I and ligated to each other, and the resulting fragment containing the H50G mutation was substituted for the corresponding fragment of wild-type pGW1-CD39L1. Soluble CD39L1 with the H50G substitution was generated by the same method as soluble CD39L1 with pGW1-CD39L1H50G as the template.

39EC was constructed by combining the extracellular domain of CD39 with the transmembrane domains and cytoplasmic tails of CD39L1. The amino acids at which CD39 and CD39L1 were attached to each other are shown in Figure 1. The CD39 extracellular domain was amplified with 5′ and 3′ primers containing part of the CD39L1 amino- and carboxy-terminal transmembrane domains, respectively. The 5′ primer contained sequence coding for the CD39L1 first transmembrane domain from upstream of the *Sfi*I site to the CCC corresponding to P28 followed by annealing sequence corresponding to T38−L43 at the beginning of the

Transmembrane domain 1

CD39

MEDIKDSKVKRFCSKN**IL I I LGFSSVLAVIALIAVGL** ↓ THNKPLPENVKYG

MAGKVRSLLPPLLLAAAG - - - - **LAGLLLLCVP** - ↓ TRDVREPPALKYG

CD39L1

Transmembrane domain 2

CD39

AEQP - LSPPLPHST ↓ **YISLMVLF - SLVLVAMV**ITGLFIFS - K - PSYFWKEAV

ADPPGLRKGTDFSS ↓ **WVVLLLLFASALLAALVLLL**RQVHSAKLPSTI

CD39L1

FIGURE 1: Splice sites of CD39 and CD39L1 used for construction of 39EC and L1EC. Arrows indicate the junctions at which CD39 and CD39L1 were attached in 39EC and L1EC (described under Materials and Methods). Residues after the arrows are T38 and Y486 for CD39 and T29 and W513 for CD39L1. Transmembrane residues are highlighted with boldface type, and extracellular residues are underlined.

CD39 extracellular domain. The 3′ primer contained sequence annealing to the sequence corresponding to CD39 extracellular amino acids P480−T485 followed by sequence coding for amino acids W513−L528 of the CD39L1 second transmembrane domain and containing silent substitutions leading to an *Nhe*I site in the sequence encoding CD39L1 amino acids L524−A526. The remaining part of the CD39L1 second transmembrane and cytoplasmic domains was amplified from pCDNA3-CD39L1 with a 5′ primer containing the same created *Nhe*I site and the Sp6 3′ primer. These PCR products were digested with *Nhe*I and ligated to each other, and the resulting fragment was digested with *Sfi*I and *Not*I and inserted into an *Sfi*I−*Not*I-digested pCDNA3-CD39L1 plasmid containing pCDNA3 and the CD39L1 coding sequence upstream of the *Sfi*I site.

39ECH59G was constructed by amplifying the extracellular domain of CD39H59G with the same primers as used above for wild-type CD39, digesting the product with *Sfi*I and *Nhe*I, and inserting it in place of the corresponding fragment of 39EC.

L1EC was constructed by combining the extracellular domain of CD39L1 with the transmembrane and cytoplasmic domains of CD39. The junctions between CD39 and CD39L1 amino acid sequences are the same as for 39EC and are shown in Figure 1. The extracellular domain of CD39L1 was amplified with a 5′ primer containing sequence encoding A34−L37 of the first CD39 transmembrane domain, including a *Hpa*I site created by silent mutation, followed by annealing sequence corresponding to T29−E34 of CD39L1 extracellular domain, and a 3′ primer composed of annealing sequence corresponding to G507−S512 of CD39L1 followed by sequence corresponding to Y486−V498 of the second CD39 transmembrane domain and including a *Spe*I site created by silent mutation in the sequence corresponding to

S494−V496. The first cytoplasmic and transmembrane domains of CD39 were amplified from pCIneo-CD39HA with T7EEV (Promega) as the 5′ primer and a 3′ primer annealing to the same region of CD39 as the 5′ primer described above and including the same introduced *Hpa*I site. The second transmembrane and cytoplasmic domains of CD39 were amplified with a 5′ primer annealing to the same region of CD39 as the 3′ primer described above and including the same introduced *Spe*I site, and T3 as the 3′ primer. These three products were ligated together by the *Hpa*I and *Spe*I sites, and the resulting fragment was inserted into pCIneo.

L1ECH50G was constructed by amplifying the first CD39 cytoplasmic and transmembrane domains and the CD39L1 extracellular domain as described above but with pGW1-CD39L1H50G instead of wild-type CD39L1 as template. These fragments were ligated via their *Hpa*I sites and inserted in place of the corresponding fragment of L1EC.

Preparation of COS7 Cell Crude Membranes. COS7 cells were transfected at 50−70% confluency by the lipofectamine method and harvested 72 h after transfection. Crude membranes were prepared by the method described in ref 33 and resuspended in 50 μL of 50 mM Tris-HCl, pH 7.8, per 100 mm plate.

Purification of Soluble CD39L1 and Soluble CD39L1H50G. Conditioned medium from three plates of transfected COS7 cells was centrifuged for 10 min at 3000 rpm in a clinical centrifuge and passed over a 1 mL ConA−Sepharose column. The column was then washed with 20 mL of a Tris-buffered saline (TBS) solution containing 1 mM each $CaCl_2$, $MgCl_2$, and $MnCl_2$. The resin was incubated in 2 mL of elution buffer (1 M α-methyl D-mannoside in the same solution) for 1 h at room temperature, and glycoproteins including soluble CD39L1 or soluble CD39L1H50G were eluted at room temperature. Protein was concentrated by centrifugation in a Centricon-30 for 90 min at 5000 rpm and diluted 1:200 for nucleotidase assays.

Detergent Solubilization. COS7 cell crude membranes were solubilized in 1% Triton X-100 or in 0.2% digitonin as described in ref 25. Solubilized protein was used immediately for nucleotidase assays or for sucrose density gradient sedimentation.

Sucrose Density Gradient Sedimentation. Proteins were loaded onto a 4.5 mL 5%−20% sucrose gradient containing 50 mM Tris-HCl, pH 7.8, and either 1% Triton X-100 or 0.2% digitonin and centrifuged in a SW50.1 rotor at 40 000 rpm for 14 h at 4 °C. Fractions (300 μL) were collected from the top of the gradient. Fraction 1 represents the least dense fraction.

Immunoblot and Antibodies. Polyclonal antibody against the extracellular domain of human CD39L1was generated in chicken by Lampire Biological Laboratories (Pipersville, PA). Antigen was produced by inserting cDNA sequence corresponding to residues T29−S512 into the bacterial expression vector pTrcHisC, transforming *Escherichia coli*, inducing expression with IPTG, extracting protein from inclusion bodies, and purifying the His-tagged protein on a nickel column. Rabbit polyclonal antibody raised against a KLH-couped peptide corresponding to the extreme C-terminus of human CD39L1 was a gift from T. L. Kirley. Rabbit polyclonal antibody generated against the extracellular domain of rat CD39 was as described in ref 31. COS7 cell membranes were solubilized in SDS buffer containing 100 mM DTT and boiled for 5 min prior to loading. Samples were run on an SDS−7.5% polyacrylamide gel, transferred to nitrocellulose membranes, blocked with 2% milk in TBS, and probed with primary antibody in 2% milk in TBS followed by goat anti-chicken or anti-rabbit horseradish peroxidase secondary antibody and visualized by chemiluminescence. Specific activities were calculated as amount of phosphate released per unit of overexpressed protein.

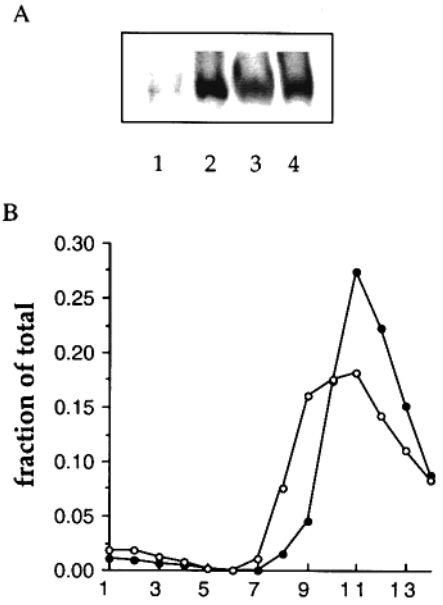

FIGURE 2: Relative activities and sedimentation properties of CD39L1 and mutants. (A) Crude membranes (for CD39L1 and CD39L1H50G) or partially purified concentrated medium (soluble CD39L1 and soluble CD39L1H50G) from COS7 cells transfected with each construct were run on an SDS−7.5% polyacrylamide gel and immunoblotted with a polyclonal antibody that recognizes the extracellular domain of CD39L1. Samples were loaded such that the ATPase activities corresponding to each lane are equal; thus the total membrane protein loaded in each lane varied inversely with the specific activity of the overexpressed protein. Relative amounts of protein determined by densitometric analysis are 1, 10, 5, and 5 for lanes 1, 2, 3, and 4, respectively. Lane 1, wild-type CD39L1; lane 2, CD39L1H50G; lane 3, soluble CD39L1; lane 4, soluble CD39L1H50G. (B) Crude membranes from CD39L1 (●) or CD39L1H50G (○) transfected COS7 cells were solubilized in 0.2% digitonin and loaded on top of a 5−20% sucrose gradient. Fractions were collected from the top and analyzed for ATPase activity. Fraction 1 represents the top of the gradient, and activities are expressed as fraction of total ATPase activity.

Nucleotidase Assays. ATPase and ADPase activities were measured at a range of calcium concentrations as described in ref 25.

RESULTS

CD39L1

Quantitation of Relative Activity per Unit of Protein. The relative amounts of overexpressed protein required for the same amount of ATPase activity were determined by densitometric analysis of an immunoblot with an antibody specific for the CD39L1 extracellular domain, as shown in Figure 2. Expression levels of wild-type and mutant proteins were equal (data not shown), so that the total protein loaded in each lane varies inversely with the specific activity of the wild-type or mutant construct. To control for possible

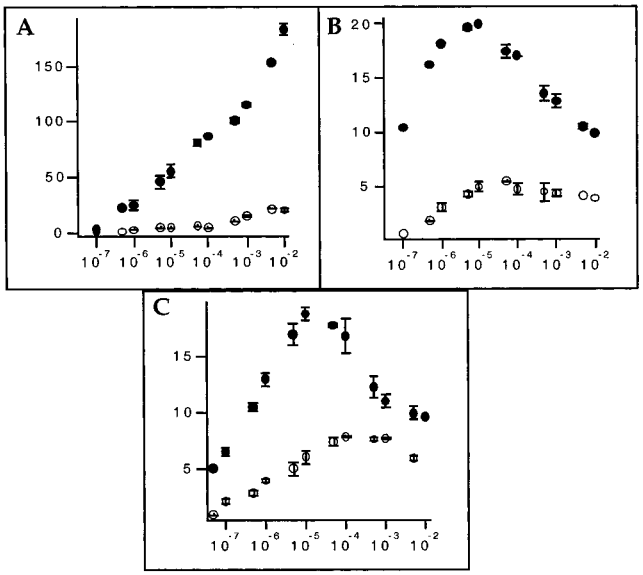

FIGURE 3: ATPase and ADPase activities of membrane-bound and soluble CD39L1. (A) Crude membranes were isolated from COS7 cells overexpressing wild-type CD39L1, and activity was determined at a series of free calcium concentrations with 2 mM ATP (●) and with 2 mM ADP (○) as substrate. Activities are expressed as percent of maximal ATPase activity of the first activation component, which has its peak at 5×10^{-4} M free calcium. (B) Membrane-bound CD39L1 was solubilized in 1% Triton X-100, and activity was determined as in panel A. (C) The extracellular domain of CD39L1 was fused to the CD4 signal sequence and secreted from COS7 cells. The resulting soluble enzyme was partially purified from COS7 cell medium, and activity was determined as in panel A.

differences in antibody recognition due to the H50G mutation, results for the two full-length proteins were verified by use of a polyclonal antibody that recognizes the C-terminal tail of CD39L1 (data not shown). The relative activity for each mutant was calculated by taking the ratio of the wild-type to the mutant band density. All activities are reported as percent of wild-type CD39L1 ATPase activity, as described for CD39 (*25*). Relative amounts of overexpressed protein shown in Figure 2 are 1, 10, 5, and 5 for CD39L1, CD39L1H50G, soluble CD39L1, and soluble CD39L1H50G, respectively.

Nucleotidase Activity of CD39L1. Since CD39 activity has been found to vary with free calcium concentration according to a biphasic curve (*34*, *25*), the ATPase and ADPase activities of CD39L1 were assayed at a series of free calcium concentrations calculated as described in ref 25. Figure 3A shows that both the ATPase and ADPase activities of CD39L1 also exhibit a biphasic dependence on calcium, but the second component for each is further activation rather than inhibition, with the juncture between the two components occurring between 10^{-4} and 10^{-3} M free calcium. The molecular basis for this second activation component is unknown; as is the case for the CD39 inhibitory component, it is unclear from these data whether the increase in free calcium, the increase in calcium−nucleotide complex, the decrease in free nucleotide, or another factor such as calcium-regulated interaction with another protein are responsible for the results. However, as discussed below, the second activation component exhibits one striking difference from the inhibitory component of CD39: it reverses when interactions with the membrane are interrupted. Crude membranes prepared from mock-transfected COS7 cells have no detectable ATPase or ADPase activity when assayed under the same conditions and at the same concentration as used in the overexpressed constructs. Therefore, the biphasic activity cannot be explained by endogenous ATPase or ADPase activity.

Although the presence of the second activation component that does not plateau under the conditions examined makes it difficult to define V_{max}, the relative hydrolysis rates for ATP and ADP can nevertheless be defined as approximately 10:1 for the respective first-component maxima as well as at corresponding free calcium concentrations along the second activation component. These results verify that the distinction between CD39 and CD39L1 substrate specificities is valid across the range of calcium concentrations. For comparison between wild-type and mutant CD39L1 activities, we have defined 100% as V_{max} for the first component of wild-type ATPase activity

Solubilization of CD39L1 in 1% Triton X-100. We then asked how disruption of membrane interactions and intersubunit transmembrane domain associations affects CD39L1 activity. Figure 3B shows that, similar to CD39 (*25*), CD39L1 loses approximately 75% of its ATPase activity when solubilized in 1% Triton X-100, based on comparison of V_{max} for the first component. In contrast, Triton X-100 solubilization has little effect on the first component of ADPase activity, unlike CD39, which loses more than 90% of its ADPase activity when solubilized. As a result, the ratio of ATPase to ADPase activities becomes 3:1. Furthermore, the calcium profiles show that the second activation components for both ATPase and ADPase activities of CD39L1 are reversed by detergent, so that in Triton X-100 each exhibits one activation component followed by an inhibitory component at higher free calcium concentrations.

These results have several implications. First, they demonstrate that intact quaternary structure and membrane associations are required for the full activities of both CD39 and CD39L1. Second, they indicate that two key distinctions between CD39 and CD39L1, the nature of the second component of the calcium titration curve and the relative hydrolysis rates of ATP and ADP, disappear when the native membrane structures are disrupted. CD39 starts with a 1.4:1 ATPase:ADPase ratio and loses a greater percentage of ADPase than ATPase activity to end up with a 3:1 ratio in its solubilized form (*25*), while CD39L1 loses ATPase but little ADPase activity to arrive at the same 3:1 ratio in its solubilized form. Thus, transmembrane interactions are required to manifest the different substrate specificities of CD39 apyrase and CD39L1 ATPase.

Soluble CD39L1. Since CD39L1 has a region of hydrophobic residues in its extracellular domain, we constructed a secreted soluble form of the enzyme containing only the extracellular domain to verify that the observed effects of Triton solubilization were not due to interactions of Triton X-100 with the extracellular domain. As shown in Figure 3C, relative ATP:ADP hydrolysis rates were the same as those observed for Triton X-100-solubilized CD39L1, as were the presence of activation and inhibition components of calcium dependence. Thus, in the absence of detergent and with their transmembrane domains and cytoplasmic tails removed, CD39 and CD39L1 have similar ATPase:ADPase ratios and calcium profiles.

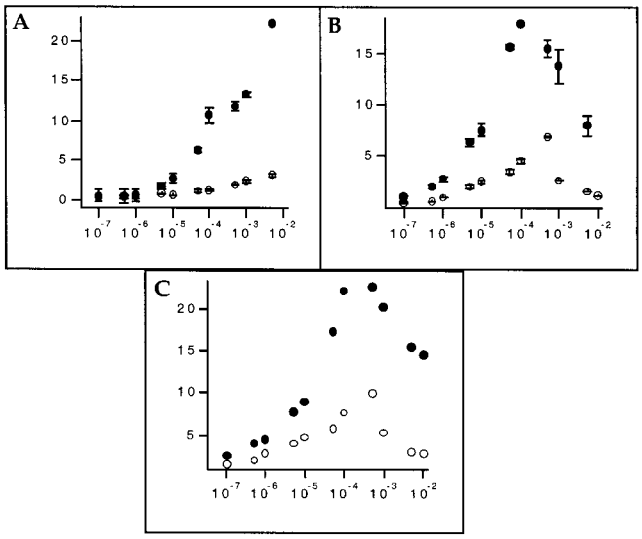

FIGURE 4: ATPase and ADPase activities of membrane-bound and soluble CD39L1H50G. (A) Crude membranes were isolated from COS7 cells overexpressing CD39L1H50G, and activity was determined at a series of free calcium concentrations with 2 mM ATP (●) and with 2 mM ADP (○) as substrate. Activities are expressed as percent of maximal ATPase activity of the first activation component, which has its peak at 5×10^{-4} M free calcium. (B) Membrane-bound CD39L1H50G was solubilized in 1% Triton X-100, and activity was determined as in panel A. (C) The extracellular domain of CD39L1H50G was fused to the CD4 signal sequence and secreted from COS7 cells. The resulting soluble enzyme was partially purified from COS7 cell medium, and activity was determined as in panel A.

H50G Substitution in Full-Length CD39L1. Given our previous observation that H59, an ACR1 residue, plays dramatically different roles in the relative ATPase:ADPase activities of CD39 depending on whether the two transmembrane domains are intact or disrupted (*25*), we asked how the transmembrane domains of CD39L1 regulate the role of the corresponding residue H50 in ACR1 of CD39L1. In particular, we wondered whether transmembrane domains might contribute to specificity by conferring a role on H50 that differs from that of H59 in native CD39. Since H59G substitution converts CD39 into an ADPase by diminishing primarily ATPase activity, we expected that if the transmembrane domains of CD39L1 regulate the role of H50 in a similar manner, then introducing an H50G substitution into native CD39L1 would likewise decrease both the total ATPase activity and the ATPase:ADPase activity ratio.

Instead, we found that in the full-length membrane-associated CD39L1, H50G substitution has no effect on the ATPase:ADPase activity ratio. Figure 4A shows that CD39L1 with the H50G substitution looks almost identical to CD39L1 in terms of substrate specificity and calcium-dependent two-component activation profile. In contrast, the specific activity as determined by Western blot analysis of crude membranes appears to be diminished by approximately 90% for both ATPase and ADPase activities. As shown in Figure 2B, sucrose density gradient sedimentation of membranes solubilized in 0.2% digitonin, a detergent that has been shown to preserve the intersubunit associations of CD39 and CD39L1 oligomers (*31*), indicated that CD39L1H50G forms oligomers of the same size as CD39L1. Although a small portion of CD39L1H50G activity sediments at a slightly lower density than CD39L1, neither the shift nor the amount of activity is sufficient to account for the effects of the mutation. These data suggest that although H50 is conserved throughout both the ectoapyrase and ecto-ATPase subfamilies, the mechanistic role of this residue is conserved with respect to ATPase activity but not with respect to ADPase activity. It is required for ATP hydrolysis by both CD39 and CD39L1 but is required for ADP hydrolysis only in CD39L1.

Table 1: Summary of V_{max} and ATPase:ADPase Ratios for CD39L1 and Derivatives[a]

	ATP	ADP	ATP:ADP
CD39L1	100	9.0 ± 0.5	11
CD39L1 in 1% Triton	20 ± 0.4	5.5 ± 0.3	3.6
soluble CD39L1	19 ± 0.6	7.1 ± 0.3	2.6
CD39L1H50G	12 ± 0.9	1.3 ± 0.1	9.2
CD39L1H50G in 1% Triton	18 ± 1.5	6.9 ± 0.2	2.6
soluble CD39L1H50G	22 ± 2.6	9.9 ± 1.2	2.3

[a] ATP and ADP values represent V_{max} for the first component of activation and are expressed as percent of wild-type CD39L1 ATPase activity.

Triton X-100-Solublized CD39L1H50G. To determine the relationship between transmembrane domains and H50, we solubilized CD39L1H50G membranes in 1% Triton X-100. As shown in Figure 4B, total ATPase and ADPase activities were approximately equal to those of Triton-solubilized CD39L1, indicating that, as is the case in CD39, H50G mutation has minimal or no effects on V_{max} activity in the solubilized context. Thus, as for CD39, the role of H50G depends on intact transmembrane associations; H50 appears to play a crucial part in both ATP and ADP hydrolysis mechanisms in membrane-bound oligomers but almost no role in either mechanism in solubilized monomers. As observed for solubilized CD39H59G (*25*), H50G substitution appears to shift the apparent K_m for free calcium slightly to the left, resulting in slight variations in the effect on ATPase:ADPase ratio at low calcium concentrations.

Nevertheless, unlike solubilized CD39, in which H59G substitution results in a 2-fold increase in ADPase:ATPase ratio, H50G substitution has little effect on the ADPase:ATPase V_{max} ratio of solubilized CD39L1. This difference suggests that the role of H50 in determining substrate specificity may also be modulated by differences intrinsic to the extracellular domain.

To verify that H50G substitution did not stabilize intersubunit interactions in the presence of Triton X-100, we performed sucrose density gradient sedimentation on Triton X-100-solubilized CD39L1 and CD39L1H50G. Both enzymes sedimented in fractions consistent with the monomeric form (data not shown).

Soluble CD39L1H50G. In addition to verifying the monomeric state of Triton X-100-solubilized CD39L1 and CD39L1H50G, we constructed a secreted form of CD39L1H50G identical to soluble CD39L1 except for the point mutation to exclude the possibility that the observed properties of solubilized monomers, in particular the differences between the ADPase:ATPase ratios of CD39L1H50G and CD39H59G, were due to interactions of the extracellular domain with Triton X-100. Figure 4C shows that the substrate specificity and kinetic properties of the secreted form were similar to those of Triton X-100-solubilized monomers, as well as to those of secreted soluble CD39L1. Table 1 summarizes the results for native and soluble CD39L1 with intact ACR1 and H50G substitution.

CD39 and CD39L1 Chimeras

Construction of Chimeric Enzymes. These results, in conjunction with our previous report on the relationships among H59, transmembrane domains, and ATPase:ADPase ratio in CD39 (*25*), suggest that transmembrane domains confer different substrate specificities, roles of H59 or H50, and ADP hydrolysis mechanisms on CD39 and CD39L1. The results thus raise the question of how their different transmembrane and cytoplasmic domains confer specificity; is the information contained in the transmembrane and cytoplasmic domains themselves, or are these domains required to manifest differences in extracellular sequence? If the second, does each enzyme require its own transmembrane and cytoplasmic domains, or is a different set of transmembrane domains sufficient to confer the high activity and substrate specificity of the native state?

To investigate how transmembrane and cytoplasmic domains contribute to the activity and specificity of CD39 and CD39L1, we constructed two chimeric enzymes in which the extracellular domains of each protein were attached to the transmembrane and cytoplasmic domains of the other. As shown in Figure 5A, 39EC is composed of the extracellular domain of CD39 and the transmembrane and cytoplasmic domains of CD39L1, while L1EC is composed of the extracellular domain of CD39L1 and the transmembrane and cytoplasmic domains of CD39. To investigate directly the relations between transmembrane domains and H59 or H50, we also constructed versions of 39EC and L1EC containing the H59G or H50G substitution, respectively.

39EC Activity. In studying 39EC, we asked whether the transmembrane domains of CD39L1 are sufficient to confer the properties of CD39L1 on the CD39 extracellular domain, whether they can replace CD39's own transmembrane and cytoplasmic domains in conferring CD39 membrane-bound properties, or whether the CD39 extracellular domain retains its soluble properties as in other situations in which it lacks its full-length membrane-bound structure.

We found that CD39L1's transmembrane and cytoplasmic domains confer nearly the same substrate specificity on the CD39 extracellular domain as do CD39's own transmembrane and cytoplasmic domains. Relative amounts of CD39, 39EC, and 39ECH50G protein required for equal ATPase activities are 1, 2, and 8 as determined by densitometric analysis of the immunoblot shown in Figure 5B. Wild-type and mutant proteins were expressed at equal levels (data not shown), so that the amount of total membrane protein loaded in each lane is inversely proportional to the specific activity of the overexpressed construct. As shown in Figure 6, the relative ATP and ADP hydrolysis rates are approximately 1.6:1, closer to the 1.4:1 ratio observed for full-length CD39 than to the 10:1 ratio of CD39L1 or the 3:1 ratio of the CD39 extracellular domain alone. 39EC also follows the calcium dependence pattern characteristic of CD39, with an inhibitory component at high free calcium concentrations, rather than that of CD39L1 (data not shown). CD39L1's transmembrane and cytoplasmic domains increase the ATPase activity of the CD39 extracellular domain relative to that of Triton X-100-solubilized CD39, although not as efficiently as CD39's own corresponding domains; 39EC ATPase activity is only 52% of full-length CD39 membrane ATPase activity.

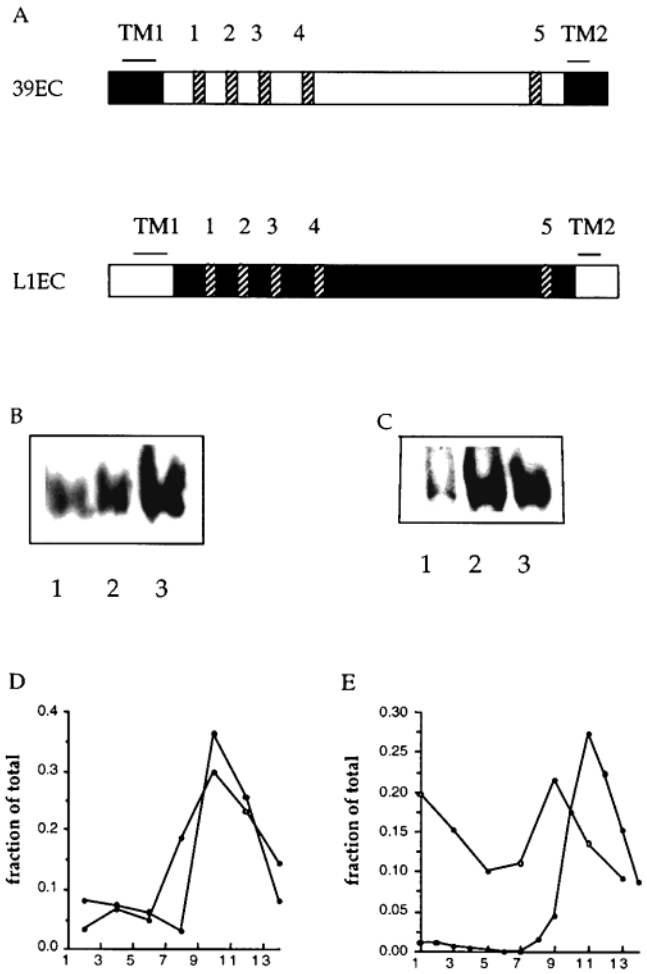

FIGURE 5: Construction and activities of 39EC and L1EC chimeras. (A) 39EC contains the extracellular domain of CD39 (white) and the transmembrane domains (TM1 and TM2) and cytoplasmic tails of CD39L1 (black). L1EC contains the extracellular domain of CD39L1 (black) and the transmembrane domains (TM1 and TM2) and cytoplasmic tails of CD39 (white). Hatched regions represent ACR1−5. (B) Crude membranes from COS7 cells transfected with CD39 (lane 1), 39EC (lane 2), or 39ECH59G (lane 3) were run on an SDS−7.5% polyacrylamide gel and immunoblotted with a polyclonal antibody that recognizes the extracellular domain of CD39. Samples were loaded such that the ATPase activities corresponding to each lane are the same; thus the total membrane protein loaded in each lane varies inversely with the specific activity of the overexpressed protein. Relative amounts of protein as determined by densitometric analysis are 1, 2, and 8 for lanes 1, 2, and 3, respectively. (C) Crude membranes from COS7 cells transfected with CD39L1 (lane 1), L1EC (lane 2), or L1ECH50G (lane 3) were run on an SDS−7.5% polyacrylamide gel and immunoblotted with a polyclonal antibody that recognizes the extracellular domain of CD39L1. The ATPase activities corresponding to lanes 2 and 3 are half that corresponding to lane 1; the total membrane protein loaded in each lane varies accordingly. Relative amounts of protein as determined by densitometric analysis are 1, 11, and 6 for lanes 1, 2, and 3, respectively. (D) Crude membranes from CD39 (●) or 39EC (○) transfected COS7 cells were solubilized in 0.2% digitonin and loaded on top of a 5−20% sucrose gradient. Fractions were collected from the top and analyzed for ATPase activity. Fraction 1 represents the top of the gradient, and activities are expressed as fraction of total ATPase activity. (E) Crude membranes from COS7 cells transfected with CD39L1 (●) or L1EC (○) were solubilized and analyzed by sucrose density gradient sedimentation as described for panel D. Activities are expressed as fraction of total ATPase activity.

To determine whether 39EC formed oligomers as effectively as CD39, we solubilized 39EC in 0.2% digitonin,

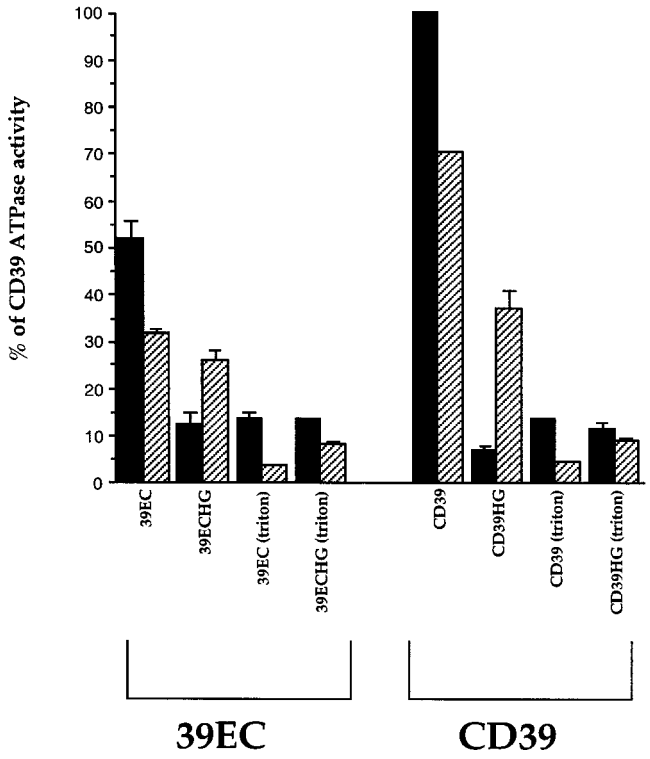

FIGURE 6: Summary of V_{max} data for 39EC and derivatives and comparison with CD39. 39EC, a chimeric enzyme composed of the extracellular domain of CD39 fused to the transmembrane and cytoplasmic domains of CD39L1, was expressed in COS7 cells. A mutant form of 39EC containing an H59G substitution in ACR1 of the CD39 extracellular domain (39ECH59G) was also expressed in COS7 cells. Maximal ATPase (black bars) and ADPase (hatched bars) activities of 39EC and 39ECH59G in membrane and solubilized with 1% Triton X-100 are expressed as percent of wild-type CD39 maximal ATPase activity. For comparison of activity, the role of H59, and detergent sensitivity of the CD39 extracellular domain in the presence of the CD39L1 transmembrane domains versus the native CD39 transmembrane domains, the corresponding data for wild-type CD39 and CD39H59G in membrane and solubilized in 1% Triton X-100 (data from ref *25*) are shown.

a detergent that has been shown not to disrupt intersubunit associations, and performed sucrose density gradient sedimentation. As shown in Figure 5D, 39EC sedimented at the same density as CD39, indicating oligomer formation.

Solubilized 39EC. We further investigated the contribution of CD39L1's transmembrane and cytoplasmic domains to the total ATPase and ADPase activities of 39EC by comparing 39EC in membrane and solubilized in 1% Triton X-100. The results of these experiments support the above findings based on immunoblot comparisons of CD39 and 39EC that the CD39L1 transmembrane and cytoplasmic domains increase CD39 extracellular domain activity. Figure 6 shows that Triton X-100 solubilization results in the loss of approximately 75% of ATPase activity and 88% of ADPase activity, compared to the loss of 86% of ATPase activity and 94% of ADPase activity that occurs when CD39 is solubilized. These results differ from other situations in which the CD39 extracellular domain is membrane-bound and oligomerized in nonnative ways, either by its amino-terminal transmembrane domain alone or by its carboxy-terminal transmembrane domain alone. Under the latter conditions CD39 activity is insensitive to Triton X-100 solubilization, indicating that the observed oligomer formation and membrane association do not enhance activity (*25*, *31*). Thus the presence of two transmembrane domains, even if they come from a different enzyme, appears to be required for membrane-associated enhancement of CD39 activity relative to its soluble form.

H59G Substitution in 39EC. We next asked whether CD39L1's transmembrane domains regulate the role of the CD39 ACR1 residue H59 in the same way as do CD39's own transmembrane domains. We therefore constructed a version of 39EC containing the same H59G substitution in the extracellular domain as that contained in CD39H59G discussed in ref 25. In contrast to 39EC, which has approximately the same ATPase:ADPase activity ratio of 1.4:1 as native CD39, CD39H59G had an ADPase:ATPase ratio of 6:1 while 39ECH59G has a ratio of only 2:1, as shown in Figure 6. This result nevertheless qualitatively resembles the effect of H59G substitution in CD39; ATPase activity is diminished to 24% and ADPase activity to 82% of 39EC activities, indicating that H59G substitution primarily diminishes ATPase activity, as in CD39H59G, which retains 7% of ATPase and 52% of ADPase activities relative to wild-type CD39.

39ECH59G Solubilized in Triton X-100. Similar to the effect of solubilization on CD39H59G, dissociation of 39ECH59G to monomers by solubilization in 1% Triton X-100 had no significant effect on ATPase activity and decreased ADPase activity to 30% of its membrane-bound activity, as shown in Figure 6. The resemblance of these changes to those of CD39H59G, as summarized in Figure 6, suggests that the transmembrane and cytoplasmic domains of CD39L1 regulate the role of H59 in a manner similar to CD39's own transmembrane and cytoplasmic domains.

LIEC Activity. While the above results demonstrate that CD39L1's transmembrane and cytoplasmic domains can substitute for those of CD39 in conferring CD39 substrate specificity and membrane-associated high activity, we sought to determine whether CD39L1 can likewise use foreign transmembrane and cytoplasmic domains to achieve its native properties. We therefore constructed the inverse chimeric enzyme, called L1EC, composed of the extracellular domain of CD39L1 and the transmembrane and cytoplasmic domains of CD39, as shown in Figure 5A. As shown in Figure 5C, relative amounts of protein required for equal amounts of ATPase activity are 1, 11, and 6 for CD39L1, L1EC, and L1ECH50G, respectively, as determined by densitometric analysis. Equal amounts of wild-type and chimeric proteins were expressed, so that the total membrane protein loaded in each lane is inversely proportional to the specific activity of the overexpressed protein. As shown in Figure 5E, sucrose density gradient sedimentation of L1EC solubilized in 0.2% digitonin indicated that oligomer formation was less efficient than that of CD39L1 and that a portion of the activity remained at the top of the gradient. Unlike 39EC, L1EC activity in membranes was not only lower than that of native CD39L1 but also approximately 4-fold lower than that of Triton X-100-solubilized CD39L1 monomers. As shown in Table 2, the substrate specificity, an approximately 2:1 ATPase:ADPase ratio, more closely resembled that of monomeric CD39L1 than that of either native CD39L1 or native CD39. Thus it appears that CD39's transmembrane and cytoplasmic domains are not sufficient to confer the substrate specificity or the high activity of either native

Table 2: V_{max} and ATPase:ADPase Ratios for CD39L1 and L1EC[a]

	ATP	ADP	ATP:ADP
CD39L1	100	9.0 ± 0.5	11
CD39L1 in 1% Triton	20 ± 0.4	5.5 ± 0.3	3.6
L1EC	4.7 ± 0.3	2.7 ± 0.6	1.8
L1EC in 1% Triton	20 ± 1.2	6.8 ± 0.8	2.9
L1ECH50G	7.9 ± 0.3	4.3 ± 0.1	1.8
L1ECH50G in 1% Triton	19 ± 0.7	7.2 ± 0.2	2.6

[a] L1EC, a chimeric enzyme composed of the extracellular domain of CD39L1 attached to the transmembrane and cytoplasmic domains of CD39, was assayed in crude membranes as well as after solubilization with 1% Triton X-100. A mutant form of L1EC containing an H50G substitution in ACR1 of the CD39L1 extracellular domain (L1ECH50G) was analyzed in the same manner. ATP and ADP values represent V_{max} for the first component of activation and are expressed as percent of wild-type CD39L1 ATPase activity.

CD39L1 or native CD39 on the CD39L1 extracellular domain.

L1EC Solubilized in Triton X-100. To confirm that CD39's transmembrane and cytoplasmic domains did not enhance CD39L1 extracellular domain activity, we compared L1EC activity in membranes and solubilized in 1% Triton X-100. Table 2 shows that, in contrast to the other membrane-bound enzymes discussed, L1EC ATPase and ADPase activities increased approximately 4-fold upon Triton X-100 solubilization. Solubilization thus brought L1EC activity up to the level of Triton X-100-solubilized CD39L1. In conjunction with the specific activity determination, these results suggest that CD39L1 requires its own transmembrane and cytoplasmic domains to exhibit its native membrane-associated substrate specificity and high activity and that substituting the corresponding domains of CD39 not only fails to replace those of CD39L1 as well as to confer CD39 properties but also reduces the activity to below the intrinsic activity of the extracellular domain.

L1ECH50G. As might be expected on the basis of monomeric CD39L1 and monomeric CD39L1H50G, L1ECH50G exhibited similar substrate specificity and total ATPase and ADPase activities to L1EC, as shown in Table 2. Triton solubilization increased L1ECH50G activity to that of Triton X-100-solubilized CD39L1H50G. These data are consistent with the observation for L1EC that CD39's transmembrane and cytoplasmic domains fail to confer native properties of either CD39L1 or CD39 and thus to regulate the role of H50G in the membrane-bound manner characteristic of either native enzyme.

DISCUSSION

These studies demonstrate that the substrate specificity and total activity of CD39L1 ecto-ATPase are regulated by its transmembrane domains and cytoplasmic tails. Like its counterpart, CD39 ectoapyrase, CD39L1 forms oligomers via intersubunit transmembrane domain interactions, and oligomer dissociation or removal of the transmembrane domains reduces total activity and changes its substrate specificity from the 10:1 ATPase:ADPase activity ratio characteristic of an ATPase to 3:1, closer to that of an apyrase. As discussed in ref 25, CD39 also undergoes a change in substrate specificity to arrive at the same 3:1 ATPase:ADPase ratio upon dissociation to monomers. These data suggest that the extracellular domains of CD39 and CD39L1 have the same substrate specificities and that their respective transmembrane domains confer the distinctive specificities of the native proteins.

Given the earlier finding that the transmembrane domains of CD39 regulate its substrate specificity by regulating the role of H59 in ACR1 (*25*), we sought to determine whether different regulation of this residue in CD39L1 might mediate the ability of transmembrane domains to confer different specificities. We found that, as in CD39, the role of the equivalent ACR1 residue H50 in CD39L1 is regulated by its transmembrane domains but that the implications for substrate specificity differ from those in CD39. As in CD39, H50 is required for ATP hydrolysis in native membrane-bound CD39L1, as indicated by the approximately 90% loss of activity with H50G substitution, but is not required for ATPase activity in solubilized monomers or in the secreted extracellular domain. These results suggest that, with respect to ATPase mechanisms, the transmembrane domains of CD39 and CD39L1 regulate the role of H59 or H50 in similar ways in the two enzymes. However, the effect of transmembrane domains on the role of H50 in the ADPase activity of CD39L1 differs significantly from that of H59 in the ADPase activity of CD39. While H50/59 is not required for ADP hydrolysis in the monomeric forms of either enzyme, it is required for ADP hydrolysis by native CD39L1 but not by native CD39. In fact, H50 seems to be involved equally in both the ATPase and ADPase activities of CD39L1, so that H50G substitution does not change the substrate specificity.

Transmembrane domains may therefore confer different specificities by conferring different ADP hydrolysis mechanisms on CD39 and CD39L1. We previously proposed that, in native CD39, ATP and ADP hydrolysis occur by different mechanisms distinguished by different substrate interactions with ACR1 (*25*), but no such difference is found for native CD39L1 on the basis of studies of the role of H50. A difference in the relation between the ATP and ADP hydrolysis mechanisms in native CD39 and CD39L1 may also be supported by studies by Heine et al. (*32*). Their work shows that when ATP is hydrolyzed to completion by CD39, no ADP accumulates in the process; only a decrease in ATP and a subsequent appearance of AMP occur, despite the fact that only one phosphate is hydrolyzed at a time as indicated by the absence of pyrophosphate. In contrast, when CD39L1 hydrolyzes ATP to completion, ADP accumulates and is subsequently hydrolyzed to AMP. These data suggest that in CD39 the ATPase and ADPase mechanisms are such that both can occur in succession without dissociation of substrate from the enzyme, while in CD39L1 the two mechanisms are such that substrate dissociation and rebinding to the enzyme are necessary. The finding by Chen and Guidotti (*35*) that the soluble extracellular domain of CD39 exhibits intermediate release and rebinding of ADP suggests that transmembrane context may play a role in regulating this distinction.

While H50G substitution experiments emphasize regulation of the ADP hydrolysis mechanism as a way for transmembrane domains to confer specificity, the calcium profiles of native and soluble CD39L1 suggest additional transmembrane-mediated differences between CD39 and CD39L1. In particular, the calcium dependence profiles for native CD39L1 ATPase and ADPase activities have two activation components, in contrast to the activation followed

by inhibition components observed for CD39 ATPase and ADPase activities. While the molecular basis for the kinetic profile remains to be determined and could represent responses to the increase in free calcium, to the increase in calcium−nucleotide complex, or to the decrease in free nucleotide by either the enzyme itself or by an associated protein, the opposite responses to high calcium concentration suggest mechanistic differences not identified by H59G and H50G substitution. How such differences might relate to substrate specificity is unclear, although the native CD39L1 calcium profile is regulated by transmembrane context in parallel with substrate specificity.

On the basis of this evidence that the transmembrane and cytoplasmic domains of CD39L1 ecto-ATPase and CD39 ectoapyrase confer the characteristic native substrate specificities on their respective extracellular domains, we asked whether membrane-associated specificity is a function of information contained in the transmembrane domains themselves. We found that the transmembrane and cytoplasmic domains of CD39L1 do not turn the CD39 extracellular domain into an ATPase; rather, they confer native CD39 properties and regulate the mechanistic role of the ACR1 residue H59 in the manner characteristic of the native transmembrane structure of CD39. In contrast, the transmembrane and cytoplasmic domains of CD39 confer neither CD39 nor CD39L1 properties on the CD39L1 extracellular domain.

The results for the first case are in contrast to previous alterations of CD39 transmembrane structure that preserved oligomer formation (*31*), namely, removal of the amino- or carboxy-terminal transmembrane domains. In both of these cases CD39 exhibited monomerlike specificity, total activity, and mechanistic role of the ACR1 residue H59 (*25*). Thus the ability of the CD39L1 transmembrane domains to confer native properties on CD39 is unlikely to be based solely on their capacity to anchor the extracellular domain in the membrane or even to direct oligomer formation. Rather, in conjunction with the results of Wang et al. (*31*) and of Grinthal and Guidotti (*25*) supporting a requirement for both CD39 transmembrane domains, these results suggest that two transmembrane domains are indeed required for native properties but that they do not have to come from CD39. Thus, for CD39, the information that determines substrate specificity and role of H59 appears to be in the extracellular domain and to have a nonspecific requirement for two transmembrane domains for manifestation.

On the other hand, CD39L1 appears to have a more specific requirement for its own transmembrane and cytoplasmic domains, despite the fact that these domains themselves do not appear to encode ecto-ATPase specificity as discussed above. The possibility has not been ruled out that a slight shift in the residues chosen as the boundaries between transmembrane and extracellular domains might significantly change the properties. Nevertheless, the apparent difference between the transmembrane requirements of CD39 and CD39L1 parallels previous observations with efforts to reconstitute Triton X-100-solubilized monomers in digitonin. While both enzymes could be induced to form oligomers in digitonin, CD39 more effectively regained native properties while CD39L1 continued to resemble the monomeric form in substrate specificity and kinetic profile (data not shown). Whether these results reflect different requirements for additional proteins or for other membrane components is unknown, but they are consistent with the possibility that CD39L1 and CD39 may depend on their transmembrane domains in different ways. CD39L1 also has a larger extracellular hydrophobic region than does CD39 (*27*); although this region is not predicted to cross the membrane, it might govern enzyme-specific membrane interactions in conjunction with the transmembrane domains. In combination with our data, two chimeras reported by Heine et al. (*36*), in which replacement of the amino-terminal transmembrane domain of CD39L1 with that of CD39 abolished activity and replacement of the carboxy-terminal transmembrane domain with that of CD39 had only a minor effect on activity, imply that the amino terminus but not the carboxy terminus may be specifically required.

The structural basis for the requirement of CD39 for two transmembrane domains is unknown. These experiments rule out a specific relation between hydrolysis mechanisms and specific features of the CD39 transmembrane or cytoplasmic domains, as well as the idea that the different transmembrane domains of the ecto-ATPase and the ectoapyrase might mediate different arrangements of subunits leading to different specificities, as is the case for two-transmembrane-domain potassium channels (*37*, *38*). One model that might explain the nonspecific contribution of two transmembrane domains to substrate specificity, at least in the case of the ectoapyrases, is suggested by the finding that residues near each transmembrane domain play significant roles in specificity determination. Smith et al. (*26*) showed that mutation of a conserved Trp in ACR5, and to a greater extent double mutation of this residue and a conserved Asp in ACR4, turned an apyrase into an ATPase. In conjunction with our finding that mutation of H59 in ACR1 turned CD39 apyrase into an ADPase, these results suggest that specificity may be determined by a combination of residues in ACR 1 and 4, near the amino-terminal transmembrane domain, and in ACR5, near the carboxy-terminal transmembrane domain. A report that one antibody to CD39 recognizes a split epitope, including regions that appear to coincide with ACR3 and ACR5 (*39*), suggests that these regions are near each other in the native enzyme. Thus, two transmembrane domains may be necessary to position their adjacent extracellular regions in the correct orientation for concerted action. Such a function could conceivably be performed by foreign transmembrane domains if the main requirement is either double rooting in the membrane or association between amino- and carboxy-terminal transmembrane domains. Since the ACR residues in both halves are conserved between ATPases and apyrases, extracellular residues outside the ACRs must also be involved directly or indirectly in these interactions and, at least in the case of CD39, are likely to carry the information for specificity determination. Consistent with this idea, Heine et al. (*36*) recently demonstrated that the region between ACR4 and ACR5 is important to the distinction between the substrate specificities of CD39 and CD39L1.

Regardless of the specific features of the extracellular domain required for native properties, the present evidence for a nonspecific requirement for two transmembrane domains supports the idea that the extracellular domain itself does not inherently assume the native conformation; rooting both ends in the membrane appears to alter the energetics

from what might putatively be considered a relaxed state to a taut state. Our results suggest that the latter is a prerequisite for the distinction between the apyrase and ATPase properties.

In summary, these studies indicate that, although the transmembrane domains confer the different substrate specificities of CD39 and CD39L1, the transmembrane domains do not independently carry sufficient information to specify one type of enzyme or the other. Rather, the information determining the apyrase properties of CD39 is intrinsic to the extracellular domain but requires two transmembrane domains for manifestation, as suggested by the finding that the transmembrane and cytoplasmic domains of CD39L1 can substitute for those of CD39. In contrast, although the transmembrane and cytoplasmic domains of CD39L1 confer no ATPase properties on CD39, they contribute in a specific manner to the ATPase specificity of CD39L1, as indicated by the inability of the transmembrane and cytoplasmic domains of CD39 to replace them. We suggest that double rooting in the membrane changes the energetic state of the extracellular domain and is a prerequisite for native properties and thus for the distinction between apyrase and ATPase substrate specificity.

ACKNOWLEDGMENT

We thank T.-F. Wang for the pGW1-CD39L1 plasmid and for the anti-CD39 antibody and T. L. Kirley for the antibody against the C-terminal tail of CD39L1. We thank Xiaotian Zhong, James Wu, and Wei Chen for technical advice and all members of the Guidotti lab for helpful discussion.

REFERENCES

1. Burnstock, G. (1971) *Nature 229*, 282−283.
2. Bankston, L., and Guidotti, G. (1996) *J. Biol. Chem. 271* (29), 17132−17138.
3. Guidotti, G. (1996) *Chem. and Biol. 3*, 703−706.
4. Watt, W. C., Lazarowski, E. R., and Boucher, R. C. (1998) *J. Biol. Chem. 273*, 14053−14058.
5. Lazarowski, E. R., Boucher, R. C., and Harden, T. L. (2000) *J. Biol. Chem. 275* (40), 31061−31068.
6. Burnstock, G., and Kennedy, C. (1985) *Gen. Pharmacol. 16*, 433−440.
7. Dubyak, G. R., and El-Moatassim, C. (1993) *Am. J. Physiol. 265*, C577−C606.
8. Zimmermann, H. (1994) *Trends Neurosci. 17*, 420−426.
9. Brake, A. J., and Julius, D. (1996) *Annu. Rev. Cell Dev. Biol. 12*, 519−541.
10. Gordon, J. L. (1986) *Biochem. J. 233*, 309−319.
11. Valera, S., Hussy, N., Evans, R. J., Adami, N., North, R. A., Surprenant, A., and Buell, G. (1994) *Nature 371*, 5516−5519.
12. Communi, D., Govaerts, C., Parmentier, M., and Boeynaems, J. M. (1997) *J. Biol. Chem. 272*, 31969−31973.
13. Fredholm, B. B., Abbrachio, M. P., Burnstock, G., Dubyak, G. R., Harden, T. K., Jacobson, K. A., Schwabe, U., and Williams, M. (1997) *Trends Pharmacol. Sci. 18*, 79−82.
14. Burnstock, G. (1998) in *The P2 Nucleotide Receptors* (Turner, J. T., Weisman, G. A., and Fedan, J. S., Eds.) pp 3−42.
15. Trams, E. G., and Lauter, C. J. (1974) *Biochim. Biophys. Acta 345*, 180−197.
16. Plesner, L. (1995) *Int. Rev. Cytolol. 158*, 141−214.
17. Zimmermann, H., Beaudoin, A. R., Bollen, M., Goding, J. W., Guidotti, G., Kirley, T. L., Robson, S. C., and Sano, K. (2000) in *Ecto-ATPases and Related Nucleotidases* (vanDuffel, L., and Lemmens, R., Eds.) pp 1−8.
18. Handa, M., and Guidotti, G. (1996) *Biochem. Biophys. Res. Commun. 218*, 916−923.
19. Schulte am Esch, J., II, Sevigny, J., Kaczmarek, E., Siegel, J. B., Imai, M., Koziak, K., Beaudoin, A. R., and Robson, S. C. (1999) *Biochemistry 38*, 2248−2258.
20. Kabsch, W., Mannherz, H. G., Suck, D., Pai, E. F., and Holmes, K. C. (1990) *Nature 347*, 37−44.
21. Flaherty, K. M., DeLuca-Flaherty, C., and McKay, D. B. (1990) *Nature 346*, 623−628.
22. Flaherty, K. M., McKay, D. B., Kabsch, W., and Holmes, K. C. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 5041−5045.
23. Bork, P., Sander, C., Valencia, A. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 7290−7294.
24. Zeng, C., Aleshin, A. E., Hardie, J. B., Harrison, R. W., and Fromm, H. J. (1996) *Biochemistry 35*, 13157−13164.
25. Grinthal, A., and Guidotti, G. (2000) *Biochemistry 39*, 9−16.
26. Smith, T. M., Lewis Carl, S. A., and Kirley, T. L. (1999) *Biochemistry 38* (18), 5849−5857.
27. Kegel, B., Braun. N., Heine, P., Maliszewski, C. R., and Zimmermann, H. (1997) *Neuropharmacology 36*, 1189−1200.
28. Kirley, T. L. (1997) *J. Biol. Chem. 272*, 1076−1081.
29. Chadwick, B. P., and Frischauf, A.-M. (1998) *Genomics 50*, 357−367.
30. Nagy, A. K., Knowles, A. F., and Nagami, G. T. (1998) *J. Biol. Chem. 273* (26), 16043−16049.
31. Wang, T.-F., Ou, Y., and Guidotti, G. (1998) *J. Biol. Chem. 273*, 24814−24821.
32. Heine, P., Braun, N., Heilbronn, A., and Zimmermann, H. (1999) *Eur. J. Biochem. 262* (1), 102−107.
33. Coppi, M. V., and Guidotti, G. (1997) *Arch. Biochem. Biophys. 346*, 312−321.
34. Handa, M. (1997) Ph.D. Thesis, Harvard University, Cambridge, MA.
35. Chen, W. and Guidotti, G. (2001) *Biochem. Biophys. Res. Commun. 282* (1), 90−95.
36. Heine, P., Braun, N., Sevigny, J., Robson, S. C., Servos, J., and Zimmermann, H. (2001) *Eur. J. Biochem. 268*, 364−373.
37. Doyle, D. A., Cabral, J. M., Pfuetzner, R. A., Kuo, A., Gulbis, J. M., Cohen, S. L., Chait, B. T., and MacKinnon, R. (1998) *Science 290*, 69−77.
38. Minor, D. L., Jr., Masseling, S. J., Jan, Y. N., and Jan, L. Y. (1999) *Cell 96*, 879−891.

BI015563H