Expression and Characterization of Soluble and Membrane-bound Human Nucleoside Triphosphate Diphosphohydrolase 6 (CD39L2)*

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Ecto-nucleoside-triphosphate diphosphohydrolase-6 (eNTPDase6), also known as CD39L2 cDNA was expressed in mammalian COS-1 cells and characterized using nucleotidase assays as well as size exclusion, anion exchange, and cation exchange chromatography. The deduced amino acid sequence of eNTPDase6 is more homologous with the soluble E-type ATPase, eNTPDase5, than other E-type ATPases, suggesting it may also be soluble. To test this possibility, both the cell membranes and the growth media from eNTPDase6-transfected COS-1 cells were assayed for nucleotidase activities. Activity was found in both the membranes and the media. Soluble eNTPDase6 preferentially exhibits nucleoside diphosphatase activity, which is dependent on the presence of divalent cations. Western blot analysis of eNTPDase6 treated with PNGase-F indicated both soluble and membrane-bound forms are glycosylated. However, unlike some membrane-bound ecto-nucleotidases, the eNTPDase6 activity was not specifically inhibited by deglycosylation with peptide N-glycosidase F. Soluble eNTPDase6 hydrolyzed nucleoside triphosphates poorly and nucleoside monophosphates not at all. Analysis of the relative rates of hydrolysis of nucleoside diphosphates (GDP = IDP > UDP > CDP >> ADP) suggests that soluble eNTPDase6 is a diphosphatase most likely not involved in regulation of ADP levels important for circulatory hemostasis.

Nucleoside triphosphate diphosphohydrolases (NTPDases) are enzymes characterized by their ability to hydrolyze nucleoside tri- and diphosphates. Although nucleotide specificity and specific activities vary among the NTPDases, other characteristics such as insensitivity to typical ATPase inhibitors and cation dependence for activity are invariant (1). The NTPDases all have their active sites outside the cytoplasm of the cell and exist as either ecto-, endo-(luminal), or soluble apyrases whose activities have been speculated to be involved in processes as diverse as neurotransmission, N- and O-glycosylation, cardiac function, platelet aggregation, cell adhesion, muscle contraction and relaxation, vascular tone, secretion of hormones, immune responses, and cell growth (2). The ecto-apyrases hydrolyze nucleoside diphosphates at rates comparable to nucleoside triphosphates and are perhaps the best studied of all the NTPDases; they include eNTPDase1 (CD39) (3) and eNTPDase3 (HB6) (4). These are integral membrane proteins with two membrane spanning regions, one at each end of the primary structure, as well as a large extracellular domain, which includes the active site of these enzymes. Intracellular luminal endo-NTPDase activities have been found associated with both the endoplasmic reticulum (ER) and the Golgi apparatus. At least two Golgi membrane-associated NTPDases have been described, a GTPase (5–7) and an UDPase, both with luminal active sites (8). The ER-UDPase lacks transmembrane domains and is believed to be a soluble luminal protein (9). In addition to the soluble ER apyrase, a soluble apyrase from potato has been characterized (10) and sequenced (8). Soluble apyrases have also been studied from mosquitoes and other hematophagous insects (11, 12), as well as from the single-celled Tetrahymena (13). In addition, human soluble eNTPDase5, also known as CD39L4 (14), has recently been expressed and characterized (15). In the present study we expressed and characterized the putative soluble human eNTPDase6, also known as CD39L2 (14). This ecto-nucleotidase has properties distinct from eNTPDase5 (CD39L4), as well as from the other ecto-, endo-, and soluble NTPDases. In clarified media from eNTPDase-expressing COS-1 cells, its specific activity is 89 μmol of P/mg/h (using GDP as substrate) with nucleotide preference for GDP = IDP > GTP > ITP > UDP > CDP > UDP > GTP > CTP > ADP > ATP. eNTPDase6 exists predominantly as an approximately 50-kDa soluble, monomeric protein. It is postulated that the membrane-bound form contains a 78-amino acid signal peptide, which is cleaved after insertion into and through the cell membrane resulting in release of the soluble ecto-nucleotidase into the extracellular space.

EXPERIMENTAL PROCEDURES

Materials—Epicurian coli ultracompetent bacteria were purchased from Stratagene. Plasmid purification kits were purchased from Qiagen, Inc. LipofectAMINE Plus reagent, Dulbecco's modified Eagle's medium, calf serum, goat anti-rabbit horseradish peroxidase-conjugated secondary antibody, and antibodies/antimyocyties were all obtained from Life Technologies. Falcon tissue culture-treated plates were from Becton-Dickinson. The mammalian expression vector pcDNA3 was obtained from Invitrogen. Restriction endonucleases and T4 DNA ligase were purchased from Promega. Ampicillin, nucleotides, diethylpyrocarbonate (DEPC), and Sephacryl S-200 were purchased from Sigma.

Human CD39L2 (eNTPDase6) cDNA in Mammalian Expression Vector pcDNA3 and Expression in COS-1 Cells—The human eNTPDase6 cDNA was isolated and sequenced and has been described previously (14). The 2762-bp eNTPDase6 cDNA insert was excised from a pLXPH retroviral expression vector and inserted into the 5.4-kb pcDNA3 mammalian expression vector using EcoRI and XbaI restriction endonucleo-
Characterization of eNTPDase6 (CD39L2)

Nucleotidase assays were performed as described under “Experimental Procedures” in the presence of 7 mM MgCl2 and 2.5 mM nucleotide (as shown). All activities are expressed in units of micromoles of Pi/mg/h, and are represented as the average of three separate transfections ± S.E.

| Nucleotide | Soluble eNTPDase6 | Membranous eNTPDase6 |
|------------|-------------------|----------------------|
| GDP        | 89 ± 27           | 9 ± 2                |
| GTP        | 8 ± 4             | 3 ± 1                |
| IDP        | 101 ± 24          | 12 ± 2               |
| ITF        | 7 ± 2             | 2.4 ± 1              |
| UDP        | 30 ± 10           | 4 ± 1                |
| UTP        | 4 ± 1             | 5 ± 2                |
| CDP        | 18 ± 8            | 1 ± 1                |
| CTP        | 10 ± 7            | 6 ± 3                |
| ADP        | 3 ± 1             | 0.6 ± 0.3            |
| ATP        | 1 ± 0.2           | 1 ± 1                |

NTPase6 was expressed in COS cells and characterized as both soluble and membrane-bound forms. The results of nucleotidase assays from three separate transfections are summarized in Table I. The soluble form of eNTPDase6 demonstrated the highest specific activity with IDP (101 μmol/mg/h) and GDP (89 μmol/mg/h). Lower nucleotidase activities were observed using UDP and CDP; 33% and 23%, respectively, of that observed with IDP. Soluble eNTPDase6 exhibited very low activity with all nucleotides triphosphates used as well as with ADP (<10 μmol/mg/h). Although 90% of the total recovered GDPase and IDPase activity was found to be associated with the soluble form of eNTPDase6, the membrane-bound form was also characterized. The membrane-bound form of eNTPDase6, from crude total membranes, also demonstrated the highest specific activity with inosine and guanosine nucleotides. Unlike soluble eNTPDase6, the membrane-bound form showed a slight preference for hydrolysis of most nucleotide triphosphates. One notable exception was a significant preference for GDP (10 μmol/mg/h) over GTP (3 μmol/mg/h).

Nucleotidase activity of both the soluble and membrane-bound forms of eNTPDase6 GDPase activities were examined for cation dependence, because this is a defining characteristic for members of the eNTPDase family (1). The soluble form was found to be highly dependent on the presence of divalent cations for optimal activity as is seen in Table II. In the absence of cations, specific activity for GDP hydrolysis was 1.1 μmol/mg/h, whereas optimal GDPase activity was 74 μmol/mg/h in the presence of 2 mM MnCl2. All concentrations of MnCl2, MgCl2, and CaCl2 tested resulted in significant increases of GDPase activity over that observed in the absence of cations. The membrane-bound form of eNTPDase6 also was found to be dependent on the presence of cations for optimal GDPase activity. In the absence of cations, GDPase activity was 0.3 μmol/mg/h, whereas GDPase activity was optimal in the presence of 10 mM MgCl2 (6 μmol/mg/h).

The soluble form of eNTPDase6 was partially purified using size exclusion chromatography, anion exchange chromatography, and cation exchange chromatography. Serum-free media was collected from COS-1 cells expressing eNTPDase6 over 3 days, as described under “Experimental Procedures.” The media was concentrated to 1 ml and loaded onto a Sephacryl S-200
The calibration plot for the standards (bovine serum albumin, ovalbumin, myoglobin, vitamin B-12), as well as the fraction containing maximum GDPase activity, is shown in Fig. 1. The fractions containing the majority of the GDPase activity corresponded to a molecular mass of ~50 kDa. This molecular mass agrees well with the theoretical weight for the purified soluble eNTPDase6 had a average (n = 2) specific activity with GDP of 9227 μmol Pi/mg/h.

An anti-eNTPDase6 polyclonal antibody was developed, affinity purified, and used for Western blot analysis and immunoprecipitation. As shown in Fig. 2, the anti-eNTPDase6 antibody reacted specifically with an approximately 50-kDa protein in both eNTPDase6-expressing COS cell membranes and media. When membranes and media were probed with pre-immune sera (not shown). Also shown in Fig. 2 is the shift in molecular mass of both the soluble and membrane-bound forms after treatment with PNGase-F, indicating both forms are N-glycosylated.

Partial purification of soluble eNTPDase6

The summation for purification of soluble eNTPDase6 is shown in Table 3. Starting material for purification was 30 ml of clarified, serum free, growth media from eNTPDase6-expressing COS-1 cells, which had been concentrated to 1.5 ml as described under “Experimental Procedures.” The average of two separate transfections and purifications is shown.

DISCUSSION

This paper describes the first expression of the soluble human nucleotidase eNTPDase6 (CD39L2), and for the first time, the characterization of both soluble and membrane-bound forms of the eNTPDase6. To date there have been several reports of soluble eNTPDases from organisms as diverse as plants (10), Tetrahymena (13), mosquitoes (11), and humans (15); however, most studies have focused strictly on the soluble forms, and comparative studies with their respective membrane-bound forms have not been reported. In this study, we found notable differences in nucleotide preference and cation dependence between the two forms of eNTPDase6, yet similarities in size, DEPC response, and lack of importance of histidine modification. As shown in Fig. 4, DEPC treatment of the soluble form of eNTPDase6 inhibited GDPase activity 60%. However, hydroxylamine treatment was unable to reverse the inhibition caused by DEPC treatment. Similar results were obtained with the membrane-bound form of eNTPDase6 (Fig. 4).

The dependence of eNTPDase6 activity on glycosylation was determined by treating both the membrane-bound and soluble forms with PNGase-F under conditions, which previously resulted in significant loss of glycan chains and activity of eNTPDase1 and eNTPDase3 (HB6 (16)). There was no significant difference in activity between those eNTPDase6 samples treated with active PNGase-F and those control samples treated with heat-inactivated PNGase-F (data not shown). However, Western blot analysis of eNTPDase6 treated with active PNGase-F indicated the presence of two glycosylation sites (Fig. 3), as is evidenced by the presence of three discrete protein bands corresponding to eNTPDase6 protein with 2, 1, or 0 glycan chains attached (see arrows in Fig. 3 between the "-" and "+" lanes incubated for 3.5 h).

Diethylycarbonate (DEPC) is an inhibitor of eNTPDases, presumably due to histidine modification (23, 24). Both the soluble and membrane-bound forms of eNTPDase6 were treated with DEPC followed by hydroxylamine to reverse histidine modification. As shown in Fig. 4, DEPC treatment of the soluble form of eNTPDase6 inhibited GDPase activity 60%. However, hydroxylamine treatment was unable to reverse the inhibition caused by DEPC treatment. Similar results were obtained with the membrane-bound form of eNTPDase6 (Fig. 4).

**TABLE II**

Characterization of eNTPDase6 (CD39L2)

Nucleotidase assays were performed as described under “Experimental Procedures” in the presence of 2.5 mM GDP and varying concentrations of divalent cations (as shown). All activities have been corrected for background and are represented in micromoles of Pi/mg/h as well as percentages of the highest activity achieved with the soluble and membrane-bound eNTPDase6, respectively.

| Cation   | Soluble eNTPDase6 | Membranous eNTPDase6 |
|----------|-------------------|----------------------|
|          | μmol of P_i/mg/h  | -fold                |
| None     | 1.1 (1.4%)        | 0.3 (5%)             |
| 2 mM MgCl_2 | 58 (76%)        | 1 (17%)              |
| 10 mM MgCl_2 | 58 (76%)        | 6 (100%)             |
| 2 mM CaCl_2 | 68 (89%)        | 0                    |
| 10 mM CaCl_2 | 76 (100%)       | 2 (33%)              |
| 2 mM MnCl_2 | 74 (97%)        | 3 (50%)              |
| 10 mM MnCl_2 | 42 (55%)        | 4 (67%)              |

**TABLE III**

Partial purification of soluble eNTPDase6

The calibration plot for the standards (bovine serum albumin, ovalbumin, myoglobin, vitamin B-12), as well as the fraction containing the maximum GDPase activity (the larger open circle) is shown. From the elution profile, the molecular mass of the soluble eNTPDase6 (CD39L2) was calculated to be 45 kDa.

**FIG. 1.** Sephacryl S200 size exclusion column elution profile. The calibration plot for the standards (bovine serum albumin, ovalbumin, myoglobin, and vitamin B-12; all represented by smaller filled circles) as well as the fraction containing the maximum GDPase activity (the larger open circle) is shown. From the elution profile, the molecular mass of the soluble eNTPDase6 (CD39L2) was calculated to be 45 kDa.
Characterization of eNTPDase6 (CD39L2)

Form of eNTPDase6 differed in that it hydrolyzed some nucleoside triphosphates slightly better than nucleoside diphosphates with a notable exception in the case of guanosine nucleosides, where the GDPase:GTPase activity ratio was 3.3:1. As shown in Table II, both forms of eNTPDase6 were dependent on the presence of cations for optimal activity, a characteristic universally found in other members of the eNTPDase family (1, 7, 8, 25).

Soluble eNTPDase6 was partially purified using size exclusion, anion exchange, and cation exchange chromatography. Fractions from the S-200 size exclusion column containing the majority of the GDPase activity were found to correspond with a molecular size of approximately 50 kDa (Fig. 1), as is predicted from the cDNA sequence of eNTPDase6. This molecular size is consistent with a monomer, and not consistent with a homo-oligomer of soluble enzyme. This is concordant with previous findings that soluble eNTPDase enzymes are monomeric (10, 26, 27), whereas membrane-bound forms are tetrameric (8, 28).

It has been speculated (14) that eNTPDase6 (CD39L2) would be released as a soluble enzyme after cleavage of the NH₂-terminal signal sequence. The theoretical pI of the deduced amino acid sequence of eNTPDase6, including the hydrophobic NH₂-terminal signal peptide sequence, is 9.91. If the soluble eNTPDase6 is generated by cleavage after Ala-78 as predicted by computer analysis of the sequence, then the resultant soluble form is predicted to have a theoretical, non-glycosylated pI = 7.52, and therefore should not bind to either anion or cation exchange resins at physiological pH. Chromatography was therefore performed under conditions (pH 7.4) that would not promote binding to either cation or anion exchangers. Using both the anion and cation columns, soluble GDPase activity was found in fractions that did not bind to either column (see “Experimental Procedures”). This indicates that the prediction of cleavage of a signal sequence after Ala-78 is most likely correct, because this would eliminate many positively charged amino acids, resulting in a 45-kDa soluble protein with a greatly reduced pI, close to 7.4, consistent with the purification results.

A polyclonal anti-peptide antibody (against a COOH-terminal non-conserved region of eNTPDase6) was developed to further study both the soluble and membrane-bound forms of eNTPDase6. As shown in Fig. 2, a single protein at approximately 50 kDa was recognized in the eNTPDase6-expressing cells and media that was not observed in control cell membranes.

Computer sequence analysis predicts two potential N-glycosylation sites for eNTPDase6. PNGase-F was used to deglycosylate eNTPDase6 to determine the extent of glycosylation required for GDPase activity. From activity data we found no difference in GDP hydrolysis between those samples treated with active PNGase-F as compared with those treated with heat-inactivated PNGase-F. These experiments were done under identical conditions used to demonstrate both deglycosyla-

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**Fig. 2.** Western blot of PNGase-F-treated soluble and membrane-bound eNTPDase6 with polyclonal anti-eNTPDase6 antibody. eNTPDase6-transfected COS-1 cell media (40 µg, lanes 1 and 2) and membrane proteins (40 µg, lanes 3 and 4) and membrane proteins (40 µg, lanes 7 and 8), with or without PNGase-F treatment, were probed with affinity purified anti-eNTPDase6 antibody. A single band was differentially present in the eNTPDase6-expressing media and membranes that had been treated with active PNGase-F, at approximately 50 kDa (lanes 2 and 6). The eNTPDase6 antibody detected a slightly larger, glycosylated eNTPDase6 protein band in both cell media and membranes expressing eNTPDase6 (lanes 1 and 5). The eNTPDase6 antibody did not react with any proteins present in the mock transfected COS-1 cell media (lanes 3 and 4) or membranes (lanes 7 and 8).

**Fig. 3.** Time course treatment of soluble eNTPDase6 with PNGase-F. eNTPDase6-transfected COS cell media were incubated in the presence of boiled, inactivated PNGase-F (-) or active PNGase-F (+), and aliquots were taken at the indicated times of incubation at 37 °C for analysis of nucleotidase activity and Western blotting. Arrows on the Western blot in between the two 3.5-h lanes indicate the presence of discrete bands corresponding to the eNTPDase6 proteins possessing 2, 1, and 0 N-glycan chains.
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Fig. 4. Inhibition by 1 mM DEPC of soluble and membrane-bound eNTPDase6. A, soluble eNTPDase6 treated with DEPC in 2% ethanol (filled circles) or 2% ethanol as the control (open circles). B, membrane-bound eNTPDase treated with DEPC in 2% ethanol (filled squares) or 2% ethanol as the control (open circles). Experiments were performed as described under “Experimental Procedures.” The average of three separate experiments is shown with standard deviations. Hydroxylamine was added, to a final concentration of 100 mM, at t = 16 min to initiate recovery from DEPC inhibition due to histidine modification. The soluble and membrane-bound eNTPDase6 activities were inhibited approximately 60% each, followed by recovery of activity of only approximately 5% and 10%, respectively.

In summary, this report describes the expression and characterization of both the membrane-bound and soluble forms of eNTPDase6 (CD39L2). The expressed protein possesses shared characteristics of the ecto-apyrases such as cation dependence and sensitivity to DEPC. eNTPDase6 is unique in that it is the first soluble eNTPDase that prefers GDP and IDP as substrates. Human eNTPDase6 is much more widespread in its tissue distribution than the only other soluble human ectonucleotidase described to date, eNTPDase5 (CD39L4 (15)). eNTPDase5 (CD39L4) was shown in two studies to be rather limited in its tissue distribution (14, 15), unlike the eNTPDase6 described here (CD39L2), for which mRNA has been found for all tissues tested (14). The broad tissue distribution of eNTPDase6 (CD39L2) suggests the possibility that the range of functions or the importance of the physiological function(s) for this soluble nucleotidase might exceed that of the narrowly expressed, soluble eNTPDase5 (CD39L4).

Note Added in Proof—After acceptance of this work, N-terminal amino acid sequencing of soluble eNTPDase6 indicated that cleavage between Arg-76 and Ala-77 separates the N terminus signal peptide from the remainder of the soluble protein.

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