Sequencing, functional expression and characterization of rat NTPDase6, a nucleoside diphosphatase and novel member of the ecto-nucleoside triphosphate diphosphohydrolase family

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We have isolated and characterized the cDNA encoding nucleoside triphosphate diphosphohydrolase 6 (NTPDase6), a novel member of the ecto-nucleoside triphosphate diphosphohydrolase family. The rat-brain-derived cDNA has an open reading frame of 1365 bp encoding a protein of 455 amino acid residues, a calculated molecular mass of 49971 Da and a predicted N-terminal hydrophobic sequence. It shares 86% sequence identity with the human CD39L2 sequence and 48% and 51% identity respectively with sequences of the two related human and murine nucleoside diphosphatases (CD39L4, NTPDase5/ER-UDPase). The mRNA was expressed in all tissues investigated, revealing two major transcripts with differing abundances. PCR analysis suggests a single open reading frame. A Myc-His-tagged NTPDase6 was expressed in Chinese hamster ovary (CHO) and PC12 cells for immunological analysis and protein isolation. The protein was contained in membrane fractions of transfected CHO cells and occurred in a soluble form in the cell culture supernatants. NTPDase6 preferentially hydrolysed nucleoside 5′-diphosphates. With different substrates the order of activity was GDP > IDP ≫ UDP, CDP ≫ ADP. Nucleoside 5′-triphosphates were hydrolysed only to a minor extent and no hydrolysis of nucleoside 5′-monophosphates was observed. The enzyme was strongly and equally activated by Ca^{2+} and Mg^{2+} and had a K_m for GDP of 211 μM. The immunohistochemical analysis of transfected CHO and PC12 cells suggests that NTPDase6 is associated with the Golgi apparatus and to a small extent also with the plasma membrane. The enzyme might support glycosylation reactions in the Golgi apparatus and, when released from cells, might catalyse the hydrolysis of extracellular nucleotides.

Key words: GDPase, glycosylation, Golgi apparatus.

INTRODUCTION

The recently identified family of ecto-nucleoside triphosphate diphosphohydrolases (E-NTPDase family) contains multiple members that differ in their substrate specificities and cellular locations. They hydrolyse nucleoside 5′-triphosphates and nucleoside 5′-diphosphates, albeit with differing nucleotide preferences. Their catalytic site is in the ecto-position and faces the extracellular medium or the lumen of intracellular organelles such as the Golgi apparatus or the endoplasmic reticulum (ER). In vertebrates, cell-surface-located or extracellular members of the enzyme family are considered to function in the extracellular hydrolysis of nucleotides [1] that act as signalling substances [2,3]. In contrast, the Golgi-located and ER-located enzymes might be important in the hydrolysis of nucleoside diphosphates to the corresponding nucleoside monophosphates and consequently in the import of nucleotide sugars from the cytosol into Golgi cisternae or the ER [4,5].

The gene family has members in vertebrates, invertebrates, plants, yeast and protozoans (references in [1,6–11]). Family members share five highly conserved sequence domains (‘apyrase conserved regions’) [8,12] that are presumably of major relevance for their catalytic activity [12–15]. The nomenclature applied here is that of Zimmermann et al. [16]. To facilitate comparison with previous papers, alternative nomenclature is given in parentheses when appropriate.

The mammalian members of the E-NTPDase family reveal two different forms of membrane association. Members with predicted transmembrane domains at the N-terminus and at the C-terminus include nucleoside triphosphate diphosphohydrolase 1 (NTPDase1) (CD39, ecto-apyrase, ecto-ATP diphosphohydrolase), NTPDase2 (ecto-ATPase, CD39L1), NTPDase3 (HB6) and NTPDase4 (UDPase). The other forms have a predicted N-terminal hydrophobic sequence but lack the C-terminal transmembrane domain. They include the functionally characterized NTPDase5 (CD39L4) and the human CD39L2 sequence that was hypothesized to encode an additional member of the E-NTPDase family (putative NTPDase6) [17].

Only the closely related NTPDase1 to NTPDase3 are bound to the cell surface. They have a wide tissue distribution and hydrolyse nucleoside 5′-triphosphates and nucleoside 5′-diphosphates but differ considerably in their preferences for nucleoside diphosphates. NTPDase1 hydrolyses ATP and ADP to similar extents [18–20], NTPDase3 has a threefold preference for the hydrolysis of ATP over ADP [13,21], and NTPDase2 hydrolyses ADP only to a marginal extent [22–24]. The physiological relevance of the differences in substrate specificity between the three ectonucleotidases is not understood.

The other functionally characterized mammalian members of the enzyme family have been allocated to intracellular organelles. The two closely related forms of NTPDase4 were assigned to the Golgi apparatus (UDPase) [4] and to lysosomal/autophagic

Abbreviations used: CHO, Chinese hamster ovary; ER, endoplasmic reticulum; E-NTPDase, ecto-nucleoside triphosphate diphosphohydrolase; Ni-NTA, Ni^{2+}-nitrilotriacetate; NTPDase, nucleoside triphosphate diphosphohydrolase; ORF, open reading frame.

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The cDNA sequence of clone L2-6 has been submitted to the GenBank®, EMBL, DDBJ and GSDB Nucleotide Sequence Databases under the accession number AJ277748.

vacuoles (LALP70) [25]. The Golgi enzyme has the greatest activity with UDP as a substrate. It also hydrolyses a number of other nucleoside 5′-diphosphates and triphosphates but not ATP or ADP. NTPDase5 (CD39L4) has a marked preference for nucleoside 5′-diphosphates, especially for UDP and GDP. The murine orthologue of the enzyme was allocated to the ER [5], suggesting an intracellular localization. However, expression of the human NTPDase5 in COS-7 cells resulted in a secreted and soluble form of the enzyme [26].

Here we report the molecular cloning, functional expression and characterization of NTPDase6, a mammalian member of the E-NTPDase family that has until now been functionally uncharacterized. After heterologous expression the enzyme is localized in the Golgi apparatus but it also occurs in a soluble extracellular form. It hydrolyses nucleoside diphosphates, in particular GDP and IDP. It might support glycosylation reactions in the Golgi apparatus and simultaneously serve the hydrolysis of extracellular nucleotides after its cellular release.

EXPERIMENTAL

Materials

The Clon Capture® cDNA selection kit was obtained from Clontech (Heidelberg, Germany). Rat brain pCMV-SPORT 2 cDNA library, Trizol reagent, reverse transcriptase SuperScript®II, cell culture media Ham's FC-12 and DMEM (Dulbecco's modified Eagle's medium), fetal calf serum, horse serum, penicillin and streptomycin were from Life Technologies (Karlsruhe, Germany). Cloning vector pcDNA3.1(−)/Myc-His A and oligo(dT) cellulose were purchased from Invitrogen (Leek, The Netherlands). Hybond N membrane, [α-^{32}P]dCTP and the enhanced chemoluminescence system were from Amersham Pharmacia Biotech (Freiburg, Germany). Anti-digoxigenin alkaline phosphatase-conjugated antibody and a chemoluminescent substrate [disodium 3-(4-methoxyspiro-{1,2-dioxoethane-2′,3-(5′-chloro)tricyclo[3.3.1.1$^{3',7}$]decan}-4-yl)phenyl phosphate] were obtained from Roche Molecular Biochemicals (Mannheim, Germany). Sawady *Pwo* DNA polymerase was from Peqlab, Biotechnologie GmbH (Erlangen, Germany). *Taq* DNA polymerase and restriction endonucleases were purchased from MBI Fermentas (St Leon-Rot, Germany). Ni^{2+}-nitrilotriacetate (Ni-NTA) resin was from Qiagen (Hilden, Germany). Nucleoside triphosphate, diphosphate and monophosphate sodium salts, poly(D-lysine), β-subunit of nerve growth factor ('NGF') and the monoclonal antibody against the Golgi 58K protein (clone 58K-9) were obtained from Sigma (Deisenhofen, Germany). Indocarbocyanin-3-labelled anti-mouse IgG antibody was from Dianova (Hamburg, Germany). The Golgi marker 4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene (BODIPY®) FL C_5-ceramide was from Molecular Probes (Leiden, The Netherlands). The Nucleobond X-500 plasmid purification kit was purchased from Macherey and Nagel (Düren, Germany). Protease inhibitors chymostatin, pepstatin, benzamidine, antipain and leupeptin were obtained from Calbiochem (Schwalbach, Germany).

Screening of the cDNA library

For isolation of the NTPDase6 clone from a rat brain library, the Clon Capture® cDNA selection kit, a RecA-based system to enrich specific cDNA clones, was applied. For designing an oligonucleotide primer and the generation of a biotinylated NTPDase6 cDNA fragment as a probe for cDNA selection, an entry of the mouse EST (expressed sequence tag) database at the NCBI (National Centre for Biotechnology Information) (accession no. AA647051) was used, which is similar to the putative human NTPDase6 (CD39L2) [17]. A 157 bp fragment was amplified by PCR from a rat brain cDNA with the forward primer 5′-GAATTCCTTGTCGGGGATGACTGTGTT-3′ and the reverse primer 5′-GATCTGAGTGGATCCTCCGCCCAA-3′, and cloned into the *Eco*RI and *Bam*HI restriction site of pBluescript SK(−), resulting in the plasmid pBSK(−)-NTPDase6. The plasmid was used as a template for synthesizing a biotinylated PCR fragment. The labelled fragment was used for enriching NTPDase6 cDNA clones in a rat brain pCMV-SPORT 2 cDNA library, in accordance with the manufacturer's instructions (Clon Capture®, cDNA selection kit). Electrocompetent *Escherichia coli* DH5α were transformed with enriched plasmid amplified and plated on Luria–Bertani/ampicillin agar plates. The resulting transformants were screened by colony hybridization with the 157 bp cDNA fragment labelled with [α-^{32}P]dCTP by PCR. Positive colonies were amplified and the insert length of the plasmids was analysed by colony PCR with insert-flanking primers (T7 and SP6) of the vector pCMV-SPORT 2.

cDNA sequencing and computer-aided sequence analysis

The plasmids of the selected clones were prepared with Nucleobond X-500 plasmid purification columns. DNA sequencing was performed by Scientific Research and Development GmbH (Oberursel, Germany). Primer walking was employed for obtaining the complete full-length sequence of the cDNA clone L2-6 in both directions. The PC/GENE program release 6.85 (Oxford Molecular Ltd., Oxford, England) was used for assembling sequencing fragments, for generating hydrophobicity plots [27], for detecting secretory signal peptides and protein motifs (located in the Prosite database), for calculating the identity between two sequences and for establishing the dendrogram of the amino acid alignment. Transmembrane helices were identified with the TopPred 2 algorithm (http://www.biokemi.su.se/~server/toppred2/toppredServer.cgi). The DNA sequence and the deduced amino acid sequence were analysed for similarity to known sequences by using the NCBI Blast Network Service. Translation of the cDNA sequence to an amino acid sequence, the nucleic acid motif search and the amino acid sequence alignment (CLUSTAL W algorithm) were performed with the program OMIGA 2.0 (Oxford Molecular Ltd.).

Northern-blot analysis

Wistar rats (200–250 g) were obtained from Charles River Wiga (Sulzfeld, Germany). Total RNA from rat spleen, thymus, lung, skeletal muscle, heart, kidney, liver and brain was isolated with Trizol reagent. Northern-blot analysis with polyadenylated RNA was performed essentially as described previously [22]. The 157 bp rat NTPDase6 cDNA fragment of the plasmid pBSK(−)NTPDase6 was used as template for the cRNA probe. The SP6/T7-polymerase and transcription kit from Roche was used to synthesize digoxigenin-labelled single-stranded anti-sense cRNA probes in accordance with the supplier's instructions.

Reverse-transcriptase-mediated PCR analysis of the NTPDase6 open reading frame (ORF)

Total RNA and mRNA were isolated from adult rat brain and heart as described for Northern analysis. The cDNA species were synthesized with SuperScript®II from 0.5 μg of mRNA with an oligo(dT)$_{18}$ primer in accordance with the manufacturer's instructions. For amplification, 2% of the reverse transcription was used with the following sets of primers: 1, 5′-ATGGGACC-TTGCGGATGACGA-3′ (nt 148–168); 2, 5′-AACACAGTC-

ATCCCCTAC-3′ (nt 692–675); 3, 5′-CGCCAAGCAACACA-TTCCATA-3′ (nt 530–550); 4, 5′-AGGTCCATGCAAGC-AAAG-3′ (nt 1339–1322); 5, 5′-AGCACGTGGACTTCTA-TGC-3′ (nt 1165–1183); 6, 5′-TAGTGTGGTGTAGCAGAG-3′ (nt 1686–1669).

Construction of an expression plasmid with a C-terminal tag

The ORF of the cDNA clone L2-6 encoding NTPDase6 was ligated into the pcDNA3.1(−)/Myc-His A vector. This vector contains a *myc* epitope and polyhistidine-encoding sequences for tags located at the C-terminus. To ligate the cDNA in frame with the tags, a fragment containing the ORF was amplified with PCR. The binding site for the forward primer 5′-TCTG-CCTCGAGCACCGGCTA-3′ was located in the 5′-untranslated region of the cDNA, inserting a *Xho*I site by point mutation at nt 94. The binding site for the reverse primer 5′-AAGGC-AGGAAGCTTCTGTCT-3′ was located at the 3′ end of the ORF and inserted a *Hin*dIII restriction site by point mutation at nt 1482. The success of cloning was confirmed by sequencing. In the final construct, pcDNA3/NTPDase6, the last four C-terminal residues of NTPDase6 were exchanged for the C-terminal tags.

Expression of recombinant NTPDase6

PC12 cells and Chinese hamster ovary (CHO) cells were cultured as described previously [22,28]. They were transfected by electroporation with the plasmid pcDNA3/NTPDase6 in electroporation buffer [137 mM NaCl/5 mM KCl/0.7 mM Na_2HPO_4/6 mM dextrose/20 mM Hepes (pH 7)] with a BTX Electrocell manipulator 600. In control experiments, CHO and PC12 cells were transfected with the empty vector alone.

Preparation of culture supernatants and membrane fractions

At 24 h after the electroporation of CHO cells, the culture medium was exchanged to remove dead cells and debris. After an additional 24 h, the conditioned culture medium was removed from cells, centrifuged at 300 ***g*** to sediment floating cells and stored at −20 °C for further processing. The remaining cells were washed twice with ice-cold buffer A [150 mM NaCl/10 mM Hepes (pH 7.4)] and scraped from the plates in ice-cold buffer A supplemented with protease inhibitors (2 μg/ml chymostatin, 1 μg/ml pepstatin, 1 mM benzamidine, 2 μg/ml antipain and 2 μg/ml leupeptin). Homogenized cells were centrifuged for 10 min at 300 ***g*** and 4 °C and the pellets were resuspended in phosphate-free physiological saline solution [140 mM NaCl/5 mM KCl/5 mM $CaCl_2$/2 mM $MgCl_2$/10 mM glucose/10 mM Hepes (pH 7.4)]. The supernatant fractions were re-centrifuged at 100000 ***g*** for 1 h and the pellets were resuspended in an equal volume of physiological saline solution and sonified. The 300 ***g*** and 10000 ***g*** pellet fractions were used for enzyme activity assays.

Ni-NTA purification of recombinant NTPDase6 from the culture medium

The Ni-NTA resin was used to purify the Myc-His-tagged NTPDase6 from the cell culture medium. The conditioned cell culture supernatant was adjusted with Tris to pH 8.0. The bound protein was eluted with 150 mM NaCl/10 mM Hepes (pH 8.0) containing 250 mM imidazole. The Ni-NTA eluate was used for nucleotidase activity assays and for Western blotting. The protein contents of the eluate fraction were determined by the technique of Spector [29]. In additional experiments the culture medium was centrifuged at 150000 ***g*** for 1 h. The supernatant and resuspended pellet fractions were incubated with Ni-NTA resin and subjected to enzyme activity assays. For Western blotting, culture supernatant fractions were precipitated with $HClO_4$ and subjected to SDS/PAGE [10 % (w/v) gel]. Immunoblotting with the monoclonal anti-Myc antibody (clone 9E10) was performed with the enhanced chemoluminescence system in accordance with the manufacturer's instructions.

Measurement of nucleotidase activities

Nucleotidase activity was determined by measuring the formation of P_i [30]. Membrane fractions were incubated in phosphate-free physiological salt solution and Ni-NTA eluates or Ni-NTA–enzyme complexes were diluted with 10 mM Hepes, pH 7.4, containing 0.5 mM $CaCl_2$ and 0.5 mM $MgCl_2$. The sodium salt of the appropriate nucleoside triphosphate, diphosphate or monophosphate was added at a concentration of 500 μM. To investigate the dependence of the enzyme activity on metal ions, 0.05–5 mM $CaCl_2$ or $MgCl_2$ was added, or $CaCl_2$ and $MgCl_2$ were replaced by 1 mM EDTA. For the determination of K_m, GDP and $MgCl_2$ were added at a molecular ratio of 1:2.

Immunofluorescence staining

At 24 h after transfection, CHO or PC12 cells were seeded on poly-(D-lysine)-coated (10 μg/ml) glass cover slips 10 mm in diameter (3×10^4 cells per well) and cultured for an additional 24 h. Nerve growth factor (10 ng/ml) was added to PC12 cells. Immunofluorescent assay of viable or previously fixed cells was performed as described previously [28] with monoclonal antibodies against the Myc epitope (clone 9E10, 5 μg/ml) or the Golgi-associated 58K protein [31] diluted 1:50 with PBS containing 1 % (w/v) BSA. After the application of an indocarbocyanin-3-labelled anti-mouse IgG antibody, cells were mounted and investigated with an epifluorescence microscope equipped with an MCID 4 imaging analysis system (Imaging Research, St Catharines, Ontario, Canada).

The fluorescent Golgi marker BODIPY® FL C_5-ceramide was used to reveal the Golgi apparatus in viable PC12 cells. The stock solution (1 mM in ethanol) was diluted 1:1000 in PC12 cell culture medium and the cells were incubated with the fluorescent probe for 30 min at 37 °C. After being washed with fresh medium, cells were fixed with 4 % (w/v) paraformaldehyde in PBS for 10 min at room temperature and mounted for fluorescence microscopy.

RESULTS

Isolation and characterization of the cDNA clone

By using colony hybridization, eight positive cDNA clones were isolated from a rat brain pCMV-SPORT 2 cDNA library after enrichment of NTPDase6 cDNA clones with the Clon Capture® cDNA selection kit. One of the clones, L2-6, was completely sequenced in both directions by primer walking. The cDNA of clone L2-6 had a length of 2458 bp and revealed a deduced ORF of 1365 bp from nt 129–1493, encoding 455 amino acid residues. A single polyadenylation signal (AAUAAA) was identified at nt 2420, which is 15 nt towards the 5′ end of the poly(A) tail of clone L2-6. The deduced amino acid sequence (Figure 1) contains two potential N-glycosylation sites situated in the middle of the polypeptide chain (Asn^{192}, Asn^{256}) and six cysteine residues. In addition, potential sites for protein kinase C phosphorylation are predicted for residues 9, 106, 210, 258, 273, 299, 312 and 446. A casein kinase II phosphorylation site is predicted for residue 61. The calculated molecular mass of the encoded protein is 49971 Da, with an isoelectric point of 8.52 and a calculated

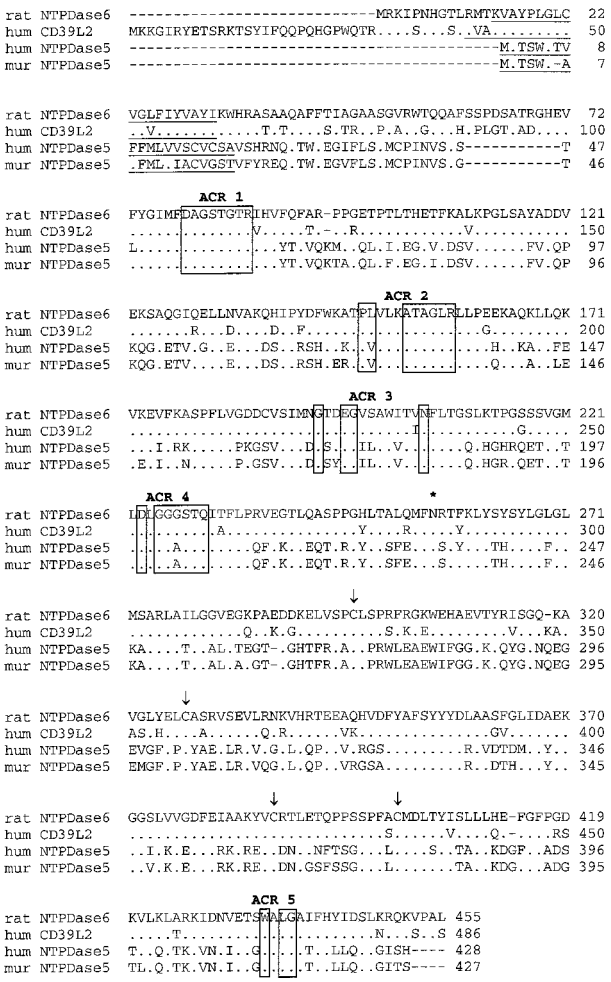

Figure 1 Alignment of the NTPDase6 amino acid sequence with the most closely related human and murine sequences

Alignment was performed with the CLUSTAL W algorithm. Dots represent identity with the amino acid residues of the rat NTPDase6 sequence. The five 'apyrase conserved regions' (ACR) are indicated by boxes and numbered. Cysteine residues and N-glycosylation sites conserved throughout the four sequences are indicated by arrows and stars respectively. Predicted N-terminal hydrophobic sequences are underlined. Accession numbers: rat NTPDase6, AJ277748; human CD39L2, AF039916; human NTPDase5 (CD39L4), AF039918; murine NTPDase5 (ER-UDPase), AJ238636.

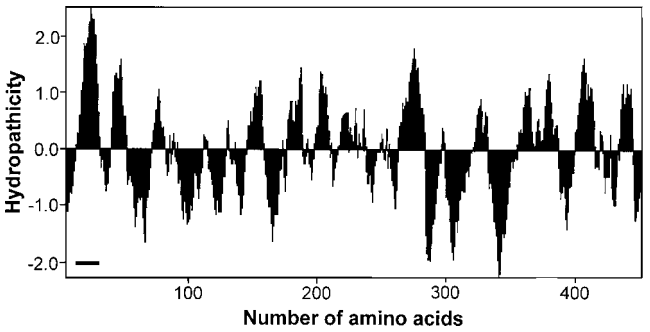

Figure 2 Hydrophobicity plot of the protein sequence deduced from rat NTPDase6

The plot was prepared by the method of Kyte and Doolittle [27] (window size: 11 residues). The bar indicates the putative N-terminal transmembrane region.

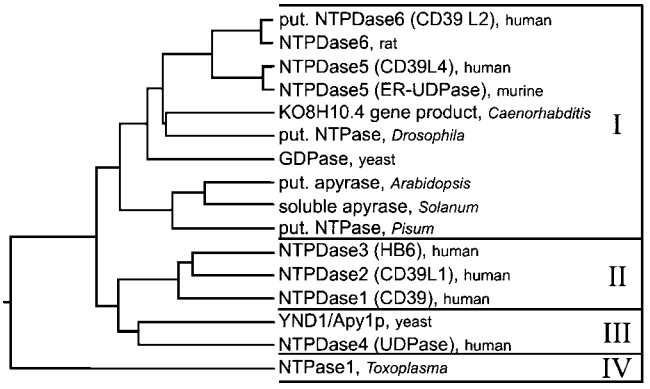

Figure 3 Hypothetical phylogenetic tree derived for 16 selected members of the E-NTPDase family

Major groups of sequences are boxed and numbered. The GenBank® accession numbers of the sequences are as follows: human putative (put.) NTPDase6 (CD39L2), AF039916; rat NTPDase6, AJ277748; human NTPDase5 (CD39L4), AF039918; murine NTPDase5 (ER-UDPase), AJ238636; KO8H10.4 gene product of *Caenorhabditis elegans*, Z83113; putative NTPDase of *Drosophila melanogaster*, AF041048; GDPase of bakers yeast, *Saccharomyces cerevisiae*, P32621; putative apyrase of *Arabidopsis thaliana*, AC004138; soluble apyrase of *Solanum tuberosum*, U58597; putative NTPase of *Pisum sativum*, Z32743; human NTPDase3 (HB6), AF034840; human NTPDase2 (CD39L1), U91510; human NTPDase1 (CD39), S73813; YND1/Apy1p of the yeast *Saccharomyces cerevisiae*, P40009; human NTPDase4 (UDPase), AF016032 and NTPase1 of *Toxoplasma gondii*, U14322.

charge at pH 7 of +5.74. Hydrophobicity analysis predicts a single hydrophobic stretch in the polypeptide chain between residues 14 and 32 (Figure 2). Computer analysis indicated no cleavage site for the N-terminal hydrophobic sequence. The protein domain structure lacks the C-terminal hydrophobic domain present in NTPDase1 to NTPDase4 and is similar to that of human and murine NTPDase5 [5,26].

Figure 1 compares the amino acid sequence of the rat NTPDase6 with that of putative human NTPDase6 (CD39L2) and human and murine NTPDase5 (CD39L4, ER-UDPase). The cDNA species of human CD39L2 and rat NTPDase6 share ATG codons at nt 232 and 129 respectively. The N-terminus of the deduced ORF of the human CD39L2 sequence is 28 residues longer. It starts at the ATG codon at nt 148, which is not found in the rat cDNA sequence. The N-terminus of the rat sequence corresponds to that of a mouse EST sequence (accession number AA611283), a sequence related to the human CD39L2 sequence [17]. All four sequences have a predicted hydrophobic domain at the N-terminus and contain the five 'apyrase conserved regions'. Only human CD39L2 and rat NTPDase6 contain a predicted N-terminal cytosolic domain. One of the two glycosylation sites is conserved between the four sequences.

Relation to other members of the E-NTPDase family

The deduced amino acid sequence of rat NTPDase6 cDNA shares 86.4% amino acid identity with that of CD39L2, the putative human NTPDase6 [17] and 48.8% and 50.8% with those of human (CD39L4) and murine (ER-UDPase) NTPDase5 respectively [5,17,26]. It is distantly related to mammalian members of the protein family with two predicted transmembrane domains, such as rat NTPDase1 and NTPDase2 [22] (21.8% and 24.2% similarity respectively), human NTPDase3 (HB6) [21]

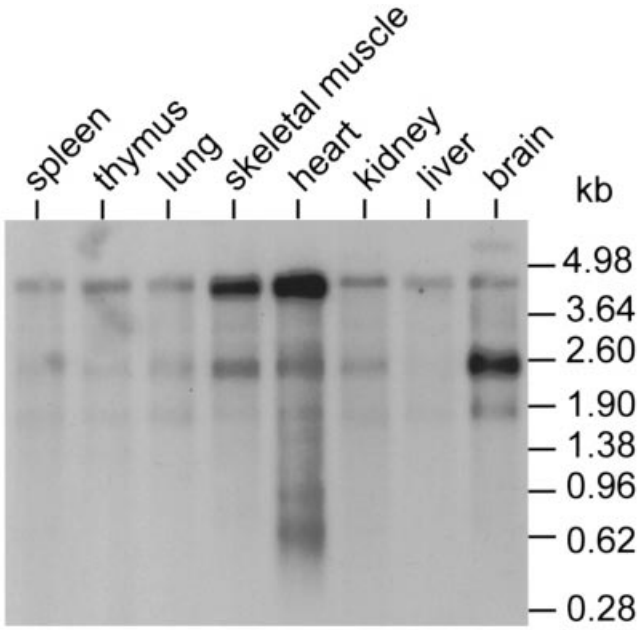

Figure 4 Northern-blot analysis of expression of NTPDase6 in rat tissues

Polyadenylated RNA (1 μg per lane) isolated from adult rat tissues was electrophoretically separated on 1% (w/v) agarose/formaldehyde gel, transferred to a nylon membrane and hybridized with a 157 bp digoxigenin-labelled riboprobe derived from clone pBSK(−)NTPDase6.

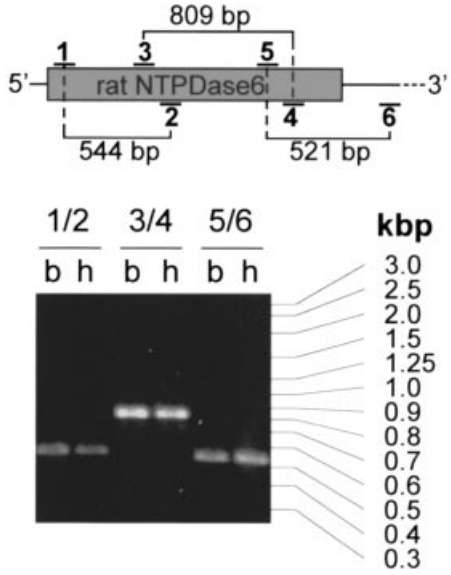

Figure 5 Characterization of the NTPDase6 ORF in brain and heart by reverse-transcriptase-mediated PCR analysis

The cDNA species employed for PCR were derived from brain (b) and heart (h) tissue. The amplification products resulting from primer combinations 1/2, 3/4 and 5/6 form overlapping frames. The relative locations of the primers (1, 2, 3, 4, 5 and 6) are shown in the upper panel. The rat NTPDase6 ORF is presented as a shaded box. DNA fragments were analysed on a 1% (w/v) agarose gel.

(23.7%), or the Golgi-allocated NTPDase4 (UDPase) [4] (26.2%).

A multiple sequence alignment of 16 selected members of the E-NTPDase family depicts four major groups (Figure 3). NTPDase5 and NTPDase6 belong to group I, which is over-represented in Figure 3. These two sequences are related to putative *Caenorabditis elegans* and *Drosophila melanogaster* NTPDases and a yeast guanosine diphosphatase. They are more distantly related to sequences obtained for a variety of plants such as *Arabidopsis thaliana*, *Solanum tuberosum* and *Pisum sativum*. The sequences in group I have an origin distinct from those forming group II and group III. The members of group II, NTPDase1 to NTPDase3, represent surface-located ectoenzymes. The members of group III, NTPDase4 and the yeast protein YND1/Apy1p, are Golgi-localized intracellular enzymes. The secreted enzymes of the protozoan parasite *Toxoplasma gondii* forming group IV are distantly related to those in the three other groups.

Northern-blot analysis

To analyse the tissue distribution of NTPDase1, polyadenylated RNA was purified from several rat tissues and probed with an 157 bp anti-sense probe. Signals were obtained for all tissues analysed, including spleen, thymus, lung, skeletal muscle, heart, kidney, liver and brain (Figure 4). Two major bands were detected at 2.5 and 4.6 kb. The preponderance of the two types of mRNA varied between tissues. Whereas in most tissues the 4.6 kb form predominated, the signal for the 2.5 kb form was strongest in the mRNA isolated from brain. The NTPDase6 cDNA of 2458 bp isolated in our experiments would correspond to the 2.5 kb band detected in the Northern blot. To investigate whether the different mRNA species encoded splice variants of NTPDase6 or identical reading frames, mRNA species obtained from brain (revealing a strong 2.5 bp band) and heart (revealing a strong 4.6 bp band) were subjected to PCR. By using three different primer pairs, three amplification products with overlapping frames covering the ORF of NTPDase6 were obtained (Figure 5). In each case the amplification products for brain and heart were identical. We conclude that heart and brain tissues express only a single ORF for NTPDase6 and that the two types of mRNA obtained in Northern blots differ in the lengths of their untranslated sequences.

Expression and biochemical characterization of NTPDase6

NTPDase6 with a Myc-His tag added to the C-terminus was expressed in CHO or PC12 cells. Cells transfected with the empty vector alone were used as controls. The Myc tag was used for immunological detection and the His tag for affinity isolation of the protein.

Increased catalytic activity for the hydrolysis of nucleoside diphosphates was observed in the culture supernatants of cells transfected with the pcDNA3/NTPDase6 construct. We therefore used an Ni-NTA resin to isolate the tagged protein from culture supernatants of CHO cells 2 days after transfection and 1 day after the final exchange of medium. The protein was eluted from the resin with 250 mM imidazole, pH 8.0, for analysis of its catalytic properties. The enzyme revealed highest activities for the hydrolysis of GDP and IDP (Table 1). Hydrolysis rates with UDP and CDP as substrates were considerably lower; the hydrolysis of ADP was only one-tenth of that of GDP. The hydrolysis of nucleoside triphosphates amounted to 10% or less of GDP hydrolysis. We analysed all nucleoside triphosphates by HPLC for possible contamination with nucleoside diphosphates. The relative contributions of GDP, IDP, UDP, CDP and ADP in the corresponding commercial nucleoside triphosphate samples were 5.7%, 2.8%, 9.4%, 14.0% and 0% respectively. It is therefore possible that the nucleoside diphosphates con-

Table 1 Substrate specificity of recombinant NTPDase6 in Ni-NTA eluates of cell culture supernatants

CHO cells were transfected with the pcDNA3/NTPDase6 construct or with the empty vector. The tagged protein in the cell culture supernatant was bound to the Ni-NTA resin and eluted with imidazole. Activities are expressed as percentages of the GDP hydrolysis rate. Values are means ± S.E.M. for three experiments with duplicate determinations in each. The 100% value corresponds to 6.3 ± 2.1 (mean ± S.E.M.) μmol of P_i/min per mg of protein.

	Activity (% of GDP hydrolysis rate)	
Substrate	NTPDase6	Vector
GDP	100	3 ± 1.9
IDP	73 ± 10.4	2 ± 1.8
UDP	27 ± 6.3	4 ± 2.4
CDP	26 ± 6.2	2 ± 1.8
ADP	10 ± 3.8	6 ± 2.9
GTP	10 ± 4.5	2 ± 1.9
ITP	5 ± 2.6	0 ± 0.6
UTP	9 ± 3.7	4 ± 2.5
CTP	8 ± 3.6	0 ± 0.9
ATP	1 ± 1.3	0 ± 1.2
GMP	2 ± 1.6	0 ± 0.8
UMP	0 ± 0	2 ± 2.1
AMP	0 ± 0	1 ± 1.2

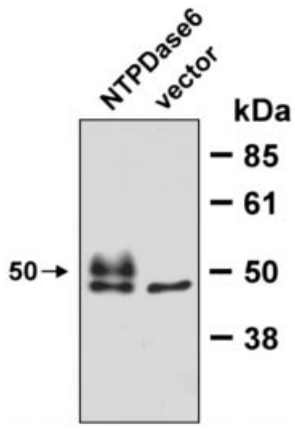

Figure 6 Western blot analysis of NTPDase6

Culture supernatants of CHO cells transfected with the pcDNA3/NTPDase6 construct (NTPDase6) or the empty vector (vector) were incubated with Ni-NTA resin. Bound protein was released with imidazole buffer and precipitated with $HClO_4$. Each lane was loaded with 60 μg of protein. Blotted proteins were reacted with anti-Myc antibody.

tributed to the apparent nucleoside triphosphatase activities determined. Nucleoside monophosphates were not substrates of NTPDase6. Catalytic activities in culture supernatants of vector-transfected controls subjected to the same protocol were very low or absent.

To determine whether the enzyme pool detected in the culture supernatant was in a soluble form or associated with membrane micelles shed from the surface of transfected CHO cells, the culture supernatants were subjected to a high-speed centrifugation step (150000 ***g*** for 1 h). As analysed for GDP as a substrate (0.5 mM and 2 mM Ca^{2+}, 2 mM Mg^{2+}), the catalytic activity remained entirely in the supernatant fraction, suggesting that the enzyme was in a soluble form. The fractionation of transfected CHO cells under isotonic conditions suggested that the enzyme can also be allocated to intracellular membrane compartments. Both the 300 ***g*** and subsequent 100000 ***g*** pellets of the cell homogenates obtained under isotonic conditions contained increased catalytic activity for the hydrolysis of nucleoside diphosphates. The pattern of substrate specificities corresponded to that determined for the soluble enzyme. The membrane fractions of vector-transfected cells contained significant endogenous nucleoside diphosphatase activity. However, there was an approx. 4-fold increase in the hydrolysis rate for GDP in both the 300 ***g*** and 100000 ***g*** pellet fractions obtained from CHO cells transfected with the pcDNA3/NTPDase6 construct.

As analysed for GDP, the substrate yielding the highest catalytic activity, the enzyme isolated from the culture supernatant was strongly and equally activated by either Ca^{2+} or Mg^{2+}. At a GDP concentration of 0.5 mM, maximal activity was obtained at a 1 mM concentration of either Ca^{2+} or Mg^{2+} (4.3 ± 2.8 μmol of Pi/min per mg of protein; mean ± S.E.M., n = 3). The catalytic activity in the absence of added bivalent cations was 5.5% of the maximal activity and was decreased to 1.8% in the presence of the chelator EDTA. The K_m for GDP in the presence of Mg^{2+} was 211 ± 62 μM (mean ± S.D., n = 3).

Western blots of the culture supernatant of CHO cells transfected with the pcDNA3/NTPDase6 construct revealed an immunoreactive protein at 50 kDa (Figure 6). A 47.6 kDa band that could also be detected in culture supernatants of vector controls presumably resulted from a non-specific interaction of the anti-Myc antibody with a protein contained in the culture medium. The predicted molecular mass of the tagged recombinant NTPDase6 is 52.3 kDa. The amino acid residues of the Myc-His tag are expected to contribute 2.3 kDa to the apparent molecular mass. This suggests that the soluble form of NTPDase6 is cleaved close to the N-terminal hydrophobic domain.

Cellular localization of NTPDase6

The tagged NTPDase6 was localized in transfected CHO and PC12 cells by immunocytochemistry with a monoclonal antibody against the Myc epitope (Figure 7). When the antibody was applied to CHO cells fixed with methanol 2 days after transfection, the protein was detected in close proximity to the cell nucleus. It was associated with distinct interconnected structural elements (Figures 7A and 7A′). The shape of the immunofluorescent structure revealed some variability between cells. In some cases it was located around the nucleus. In other cases it was restricted to a smaller cytoplasmic domain. When the primary antibody was applied to viable transfected cells, surface-located immunofluorescence could be detected (Figures 7B and 7B′). The intensity of surface-located immunofluorescence was very low in most immunopositive cells. A comparison of the contribution of immunopositive cells revealing surface labelling with intracellularly labelled cells seeded from the identical electroporation cuvette revealed that approx. 20% of the transfected cells were surface-labelled. Vector-transfected cells revealed no immunofluorescence (results not shown).

The cellular localization of NTPDase6 was also analysed in PC12 cells. PC12 cells, derived from a tumour of the rat adrenal medulla [32] are immunoreactive for the anti-58K antibody that binds to the Golgi apparatus. At 2 days after transfection with the pcDNA3/NTPDase6 construct, PC12 cells revealed an intracellular labelling corresponding to that obtained for CHO

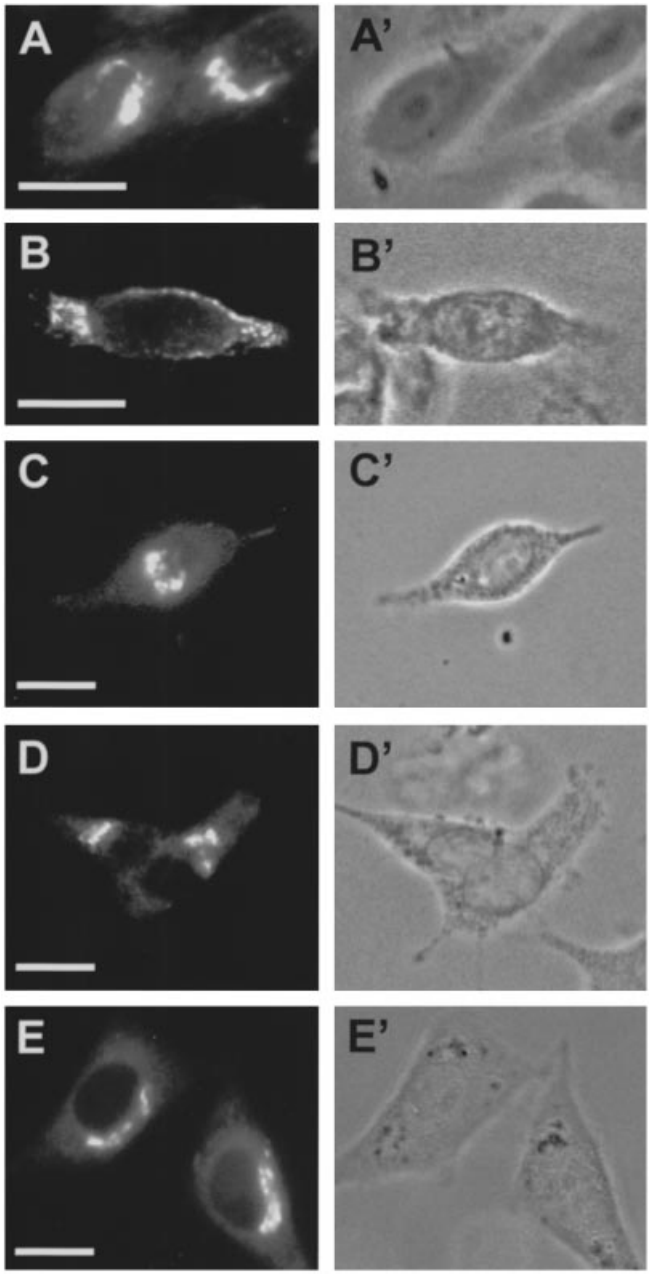

Figure 7 Localization of tagged NTPDase6 in transfected CHO and PC12 cells

CHO cells (**A**, **B**) or PC12 cells (**C**, **D**, **E**) were transfected with the pcDNA3/NTPDase6 construct and analysed 2 days after transfection. Immunolocalization of tagged NTPDase6 with the anti-Myc monoclonal antibody was performed after fixation with methanol (**A**, **C**) or on the surface of viable cells (**B**). (**D**) Immunolocalization of the Golgi apparatus with the anti-58K antibody. (**E**) Localization of the Golgi apparatus after application of the fluorescent Golgi marker BODIPY® FL C_5-ceramide. (**A′**–**E′**) Corresponding phase-contrast images. Scale bars, 20 μm.

cells (Figures 7C and 7C′). The Golgi-specific anti-58K antibody revealed an identical pattern of immunolabelling (Figures 7D and 7D′). Because both the anti-Myc antibody and the anti-58K antibody were monoclonal, double-labelling experiments could not be performed. Vector-transfected cells or cells analysed with only the secondary antibody were immunonegative. In addition, the fluorescent BODIPY® FL C_5-ceramide Golgi marker was applied to viable PC12 cells. It revealed the same staining pattern within the perinuclear region as the two antibodies (Figures 7E and 7E′).

DISCUSSION

NTPDase6, a novel member of the E-NTPDase family

In the present study we describe the primary structure of rat NTPDase6 and show that it is a novel enzyme member of the E-NTPDase family that hydrolyses nucleoside diphosphates, in particular GDP and IDP. The rat NTPDase6 sequence shares 86 % amino acid identity with the human CD39L2 sequence [17]. CD39L2 presumably encodes the human orthologue of NTPDase6 but has not previously been expressed or characterized. The closest functionally identified relative of rat NTPDase6 is murine NTPDase5. The two sequences share 50.8 % amino acid identity, an N-terminal hydrophobic region and one potential N-glycosylation site.

The simultaneous presence of NTPDase6 in the Golgi apparatus, at the cell surface and in the culture supernatant seems puzzling. Our results suggest that NTPDase6 can be cleaved to form a soluble enzyme. The cellular site of protein cleavage is unknown. The observation that the protein can be detected at the surface of a small percentage of cells indicates that not all of the pool of this protein is cleaved inside the ER. The cellular localization of NTPDase6 is similar to that of NTPDase5, the most closely related member within the E-NTPDase family. Murine NTPDase5 (ER-UDPase) was identified as a soluble nucleoside diphosphatase in the lumen of the liver ER [5], whereas the human orthologue (CD39L4) was found to be released from transfected COS-7 cells in soluble form [26]. This suggests that NTPDase6, like NTPDase5, might be cleaved at the N-terminus, retained in an intracellular membrane compartment and in addition be released into the extracellular medium.

Catalytic properties

As with NTPDase5 [5,26], NTPDase6 has a strong preference for nucleoside diphosphates but the two enzymes differ in their substrate specificities. The rat NTPDase6 described here hydrolyses GDP > IDP $\gg$ UDP, CDP $\gg$ ADP, whereas murine NTPDase5 [5] hydrolyses UDP > GDP, IDP $\gg$ ADP, CDP. The catalytic properties of NTPDase5 and NTPDase6 differ from those of the Golgi-located NTPDase4 (human UDPase). This enzyme preferentially hydrolyses UDP and GDP but also the corresponding nucleoside triphosphates [4].

Members of the E-NTPDase family with a significant intracellular localization differ in general substrate specificity from the corresponding ectoenzymes. The intracellular enzymes have a preference for nucleoside 5′-diphosphates, whereas the ectoenzymes NTPDase1 to NTPDase3 hydrolyse nucleoside 5′-triphosphates preferentially [13,18–22]. It can be expected that these principal differences in catalytic properties reflect the nucleotide substrates available to the enzymes *in vivo*. Furthermore, it is noteworthy that the degree of putative glycosylation varies between the intracellular and surface-located members of the enzyme family. NTPDase4 to NTPDase6 have two potential N-glycosylation sites, whereas the surface-located NTPDase1 to NTPDase3 contain six or seven potential N-glycosylation sites.

Functional relevance

The functional role of NTPDase6 has not yet been investigated. The function of the previously identified nucleoside-

diphosphate-hydrolysing enzymes of the ER (NTPDase5, ER-UDPase) and the Golgi apparatus (NTPDase4, UDPase) has been related to the glycosylation reactions that occur in these cellular compartments. Nucleotide sugars synthesized in the cytosol can be transported via specific transport systems into either the rough ER or the Golgi apparatus. There they are used by luminal glycosyltransferases and serve the glycosylation of proteins and lipids. The resulting nucleoside diphosphates, which cause product inhibition of the glycosyltransferases, are cleaved by luminal nucleoside diphosphatases. The resulting nucleoside monophosphates are then exchanged for more nucleotide sugars via an anti-porter system [33,34].

There is a distinct topography of glycosylation reactions in these endomembrane systems. Whereas UDP derived from imported UDP-sugars is present in the ER lumen, GDP is not known to be present in this compartment [35]. It has therefore been suggested that the soluble NTPDase5 of the ER (ER-UDPase) mainly supports glycoprotein re-glycosylation reactions by hydrolysing UDP derived from UDP-glucose [5]. The Golgi-associated NTPDase6 has a preference for the hydrolysis of GDP and might thus have a role in the hydrolysis of GDP located in the Golgi lumen, and consequently in the transport of GDP-fucose, the only GDP nucleotide sugar known to be transported into the Golgi apparatus of mammalian cells [33,34]. No inosine nucleotides are known to participate in glycosylation reactions. The IDPase activity of NTPDase6 therefore remains unexplained.

NTPDase6 hydrolyses UDP less than one-third as efficiently as GDP and might therefore be less relevant for the hydrolysis of UDP and the import of UDP sugars than the ubiquitously Golgi-located and membrane-anchored NTPDase4 (UDPase) [4]. It should be noted that NTPDase5 and NTPDase6 have identical dependences on Ca^{2+} or Mg^{2+}, whereas NTPDase4 is preferentially activated by millimolar Ca^{2+} and only to a small extent by Mg^{2+} [4]. The intraluminal concentrations of Ca^{2+} or Mg^{2+} might thus influence the effect of the individual nucleotidases in the hydrolysis reactions. It remains to be determined whether the Golgi-located nucleotidases can be involved in functions additional to the transport of nucleotide sugars and glycosylation.

Possible role as an exoenzyme

The released and soluble NTPDase6 could in principle contribute to the hydrolysis of extracellular nucleotides. Release of the Golgi-allocated enzyme might be due to incomplete sorting or retention mechanisms or represent a constitutive and physiologically relevant secretory mechanism. Interestingly, soluble glycosyltransferases derived from the Golgi membrane-bound forms by proteolysis are also secreted from cells in active form [33]. In contrast with ADP and UDP [2,3], GDP and IDP have not been recognized as extracellular mediators. However, both guanosine and inosine elicit cellular responses [36,37]. UDP and ADP are hydrolysed by NTPDase6 3-fold and 10-fold more slowly than GDP. The K_m of 211 μM for the hydrolysis of GDP by NTPDase6 is higher than that determined for ATP and the prototype ectonucleotidase NTPDase1 (CD39) (K_m values 10–100 μM) [1].

Relation to other members of the E-NTPDase family

Yeast (*Saccharomyces cerevisiae*) expresses two members of the E-NTPDase family, both located in the Golgi apparatus. Yeast GDPase is related to the mammalian nucleoside diphosphatases NTPDase5 and NTPDase6 [38], whereas the other yeast enzyme (YND1/Apy1p) [39,40] is related to NTPDase4, the human Golgi-located UDPase [4]. The yeast enzymes have similar general catalytic properties to those of the related mammalian enzymes. Double deletions of the two yeast genes reveal that the enzymes are required for Golgi glycosylation and cell wall integrity [39]. It is noteworthy that the genome of *S. cerevisiae* contains only these two copies of the E-NTPDase family genes and that surface-located members of this enzyme family are apparently lacking. It is tempting to speculate that, during the course of evolution, members of the E-NTPDase family were first located in intracellular membrane compartments such as the Golgi apparatus, where they had an important function in facilitating glycosylation reactions by hydrolysing nucleoside diphosphates. After gene duplication in multicellular organisms, members of the enzyme family might have reached a surface location with partly altered catalytic properties.

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