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Golgi Localization and Functional Expression of Human Uridine Diphosphatase*

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A full-length E(ecto)-ATPase (Plesner, L. (1995) *Int. Rev. Cytol.* 158, 141–214) cDNA was cloned from a human brain cDNA library; it encodes a 610-amino acid protein that contains two putative transmembrane domains. Heterologous expression of this protein in COS-7 cells caused a significant increase in intracellular membrane-bound nucleoside phosphatase activity. The activity was highest with UDP as substrate and was stimulated by divalent cations in the following order: $Ca^{2+} \gg Mg^{2+} > Mn^{2+}$. The results of immunofluorescence staining indicate that this protein is located in the Golgi apparatus. UDP hydrolysis was increased in the presence of Triton X-100 or alamethicin, an ionophore that facilitates movement of UDP across the membrane, suggesting that the active site of this UDPase is on the luminal side of the Golgi apparatus. This is the first identification of a mammalian Golgi luminal UDPase gene. Computer-aided sequence analysis of the E-ATPase superfamily indicates that the human UDPase is highly similar to two hypothetical proteins of the nematode *Caenorhabditis elegans* and to an unidentified 71.9-kDa yeast protein and is less related to the previously identified yeast Golgi GDPase.

There is evidence that a UDPase is present on the luminal side of the Golgi apparatus of mammalian cells (1, 2). Proteins and lipids are glycosylated in the lumen of the Golgi apparatus. UDP-Gal, a sugar donor in glycosylation, is synthesized in the cytosol and translocated into the Golgi lumen via specific membrane carriers (3). After transfer of sugar residues to proteins and lipids by galactosyltransferases, the resulting UDP is hydrolyzed to UMP by UDPase (4). In this way, UDP, which is highly inhibitory to galactosyltransferases (5), does not accumulate in the lumen of the Golgi apparatus. UMP then exits the Golgi lumen by exchange with cytosolic UDP-galactose (for review, see Ref. 6).

A highly specific *Saccharomyces cerevisiae* Golgi GDPase has been described and purified to homogeneity (7). This enzyme appears to be involved in protein and lipid mannosylation, as the GDP is generated from the mannose donor, GDP-mannose, by mannosyltransferase. Null mutants of yeast GDPase accumulate GDP in the Golgi lumen and decrease mannosylation of proteins and lipids in this compartment (8). These mutant cells also have defects in the transfer of GDP-mannose into the Golgi lumen (9) because GMP exit is required for the entry of GDP-mannose. The gene encoding the yeast GDPase, *GDA1* (8), is a member of the E-ATPase protein family (10) since it is similar in amino acid sequence to potato apyrase (11) and to animal ectoapyrase CD39 (12, 13). The genes encoding mammalian Golgi UDPase and GDPase have not been identified yet. Through a homology search of the GenBank™ Data Bank, we found a newly identified human partial cDNA (Kiaa0392) that encodes a novel E-ATPase (14). In this study, we show that this new E-ATPase is a Golgi luminal UDPase.

EXPERIMENTAL PROCEDURES

Materials—Nucleoside phosphates and *Trichoderma viride* alamethicin were purchased from Sigma. RPMI 1640 medium, fetal bovine serum, penicillin/streptomycin, Dulbecco's modified Eagle's medium, and L-glutamine were purchased from Life Technologies, Inc. Brefeldin A (BFA)[1] was purchased from Molecular Probes, Inc. (Eugene, OR). Anti-Myc monoclonal antibody (9E10) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-p58 monoclonal antibody that recognizes an epitope located on the 58-kDa Golgi peripheral membrane protein (15) was purchased from Sigma.

Cloning of a Full-length UDPase cDNA—The rapid amplification of 5′-cDNA ends (5′-RACE) method (16) was used to amplify the 5′-end upstream sequence of the human Kiaa0392 cDNA (GenBank™ accession number AB002390) (14) from a human brain Marathon-Ready cDNA library (CLONTECH, Palo Alto, CA). The sense PCR primer AP1 (5′-CCATCCTAATACGACTCACTATAGGGC) was provided by the manufacturer for amplifying the 5′-end adapter sequence (5′-CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGGCAGGT). The antisense primer A1 (5′-GTGGCACATGCTCTGCAGCAAAGTTCAA) was complementary to nucleotides 308–335 of the human brain Kiaa0392 cDNA (14). The PCR product was subcloned into pGEM3zf⁻ (Promega, Madison, WI) with a blunt end at the 5′-end and a *Pst*I site at the 3′-end (underlined above).

The remaining coding sequence of Kiaa0392 cDNA was also amplified by PCR. The nucleotide sequence of the sense primer S1 was complementary to that of primer A1. The antisense primer A2 (5′-GCTCTAGAGTGGAGCTGTGAGCTGAATCACAAGGT), with an *Xba*I site (underlined) at the 5′-end, contained a sequence identical to nucleotides 1658–1684 of Kiaa0392 cDNA (14). Full-length cDNA was created by fusing this PCR product with the 5′-RACE PCR product using the *Pst*I and *Xba*I restriction sites and was subcloned into GW1-CMV mammalian expression vector (British Biotechnology, Oxford, United Kingdom) using *Not*I and *Xba*I sites. DNA was sequenced by the dideoxy chain termination method with a Sequenase kit from U. S. Biochemical Corp.

Two primers were used to PCR-amplify cDNA encoding Myc epitope-tagged UDPase (Myc-UDPase). The sense primer contained a sequence identical to the T7 promoter of pGEM3zf⁻ vector. The antisense primer (5′-GCTCTAGATTACAAGTCCTCTTCAGATATCAGCTTTTGCTCCAAGGTCCCTGGGGCATT) contained an *Xba*I site (underlined) at the 5′-end, a stop codon, an antisense sequence encoding the Myc epitope

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF016032.

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[1] The abbreviations used are: BFA, brefeldin A; 5′-RACE, rapid amplification of 5′-cDNA ends; PCR, polymerase chain reaction; ACR, apyrase conserved region; RER, rough endoplasmic reticulum; E-ATPase, ecto-ATPase.

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TCG AGC GGC CGC CCG GGC AGG TCA GGA AAG GAG CGC GGC CTC CAG GAA GCC GGC TGG CCG   60
TGA TGC TGC CCA CTG GTG GTG CCC CGC TCC CGG TCC CCG CCG GCC AAG CAG GAG GGC TCT  120
AAC TCC GTG AGA CCC CAG CAT TAT TTC TAT AAT TGT TTG TGA GAA GGA CTG AAT CCC AGA  180
GCA TTG CCT TGT TGC TGA CCT TTC AGT atg ggg agg att ggc atc tcc tgt ctt ttt cct  240
                                    M   G   R   I   G   I   S   C   L   F   P     11
gct tct tgg cat ttt agc ata tct cca gta ggg tgt cct cga att ctg aat acc aat tta  300
A   S   W   H   F   S   I   S   P   V   G   C   P   R   I   L   N   T   N   L     31
cgc caa att atg gtc att agt gtc ctg gct gct gct gct gtt tca ctt tta tat ttt tct  360
R   Q   I   M   V   I   S   V   L   A   A   A   A   V   S   L   L   Y   F   S     51
gtt gtc ata atc cga aat aag tat ggg cga cta acc aga gac aag aaa ttt caa agg tac  420
V   V   I   I   R   N   K   Y   G   R   L   T   R   D   K   K   F   Q   R   Y     71
ctg gca cga gtt acc gac att gaa gct aca gac acc aat aac ccc aat gtg aac tat ggg  480
L   A   R   V   T   D   I   E   A   T   D   T   N   N   P   N   V   N   Y   G     91
atc gtg gtg gac tgt ggt agc agt ggg tct cga gta ttt gtt tac tgc tgg cca agg cat  540
I   V   V   D   C   G   S   S   G   S   R   V   F   V   Y   C   W   P   R   H    111
aat ggc aat cca cat gat ctg ttg gat atc agg caa atg agg gat aaa aac cga aag cca  600
N   G   N   P   H   D   L   L   D   I   R   Q   M   R   D   K   N   R   K   P    131
gtg gtc atg aag ata aaa ccg ggc att tca gaa ttt gct acc tct cca gag aaa gtc agt  660
V   V   M   K   I   K   P   G   I   S   E   F   A   T   S   P   E   K   V   S    151
gat tac att tct cca ctt ttg aac ttt gct gca gag cat gtg cca cgg gca aaa cac aaa  720
D   Y   I   S   P   L   L   N   F   A   A   E   H   V   P   R   A   K   H   K    171
gag aca cct ctc tac att ctc tgc acg gct gga atg aga atc ctc ccc gaa agc cag cag  780
E   T   P   L   Y   I   L   C   T   A   G   M   R   I   L   P   E   S   Q   Q    191
aaa gct att ctg gaa gac ctt ctg acc gat atc ccc gtg cac ttt gac ttt ctg ttt tct  840
K   A   I   L   E   D   L   L   T   D   I   P   V   H   F   D   F   L   F   S    211
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T   P   D   M   P   Y   L   D   P   C   L   P   L   D   I   K   D   E   I   Q    371
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Q   N   G   Q   T   I   Y   L   R   G   T   G   D   F   D   L   C   R   E   T    391
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I   Q   P   F   M   N   K   T   N   E   T   Q   T   S   L   N   G   V   Y   Q    411
ccc cca att cac ttc cag aac agt gaa ttc tat ggc ttc tcc gaa ttc tac tac tgc acc1500
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P   L   R   D   I   Q   Q   E   A   F   R   A   S   H   T   H   W   R   G   V    551
tcc ttt gtc tac aac cac tac ctg ttc tct ggc tgc ttc ctg gtg gtg ctg ctg gcc atc1920
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L   L   Y   L   L   R   L   R   R   I   H   R   R   T   P   R   S   S   S   A    591
gcc gcc ctc tgg atg gag gag ggc ctt ccc gcc cag aat gcc cca ggg acc ttg tga tcc2040
A   A   L   W   M   E   E   G   L   P   A   Q   N   A   P   G   T   L   *        609
```

Fig. 1. **Nucleotide sequence of human UDPase cDNA together with the deduced amino acid sequence.** Nucleotides and amino acid residues are numbered. Before the ATG initiation codon, two stop codons are identified (*thick white letters*). The four highly conserved apyrase regions (ACR1–4) are in *boldface*. The putative membrane-spanning regions determined with the use of the algorithm of Kyte and Doolittle (23) are *underlined*. The two putative *N*-linked glycosylation sites are in *boldface* and *underlined*.

(EQKLISEEDL), and a sequence complementary to nucleotides 2017–2134 in Fig. 1. The cDNA fragment was subcloned into GW1 mammalian expression vector using *Not*I and *Xba*I sites and then partially sequenced.

Northern Analysis—Polyadenylated RNA (2 μg/lane) from human cell lines was subjected to electrophoresis on a formaldehyde-containing 1.2% agarose gel and blotted by capillary flow onto a charge-modified nylon membrane (NEN Life Science Products). A human multiple-tissue Northern blot (2 μg of polyadenylated RNA/lane; CLONTECH) was used to determine tissue specificity of UDPase mRNA. The probes were generated from UDPase cDNA by PCR and then labeled with [α-^{32}P]dATP using a random priming kit (Life Technologies, Inc.). Prehybridization, hybridization, and washing of membranes were carried out following the rapid hybridization protocol from CLONTECH. Final washes were at 68 °C in 0.1% sodium saline citrate and 0.1% SDS. The blot was exposed to Biomax Mr film (Eastman Kodak Co.) with intensifying screens at −80 °C for 48 h. A human β-actin cDNA probe was used as a control.

Genomic Southern Analysis—Human genomic DNA (10 μg) was digested with either *Eco*RI or *Nde*I, electrophoresed on a 0.8% agarose gel, and transferred to a charge-modified nylon membrane. The membrane blot was then prehybridized and hybridized under high stringency conditions (42 °C, 50% formamide and 5× SSC). For the probe, a 404-base pair DNA fragment (nucleotide sequence 1024–1428) was generated by PCR. The primer sequences were 5′-ATGGGCGGCGTGTCGACT and 5′-TTAGAGGGAAGTCTGGGTCTC. This DNA fragment has neither *Eco*RI nor *Nde*I restriction sites. PCR amplification of human genomic DNA with these two primers resulted in a 400-base pair product (data not shown), indicating that there is no intron between these two primers. Final washes were at 65 °C in 0.5% sodium saline citrate and 0.1% SDS.

Cell Culture and Transfection—COS-7 cells were grown in a humidified incubator with 5% CO_2 at 37 °C in Dulbecco's modified Eagle's medium supplemented with penicillin, streptomycin, glutamine, and 10% fetal bovine serum. COS-7 cells were transfected with cDNA encoding wild-type or Myc-tagged protein by LipofectAMINE reagent (Life Technologies, Inc.). The cells were harvested for immunoblotting or for enzymatic assay 48 h after transfection.

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Preparation of COS-7 Crude Membranes—Crude membranes of the transfected COS-7 cells were prepared by the method of Coppi and Guidotti (17). Protein concentration was determined by the method of Peterson (18).

Electrophoresis and Immunoblotting—COS-7 cells transfected with control vector or Myc-UDPase cDNA were harvested, solubilized in reducing sample buffer (2% SDS, 10% glycerol, 1% β-mercaptoethanol, and 65 mM Tris-HCl, pH 6.8), and boiled for 5 min prior to loading. Samples were analyzed on 9% SDS-polyacrylamide gel, followed by immunoblotting with anti-Myc monoclonal antibody 9E10. Immunoreactive bands were visualized with horseradish peroxidase-conjugated goat anti-mouse antibody and the Renaissance chemiluminescence reagent (DuPont).

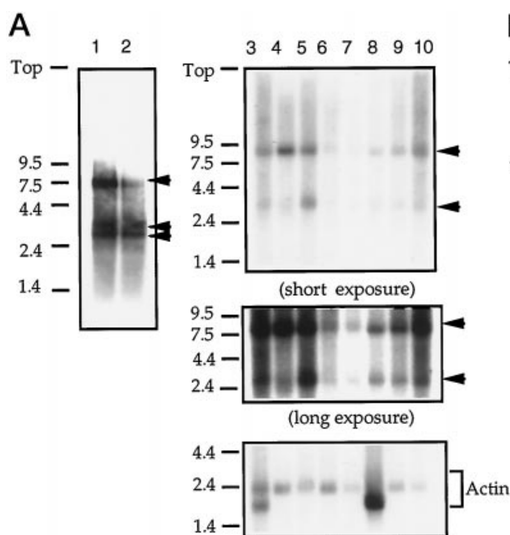

FIG. 2. **Northern and Southern analyses of the human UDPase gene.** *A*, presence of mRNA in human B lymphoblast LG2 cells (*lane 1*) and human T lymphocyte Jurkat cells (*lane 2*) and in various human tissues, including heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas (*lanes 3–10*, respectively; with short or long exposure time). The positions of UDPase mRNA in the human tissue blot are indicated by *arrowheads*. The positions of molecular size markers are shown in kilobases (*kb*). Human β-actin cDNA was used as a control probe for the human tissue blot. There is a single 2.0-kilobase band in all lanes, except for heart and skeletal muscle, which contain a second form of β-actin cDNA (1.8 kilobases). *B*, Southern analysis of *Eco*RI-digested (*lane 1*) and *Nde*I-digested (*lane 2*) human genomic DNA (10 μg).

Measurement of Nucleoside Phosphatase Activity—To measure nucleoside phosphatase activity, COS-7 crude membranes (12 μg at 0.24 mg/ml) were preincubated for 5 min at 37 °C with 1 mM NaN_3, 0.5 mM Na_3VO_4, and 5 mM $CaCl_2$ in 45 μl of buffer A (20 mM HEPES/Tris, pH 7.4, 120 mM NaCl, 5 mM KCl, and 0.2 mM EDTA). The nucleoside phosphatase reaction was initiated by addition of 0.1 volume (5 μl) of the same buffer containing nucleoside phosphate substrates, pH 7.0, to give a final concentration of 0.1–1 mM. Nucleoside phosphatase activities were linear for at least 30 min (see Fig. 3*C*). The divalent cation-stimulated nucleoside phosphatase activity was determined by measuring the inorganic phosphate released as described by Lanzetta *et al.* (19) or by Ames (20) and by subtracting values obtained with 0.2 mM EDTA alone from those obtained with various concentrations of divalent cation (*e.g.* $CaCl_2$, $MgCl_2$, or $MnCl_2$) plus chelator. To examine the membrane orientation of the UDPase activity, alamethicin (final concentration of 0.2 mg/ml) or Triton X-100 (final concentration of 0.1% (v/v)) was added to facilitate transmembrane diffusion of UDP.

Intact or lysed COS-7 cells were used to determine whether the active site of this protein is located intracellularly or extracellularly. Transfected COS-7 cells were incubated with 10 mM EDTA to detach them from culture dishes and washed twice with buffer A. The intact cells were resuspended in buffer A with 1 mM NaN_3 and 0.5 mM Na_3VO_4 to a final density of 10^6 cells/ml. Triton X-100 was added to a final concentration of 0.1% together with protease inhibitors including 1 mM phenylmethylsulfonyl fluoride and 2 μg/ml each aprotinin, chymostatin, pepstatin A, and leupeptin. The cells were allowed to lyse for 30 min on ice. In a separate experiment, cells were disrupted by homogenization with a Dounce homogenizer in the same buffer without Triton X-100. Both intact cells and cell lysates were then preincubated for 5 min at 37 °C in 180 μl of buffer A with 1 mM NaN_3 and 0.5 mM Na_3VO_4, with or without 5 mM $CaCl_2$. The nucleoside phosphatase reaction was initiated by addition of 0.1 volume (20 μl) of the same buffer containing UDP, pH 7.0, to give a final concentration of 1 mM. At the end of the incubation, cells that had not been homogenized or treated with Triton X-100 were still intact (>90%) as demonstrated by trypan blue exclusion. The calcium-activated UDPase activities were determined by measuring the inorganic phosphate released as described by Ames (20)

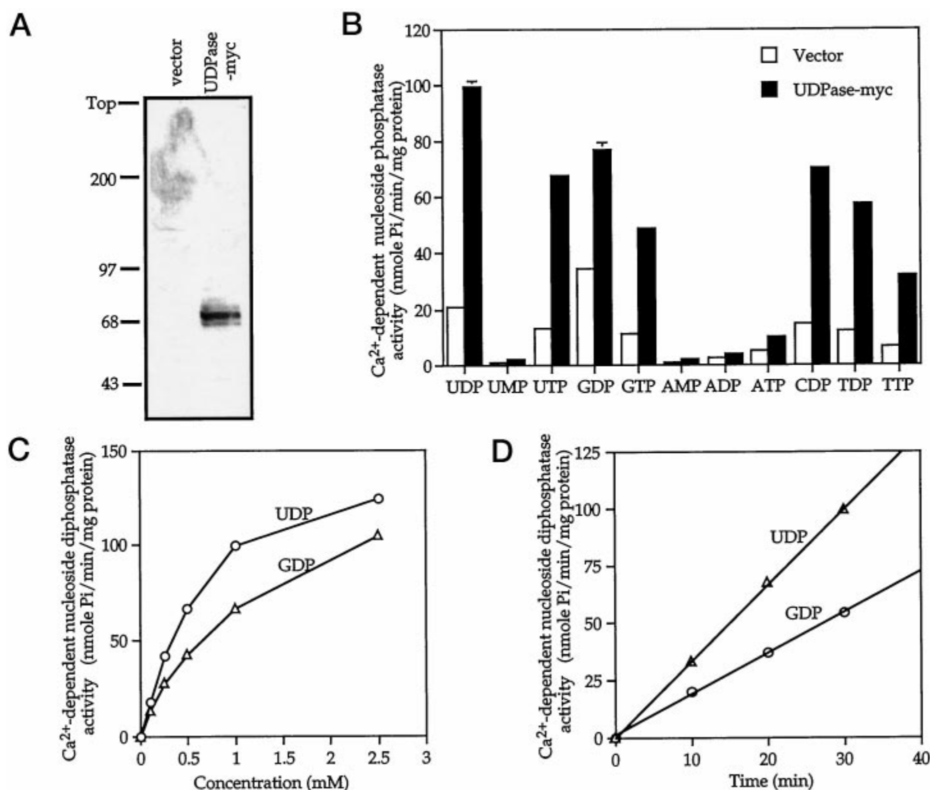

FIG. 3. **Expression of human UDPase in COS-7 cells.** *A*, immunoblot analysis of total cell lysates of COS-7 cells transfected with Myc-UDPase cDNA or GW1-CMV control vector. Cell lysates were analyzed by Western blotting using anti-Myc monoclonal antibody 9E10. The positions of molecular size markers are shown in kilodaltons. *B*, Ca^{2+}-stimulated nucleoside phosphatase activities in the crude membranes of COS-7 cells transfected with Myc-UDPase cDNA (*black bars*) or GW1-CMV control vector (*white bars*). Substrate specificity was determined with different nucleoside phosphates in all cases at a substrate concentration of 1 mM. *C*, nucleoside phosphatase assay with different concentrations of UDP and GDP (0.1–2.5 mM). Assays were carried out for 30 min as described under "Experimental Procedures." *D*, time course of nucleoside phosphatase assay with 1 mM UDP or GDP. All values are means ± S.D. of results from three independent experiments.

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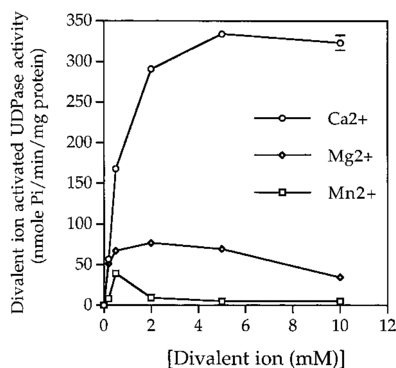

FIG. 4. **Effects of divalent cation on UDPase activity.** Crude membranes were incubated for 5 min at 37 °C with 1 mM NaN_3, 0.5 mM Na_3VO_4, and 0.2 mg/ml alamethicin in buffer A. UDPase activities stimulated by various concentrations of divalent ion (*e.g.* $MgCl_2$, $CaCl_2$, or $MnCl_2$) were determined by subtracting values obtained with 0.2 mM EDTA alone from those obtained with various concentrations of divalent cation plus chelator. Values are means ± S.D. of results from three independent experiments.

and by subtracting values obtained with 1 mM EDTA alone from those obtained with 5 mM $CaCl_2$.

Immunofluorescence Staining—COS-7 cells grown on polylysine-coated culture chamber slides (Nunc Inc., Naperville, IL) were transfected at ~40–60% confluence with Myc-UDPase cDNA using the LipofectAMINE method. After exposure of cells to BFA (20 μg/ml) for 30 min at 37 °C, cells were washed twice with phosphate-buffered saline, pH 7.4, containing 1 mM $MgCl_2$ and 0.1 mM $CaCl_2$. The immunofluorescence staining was done by modifying the method described by Yoon and Guidotti (21). Cells were fixed with 2% paraformaldehyde, permeabilized with 0.1% Triton X-100, and stained with primary antibody. For staining Myc-UDPase, monoclonal antibody 9E10 (1 μg/ml) was used to recognize the Myc epitope. Monoclonal antibodies against the 58-kDa Golgi peripheral membrane protein (1:50 dilution) were used as markers of Golgi complexes (15, 22). Fluorescein isothiocyanate-labeled secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) was used at a dilution of 1:1000. Immunofluorescence was viewed with a Zeiss Axioskop microscope.

RESULTS

DNA Cloning—We cloned the full-length coding sequence of a novel cDNA (Fig. 1) from a human adult brain cDNA library by the 5′-RACE method (16). The protein product of this cDNA is a new E-ATPase protein (14) since it contains apyrase conserved regions (ACR1–4) (Fig. 1) that are present in all known E-ATPase proteins (11). The initiation codon (ATG) at nucleotide sequence 199–201 is the most likely translational initiation site because there are two stop codons in the upstream sequence of the same reading frame (Fig. 1). The deduced polypeptide sequence from this initiation codon contains 610 amino acids. Hydrophobicity analysis using the Kyte and Doolittle algorithm (23) predicts two hydrophobic stretches in the polypeptide (amino acid residues 33–55 and 551–573), both sufficiently long to transverse the membrane (Fig. 1). There are two potential *N*-glycosylation sites between the two putative transmembrane domains (indicated in *boldface* and *underlined* in Fig. 1), suggesting that this protein may be a membrane glycoprotein.

Northern Blot Analysis—To determine whether this new E-ATPase is another ectoapyrase or ecto-ATPase, we examined its mRNA expression pattern in cells with or without ectoapyrase activity. The mRNA is expressed in cells with ectoapyrase activity (human B lymphoblast LG2) and without ectoapyrase activity (human T lymphoma Jurkat cells) (13) as determined by Northern blot analysis (Fig. 2*A*). We were able to detect at least three mRNA transcripts (3.0, 3.2, and 7.5 kilobases in length) reacting with the same probe (Fig. 2*A*). Reverse transcription-PCR analysis of human breast cancer

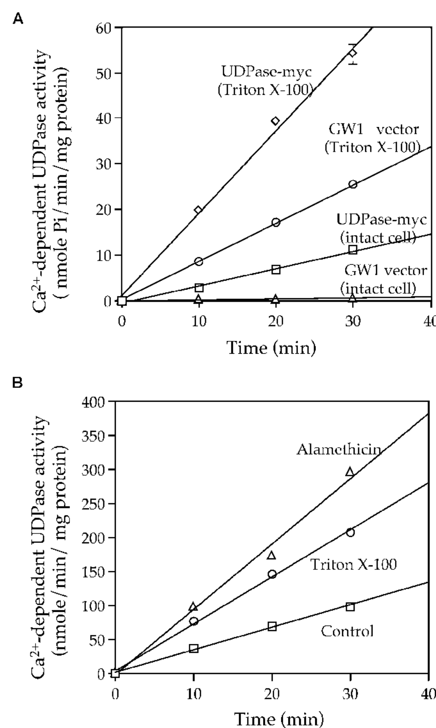

FIG. 5. **Localization of the UDPase activity.** *A*, the activity of UDPase is located inside the plasma membrane. COS-7 cells transfected with Myc-UDPase cDNA or control vector were examined for Ca^{2+}-dependent UDPase activity either with intact cells or after lysis with 0.1% Triton X-100 (see "Experimental Procedures"). *B*, membrane orientation of the UDPase activity. The UDPase activity of crude membranes of Myc-UDPase cDNA-transfected COS-7 cells was measured in the absence or presence of Triton X-100 (0.1% (v/v)) or alamethicin (0.2 mg/ml) (see "Experimental Procedures"). Values are means ± S.D. of results from three independent experiments.

MCF-7 cell lines (with ectoapyrase activity) and their multidrug-resistant derivatives, MCF-7ADR cells (ectoapyrase-deficient), indicated that both cells express this mRNA (data not shown). The presence of mRNA in these four cell lines suggests that this new E-ATPase is probably not a new ectoapyrase or ecto-ATPase.

To determine tissue-specific mRNA distribution, we analyzed RNA purified from human tissues by hybridization with a fragment of the full-length cDNA (1–667 base pairs) (Fig. 1). mRNA was expressed in all the tissues examined, including heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas (Fig. 2*A*, *lanes 3–10*).

Genomic Southern Analysis—To determine the copy number of this new E-ATPase gene, human genomic DNA was digested with *Eco*RI (Fig. 2*B*, *lane 1*) or *Nde*I (*lane 2*) and then analyzed by Southern blotting with a cDNA probe (nucleotide sequence 1024–1428) under high stringency hybridization and wash conditions. The sequence of this cDNA probe has neither *Eco*RI nor *Nde*I sites and does not contain any intron within the fragment at the level of the genomic DNA (see "Experimental Procedures"). The presence of at least two bands in both digests (Fig. 2*B*) indicates that there might be two or more copies of this new E-ATPase gene in the human genome.

Functional Analysis—To study the function of this new E-ATPase, a control expression vector or the cDNA encoding a Myc-tagged protein (Myc-UDPase) was transfected into COS-7 cells. Transfected COS-7 cells expressed an ~69-kDa protein recognized with anti-Myc monoclonal antibody 9E10 (Fig. 3*A*). No protein was detected in the immunoblot of COS-7 cells transfected with control vector.

Expression of Myc-tagged protein significantly increased the

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nucleoside phosphatase activity in COS-7 crude membranes. As shown in Fig. 3*B*, the UDPase activity (99.23 ± 2.46 nmol of P_i/min/mg) is 4.76-fold higher than the UDPase activity (20.85 ± 0.57 nmol of P_i/min/mg) of crude membranes from COS-7 cells transfected with control vector alone. The nucleoside phosphatase activity was measured in the presence of 1 mM azide (inhibitor of F-type ATPase) and 0.5 mM vanadate (inhibitor of P-type ATPase). It is known that activities of E-ATPases are not inhibited by these inhibitors (10).

Comparison of the substrate specificity of nucleoside phosphatase activities in COS-7 crude membranes is illustrated in Fig. 3*B*. It is important to note that these activities were determined under conditions where the activities were linear with respect to time and substrate concentration (Fig. 3, *C* and *D*). The activity was highest with UDP as the substrate; lower activity was obtained with GDP, CDP, and TDP. AMP, ADP, ATP, and UMP were not substrates. UTP, GTP, TTP, and CTP (data not shown) were hydrolyzed, but with rates lower than those of UDP, GDP, TDP, and CDP (Fig. 3*B*). The cleavage rates of these nucleoside triphosphates may not represent the real activity since hydrolysis of nucleoside triphosphates by other phosphatases would provide products (*i.e.* nucleoside diphosphates) that would be cleaved by the UDPase. Finally, UDP was a better substrate than GDP or other nucleoside diphosphates (data not shown) in the concentration range from 0.1 to 2.5 mM (Fig. 3*C*). We concluded that human E-ATPase is a UDPase.

The activity of this human UDPase was stimulated by Ca^{2+}, whereas Mg^{2+} and Mn^{2+} had a lower or minimal effect (Fig. 4). The maximum activation was obtained at 5 mM Ca^{2+}. We found that the enzymatic properties of human UDPase are similar to those of the previously reported rat liver cell Golgi luminal UDPase (1). First, the substrate specificities of human UDPase and rat liver Golgi UDPase are almost identical. Second, the activity of Golgi luminal UDPase in rat liver cells is also known to be activated by Ca^{2+} and not by Mg^{2+} or Mn^{2+} (1).

Cellular Localization—To study the cellular localization of this human UDPase, we compared the enzymatic activity of intact and disrupted cells to determine whether it is located intracellularly or extracellularly. The rational of the experiment is that if the enzyme is located inside the plasma membranes, the UDPase activity will increase after the cells are disrupted. On the other hand, the UDPase activity will not change if the enzyme is an ectoprotein. In fact, the UDPase activity of Myc-UDPase cDNA-transfected COS-7 cells increased significantly after the cells were treated with 0.1% Triton X-100 (~5-fold) (Fig. 5*A*) or were homogenized with a Dounce homogenizer (~4-fold) (data not shown). At the end of the incubation, cells not treated with detergent were still intact (>90%), as demonstrated by trypan blue exclusion. These results suggest that human UDPase is located inside the cell. The UDPase activity of COS-7 cells transfected with GW1 control vector increased after these cells were solubilized with 0.1% Triton X-100. Since UDPase is likely present in all mammalian cells, it is not surprising to find that COS-7 cells have endogenous UDPase activities. Interestingly, the UDPase activity of Triton-treated mock-transfected cells was even higher that of intact cells transfected with Myc-UDPase cDNA (Fig. 5*A*). These results further support the conclusion that UDPase is an intracellular enzyme.

The subcellular distribution of human UDPase was analyzed by immunofluorescence staining. COS-7 cells transfected with Myc-UDPase cDNA were stained with anti-Myc antibody in a

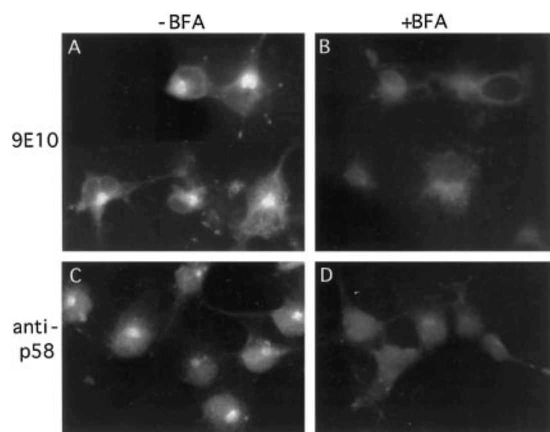

FIG. 6. **Localization of UDPase protein.** COS-7 cells were transfected with Myc-UDPase cDNA. 48 h post-transfection, cells were treated with or without BFA (20 μg/ml) for 30 min at 37 °C. BFA was used here to induce enzymes of the Golgi stacks to redistribute into the endoplasmic reticulum and to cause the Golgi cisternae to disappear (22) (*B* and *D*). Fixed cells were stained either with anti-Myc monoclonal antibody 9E10 (1 μg/ml) (*A* and *B*) or with the monoclonal antibody against the 58-kDa Golgi peripheral membrane protein (1:50 dilution) (*C* and *D*), followed by fluorescein isothiocyanate-conjugated anti-mouse IgG, and then viewed by fluorescence microscopy (see "Experimental Procedures").

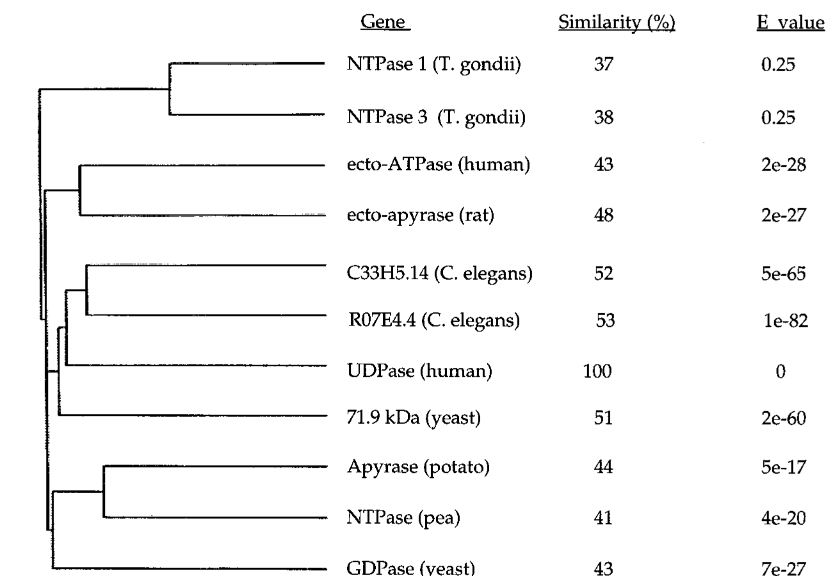

FIG. 7. **Relatedness within the E-ATPase family.** The *horizontal branch lengths* are proportional to the mean number of differences per residues along each branch. The relationships were derived from Neighbor-Joining weighted progressive alignments using the CLUSTAL W program (36). The GenBank™ accession numbers for E-ATPases are as follows: rat ectoapyrase, U81295; human ecto-ATPase, U91510; potato apyrase, U58597; pea NTPase, Z32743; yeast GDPase, U18799; hypothetical protein C33H5.14 of *C. elegans*, U41007; hypothetical protein R07E4.4 of *C. elegans*, U39652; hypothetical 71.9-kDa yeast protein, U18778; and NTPases 1 and 3 of *T. gondii*, U14322 and U14324, respectively. Sequence similarity and *E* values were obtained by comparing UDPase with other E-ATPases using the Gapped BLAST data base search program (37).

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                                                                                                                     ---
UDPase        MGRIGISCLF PASWHFSISP VGCPRILNTN LRQIMVISVL AAAAVSLLYF SVVIIRNKYG RLTRDKKFQR YLARVTDIEA TDTNN.P... NVNYGIVVDC 100
R07E4.4                                                   MRVSLRFT ILAVSAMIFF PVIVFIYVVE AHTSPKVI.. ADDQE..... R.SYGVICDA
C33H5.14                                                                      MSGGSSER VQRSVRSV.. VETKN..... NIKYGVICDA
71.9kDA                                                                                           MLIEN.T... NDRFGIVIDA
GDPase        MAPIFRNYRF AIGAFAVIML ILLIKTSSIG PPSIARTVTP NASIPKTPED ISILPVNDEP GYLQDSKTEQ NYPELADAVK SQTSQ.TCSE EHKYVIMIDA
CD39                                                   MEDIK DSKVKRFCSK NILIILGFSS VLAVIALIAV GLTHNKPLPE NVKYGIVLDA
CD39L1                                                     M AGKVRSLLPP LLLAAAG... .LAGLLLLCV P.TRDVREPP ALKYGIVLDA

              --ACR1-----    -----------------X1------------------                                     -----ACR2-----
UDPase        GSSGSRVFVY CW.....PRH NGNPHDLLDI RQMRD.KNRK PVVMKIKPGI SEFATSPEKV .SDYISPLLN FAAEHVPRAK HKETPLYILC TAGMRILPE. 200
R07E4.4       GSTGTRLFVY NW.....IST SDS..ELIQI EPVIY.DN.K PVMKKISPGL STFGTKPAQA .AEYLRPLME LAERHIPEEK RPYTPVFIFA TAGMRLIPDE
C33H5.14      GSSGTRLFVY T......LKP LSG..GLTNI DTLIH.ES.E PVVKKVTPGL SSFGDKPEQV .VEYLTPLLR FAEEHIPYEQ LGETDLLIFA TAGMRLLPE.
71.9kDa       GSSGSRIHVF KWQDTESLLH ATNQDSQSIL QSVPHIHQEK DWTFKLNPGL SSFEKKPQDA YKSHIKPLLD FAKNIIPESH WSSCPVFIQA TAGMRLLPQD
GDPase        GSTGSRVHIY KFDVC..... .TSPPTLLD. ........EK ..FDMLEPGL SSFDTDSVGA .ANSLDPLLK VAMNYVPIKA RSCTPVAVKA TAGLRLLGD.
CD39          GSSHTNLYIY KWPAEKE... ..NDTGVVQL LEECQVKG.. .......PGI SKYAQKTDEI .AAYLAECMK MSTERIPASK QHQTPVYLGA TAGMRLL..R
CD39L1        GSSHTSMFIY KWPADKE... ..NDTGIVGQ HSSCDVPG.. .......GGI SSYADNPSGA .SQSLVGCLE QALQDVPKER HAGTPLYLGA TAGMRLL..N

                                                  -----ACR3-----                                       -----ACR4-----
UDPase        ....SQQKAI LEDLLTDIPV HFDFLFSD.. SHAEVISGKQ EGVYAWIGIN FVLGRFEHIE DDDEAVVEVN IPGSESSEAI VRKRTAGILD MGGVSTQIAY 300
R07E4.4       YVLIGQKEAV LKNLRNKLPK ITSMQVLK.. EHIRIIEGKW EGIYSWIAVN YALGKF.... ...NKTATLD FPGTSPAHA. .RQKTVGMID MGGASAQIAF
C33H5.14      ....AQKDAI IKNLQNGLKS VTALRVSD.. SNIRIIDGAW EGIYSWIAVN YILGRF.... ...DK..... ........E. .NDSKVGMID MGGASVQIAF
71.9kDa       I.....QSSI LDGLCQGLKH PAEFLVEDCS AQIQVIDGET EGLYGWLGLN YLYGHF.... NDYNPEVSDH F......... ....TFGFMD MGGASTQIAF
GDPase        ....AKSSKI LSAVRDHLEK DYPFPVVEGD G.VSIMGGDE EGVFAWITTN YLLGNIGANG .......... .......... PKLPTAAVFD LGGGSTQIVF
CD39          MESKQSADEV LAAVSRSLKS .YPF...DFQ G.AKIITGQE EGAYGWITIN YLLGRF.... .......TQE QSWLNFISDS QKQATFGALD LGGSSTQVTF
CD39L1        LTNPEASTSV LMAVTHTLTQ .YPF...DFR G.ARILSGQE EGVFGWVTAN YLLENF.... .......I.K YGWVGRWFRP RK.GTLGAMD LGGASTQITF

UDPase        EVP.KTEEVA ..KNLLAEFN LGCDVHQTEH VYRVYVATFL GFGGNAARQR YEDRIFANTI QK.NRLLGKQ TGLTPDMPYL DPCLP...LD IKDEIQ.QNG 400
R07E4.4       ELP.DTDSFS ..SINVENIN LGCREDDSLF KYKLFVTTFL GYGVNEGIRK YEHMLLSKLK DQ.NGTVIQ. .......... DDCMP...LN LHKTVTLENG
C33H5.14      EIANEKESYN ..GGNVYEIN LGSIETNEDY KYKIYSTTFL GYGANEGLKK YENSL...VK SG.NS...N. .......... DSCSP...RG LNRLI....G
71.9kDa       .APHDSGEIA RHRDDIATIF LRS.VNGDLQ KWDVFVSTWL GFGANQARRR YLAQLINTLP EN.TNDYEND DFSTRNLN.. DPCMP...RG SSTDFEFKD.
GDPase        EPTFPINEKM VDGEHKFDLK FG......DE NYTLYQFSHL GYGLKEGRNK VNSVLVENAL KD.GKILKGD NTKTHQLS.. SPCLPPKVNA TNEKVTLESK
CD39          ...VPLNQTL EAPETSLQFR LYGTD..... .YTVYTHSFL CYGKDQALWQ KLAQDIQVSS GGILKDPCFY PGYKKVVNVS ELYGTPCTKR FEKKLPFN..
CD39L1        ....ETTSPA EDRASEVQLH LYGQH..... .YRVYTHSFL CYGRDQVLQR LLASALQ..T HGF..HPCWP RGFSTQVLLG DVYQSPCTMA .QRPQNFNSS

UDPase        QTIYLRGTGD FDLCRETIQP FMNKTNETQT S........L NGVYQPPI.. HF.QNSEFGY FSEFYYCTED VLRMGGDYNA AKFTKAAKDY C..ATKWSIL 500
R07E4.4       ENFVRRGTGN WNTCSNEVKK LLNPESSSEV CKAEAAKCYF GAVPAPSI.. PL.SNIEMYG FSEYWYSTHD VLGLGGQYDA ENIAKKTQQY C..SKRWSTI
C33H5.14      E.FTVNGTGE WDVCLAQVSS LIG.DKAQPS CPNPT..CFL RNVIAPSV.. NL.STVQLYG FSEYWYTTSN .FGSGGEYHY QKFTDEVRKY C..QKDWNDI
71.9kDa       TIFHIAGSGN YEQCTKSIYP LLLKN..... MPCDDEPCLF NGVHAPRI.. DF.ANDKFIG TSEYWYTAND VFKLGGEYNF DKFSKSLREF C..NSNWTQI
GDPase        ETYTIDFIGP DEPSGAQCR. FLTDEILNKD AQCQSPPCSF NGVHQPSLVR TFKESNDIYI FS.YFYDRTR PLGMPLSFTL NELNDLARIV CKGEETWNSV
CD39          .QFQVQGTGD YEQCHQSILK FFNNSH.... ..CPYSQCAF NGVFLPPLQG SFGAFSAFYF VMDFF...KK MANDSVS.SQ EKMTEITKNF C..SKPWEEV
CD39L1        ARVSLSGSSD PHLCRDLVSG LFSFSS.... ..CPFSRCSF NGVFQPPVAG NFVAFSAFFY TVDFL...RT SMGLPVA.TL QQLEAAAVNV C..NQTWAQ.

                                                     ---------X2--------
UDPase        RERFDRGLYA SHADLHRLKY QCFKSAWMFE VFHRGFSFPV .........N YKSLKTALQV YDKEVQWTLG AILYRTRFLP LRDIQQEAFR ASHTHWRGVS 600
R07E4.4       QAESKKQLY. PRADEERLRT QCFKSAWITS VLHDGFSVDK .........T HNKFQSVSTI AGQEVQWALG AMIYHMRFFP LRDSSRNLIV K.ETHSSSES
C33H5.14      QDGFKRNEF. PNADIERLGT NCFKAAWVTS VLHDGFNVDK .........T KHLFQSVLKI AGEEMQWALG AMLYHS.... .KDLKFNLLE QLEVAQSTQQ
71.9kDa       LANSDKGVYN SIPE.NFLKD ACFKGNWVLN ILHEGFDMPR IDVDAENVND RPLFQSVEKV EERELSWTLG RILLYASGSI LAG.NDDFMV GIAPSERRTK
GDPase        FSGIAGSLDE LESDSHF... .CLDLSFQVS LLHTGYDIPL .......... QRELRTGKKI ANKEIGWCLG ASLPLLKADN WKCKIQSA
CD39          KASY...... PTVKEKYLSE YCFSGTYILS LLLQGYNFTG TSWDQIHFMG ........KI KDSNAGWTLG YMLNLTNMIP AEQP.LSPPL PHSTYISLMV
CD39L1        .......... .......... .........Q LLSRGYGFDE RAFGGVIFQK ........KA ADTAVGWALG YMLNLTNLIP ADPPGLRKGT DFSSWVVLLL

UDPase        FVYNHYLFSG CFLVVLLAIL LYLLRLRRIH RRTPRSSSAA ALWMEEGLPA QNAPGTL
R07E4.4       LWAPLFFLSA VFCLFVLVCA KEQSVLCFDD KRRSSFGMSR SQYSYKMLKE NRTSSSFLEN FA
C33H5.14      I..SNFFSFF VILIIVLAVA LYRQLQSEST YKKYNFLRTD SKPDFLNV
71.9kDa       LTGKKFIPGK ....LLESDQ LRKQSSSLSN KGFLMWFAII CCIFYLIFHR SHIIRRRFSG LYNITKDFKT GIRRRLKFLR RSDPFSRLEE GELGTDVDGF 700
CD39          LF.SLVLVAM VITGLFIFS. K.PSYFWKEA V
CD39L1        LFASALLAAL VLLLRQVHSA KLPSTI
```

FIG. 8. **Amino acid sequence alignments of UDPase and other E-ATPases.** The E-ATPases are human Golgi UDPase, hypothetical protein C33H5.14 of *C. elegans*, hypothetical protein R07E4.4 of *C. elegans*, hypothetical 71.9-kDa yeast protein, yeast GDPase, rat ectoapyrase (CD39), and human ecto-ATPase (CD39L1). All these E-ATPases share the apyrase conserved regions (ACR1–4). The two amino acids in the first apyrase conserved region (ACR1) and the other two regions (X1 and X2) that are different between UDPase/GDPase and ecto-ATPase/ectoapyrase are *underlined*.

patch close to the nucleus corresponding to the Golgi complex (Fig. 6*A*). No stain was found in cells transfected with control vector (data not shown). Monoclonal antibodies against the 58-kDa Golgi peripheral membrane protein were used here as a Golgi marker (Fig. 6*C*) (15). After cells were exposed to BFA, the fluorescence signal of both Myc-UDPase (Fig. 6*B*) and the 58-kDa Golgi peripheral membrane protein (Fig. 6*D*) dramatically decreased. BFA has been shown in studies of numerous cells types to induce enzymes of the Golgi stacks to redistribute into the endoplasmic reticulum and to cause the Golgi cisternae to disappear (22). These results suggest that the UDPase protein is located in the Golgi apparatus.

This conclusion is supported by the experiment shown in Fig. 5*B*, in which the UDPase activity of COS-7 crude membranes increased ~2-fold in the presence of Triton X-100 and 3-fold in the presence of alamethicin, an ionophore that facilitates transmembrane diffusion of nucleotides. We conclude that the active site of the UDPase is on the luminal side of Golgi complexes. Similar criteria have been used to localize other Golgi luminal enzymes, including galactosyltransferase and sialyltransferase (24) as well as a Golgi luminal protein kinase (25). We conclude that the human UDPase is a Golgi luminal enzyme.

Relatedness within the E-ATPase Protein Family—Sequence alignment of the E-ATPase family members suggests the existence of four major subgroups in the E-ATPase protein superfamily (Fig. 7). Human Golgi UDPase is highly related to two nematode hypothetical proteins (C33H5.14 and R07E4.4) and to a functionally unidentified 71.9-kDa yeast protein, suggesting that the enzymes of *Caenorhabditis elegans* and yeast might be functionally similar to human Golgi UDPase. Yeast GDPase (8), pea NTPase (26), and potato apyrase (11) are less related to human UDPase, as are vertebrate ectoapyrases (27) and ecto-ATPases (28). Finally, the NTPases of the protozoan parasite *Toxoplasma gondii* (29) are more distant from all

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other E-ATPases in the dendrogram, suggesting an ancient and independent line of evolution of the *T. gondii* NTPases.

DISCUSSION

We have cloned a new human E-ATPase gene, as the protein sequence contains the four apyrase conserved regions (ACR1–4) (11). Expression of the protein in COS-7 cells resulted in an increase in Ca^{2+}-dependent nucleoside phosphatase activity. This activity was highest with UDP as substrate and was simulated by divalent cations in the following order: $Ca^{2+} \gg Mg^{2+} > Mn^{2+}$. By immunofluorescence staining and enhancement of UDP hydrolysis in the presence of alamethicin, the protein was determined to be a Golgi luminal UDPase. Since rat liver UDPase (1) and yeast GDPase (8) are involved in protein and lipid glycosylation in the Golgi apparatus, it is likely that the human UDPase might have the same function.

UDPase mRNA is present in all human tissues and all four cell lines examined here, suggesting that this UDPase is widely expressed in many human cells. Recently, the gene encoding the *S. cerevisiae* GDP-mannose transporter (Vrg4p) was identified; the lethal phenotype of a null *vrg4* mutant suggests that glycosylation of protein and lipid in the Golgi apparatus is essential for viability (30). Therefore, Golgi UDPase might be essential for most, if not all, cells. It will be of interest to know whether there are genetic diseases affecting nucleoside phosphate transport or metabolism in the lumen of the Golgi apparatus, in a manner analogous to diseases involving cysteine and sialic acid transport into lysosomes.

Genomic Southern analysis under high stringency hybridization conditions indicates that there might be at least two copies of the UDPase gene in the human genome. In fact, the presence of another UDPase activity in the rough endoplasmic reticulum (RER) has been described in rat liver cells. Like the Golgi UDPase, the RER UDPase also preferentially hydrolyzes UDP and GDP. However, these two enzymes are slightly different in at least two respects. First, the RER UDPase (which actually should be named RER GDPase) hydrolyzes nucleotides in the order GDP > UDP. Second, the activity of Golgi UDPase is preferentially activated by Ca^{2+}, whereas the activity of RER UDPase is stimulated by both Ca^{2+} and Mg^{2+} (1). Further study is required to know whether the second gene is indeed a RER UDPase. Interestingly, *C. elegans* also has two copies of a UDPase-like gene (Figs. 7 and 8). In the genome of *S. cerevisiae*, there are only two copies of an E-ATPase gene. The hypothetical 71.9-kDa protein of chromosome V (Figs. 7 and 8) also shares high similarity in amino acid sequence with human UDPase. It is likely that the two *C. elegans* proteins and this yeast protein may have functions similar to that of human UDPase. The second E-ATPase gene in *S. cerevisiae* encodes the Golgi GDPase. Although yeast GDPase is not essential for cell viability and growth, null mutants of GDPase have a partial block in *O*- and *N*-glycosylation of secreted proteins as well as a dramatic decrease in mannosylinositol phosphorylceramide (8) and a decrease in transport of GDPase-mannose into the Golgi lumen (9). Since glycosylation of proteins and lipids in the Golgi apparatus is essential for viability (30), we wonder whether the activity of the hypothetical 71.9-kDa protein may account for the viability of *gda1* null mutants. It will be interesting to know whether the yeast GDPase and the 71.9-kDa protein have different biological functions.

Members of the E-ATPase protein family all share high sequence similarity in four apyrase conserved regions (ACR1–4) (11). ACR1 and ACR4 sequences are similar to actin-hsp70-hexokinase β- and γ-phosphate-binding motifs, indicating a possible role in nucleotide binding; however, the structures and functions of these ACRs are not yet known. To understand the catalytic mechanism of E-ATPase, it is important to point out the differences between various E-ATPase enzymes. Ectoapyrase (CD39) is an ectoenzyme that hydrolyzes nucleoside tri- or diphosphates (13, 31, 32); ecto-ATPase (CD39L1) is also an ectoenzyme, but it preferentially hydrolyzes adenosine triphosphate (28, 33, 34). Ca^{2+} and Mg^{2+} have almost the same stimulatory effect on ectoapyrase (13). T-tubule ecto-ATPase is more active with Mg^{2+} than Ca^{2+} (35). The activity of human Golgi luminal UDPase is highest with UDP and GDP as substrates and is stimulated by divalent cations in the following order: $Ca^{2+} \gg Mg^{2+} > Mn^{2+}$ (Fig. 4). Interestingly, amino acid sequence alignments between UDPase and other E-ATPases (Fig. 8) revealed that some regions of these E-ATPases may be important in determining substrate specificity. First, the sequence of the ACR1 region of UDPase/GDPase, D*X*GS(S/T)G(S/T)R*XXX*, is different from that of ecto-ATPase/ectoapyrase, D*X*GSSHT(N/S)*XXX*, in two amino acid residues (underlined). Since ACR1 is similar to the β-phosphate-binding motif of actin-hsp70-hexokinase, these two amino acid residues may be responsible for distinguishing nucleoside diphosphates from nucleoside triphosphatases. Second, at least two other regions, X1 and X2, have significant differences between UDPase/GDPase and ecto-ATPase/ectoapyrase; however, the functions of these regions are still unknown.

In summary, we have cloned a new human E-ATPase gene and shown that it encodes a Golgi luminal UDPase. This is the first identification of a mammalian Golgi UDPase gene. The wide distribution of UDPase mRNA in human tissues and cells makes it evident that metabolism of nucleoside phosphates in the Golgi lumen is essential for the functions of the Golgi apparatus, *e.g.* protein and lipid glycosylation. Finally, we have shown that human UDPase is highly related to one yeast and two worm hypothetical proteins.

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