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Glycoprotein reglucosylation and nucleotide sugar utilization in the secretory pathway: identification of a nucleoside diphosphatase in the endoplasmic reticulum

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UDP is generated in the lumen of the endoplasmic reticulum (ER) as a product of the UDP-glucose-dependent glycoprotein reglucosylation in the calnexin/calreticulin cycle. We describe here the identification, purification and characterization of an ER enzyme that hydrolyzes UDP to UMP. This nucleoside diphosphatase is a ubiquitously expressed, soluble 45 kDa glycoprotein devoid of transmembrane domains and KDEL-related ER localization sequences. It requires divalent cations for activity and hydrolyzes UDP, GDP and IDP but not any other nucleoside di-, mono- or triphosphates, nor thiamine pyrophosphate. By eliminating UDP, which is an inhibitory product of the UDP-Glc:glycoprotein glucosyltransferase, it is likely to promote reglucosylation reactions involved in glycoprotein folding and quality control in the ER.

Keywords: endoplasmic reticulum/nucleoside diphosphatase/reglucosylation/UDP/UMP

Introduction

Several mechanisms operate in the cell to assist the folding and assembly of newly synthesized proteins. The endoplasmic reticulum (ER) is unique in that it contains a special molecular chaperone system devoted to the folding of glycoproteins with N-linked oligosaccharides (for a recent review see Helenius *et al*., 1997). The system is centered around two lectins, calnexin and calreticulin, which recognize monoglucosylated oligosaccharides present on newly synthesized and incompletely folded glycoproteins. By binding to these oligosaccharide trimming intermediates, calnexin and calreticulin promote proper folding and assembly of glycoproteins. They are also part of a quality control system that restricts exit of newly synthesized proteins to those that have reached their native conformations.

The monoglucosylated structures recognized by calnexin and calreticulin arise by two mechanisms. They are generated by the sequential removal of two of the three glucose residues present on core oligosaccharides. They are also formed by reglucosylation of completely deglucosylated oligosaccharides through the action of a UDP-Glc:glycoprotein glucosyltransferase (GT) (Parodi *et al*., 1983). GT is a lumenal ER enzyme that uses UDP-Glc transported from the cytosol as the glucose donor (Pérez and Hirschberg, 1986; Vanstapel and Blanckaert, 1988; Trombetta *et al*., 1991)

Studies *in vitro* and *in vivo* have shown that only glycoproteins that have failed to reach their native conformation are substrates for GT (Sousa *et al*., 1992; Trombetta and Parodi, 1992; Fernandez *et al*., 1994; Hammond *et al*., 1994; Van Leeuwen and Kearse, 1997; Wada *et al*., 1997; Cannon and Helenuis, 1999). Due to its capacity to distinguish between glycoprotein conformers, it thus serves as a folding sensor in the calnexin/calreticulin pathway (see Helenius *et al*., 1997). Reglucosylation by GT and removal of the added glucose by glucosidase II regulate a cycle of lectin binding and release that is repeated until substrate glycoproteins are correctly folded. Since glycoproteins typically contain more than one oligosaccharide chain, and given the cyclic nature of the de- and reglucosylation process, several molecules of UDP-Glc can be consumed during the folding of a glycoprotein.

For the de- and reglucosylation cycle to work, UDP-Glc has to be transported from the cytosol where it is synthesized into the ER lumen, where it is used by GT (Perez *et al*., 1986; Vanstapel *et al*., 1988). A similar topological arrangement exists in the Golgi apparatus, where nucleotide sugars made in the cytosol are utilized by lumenally oriented glycosyltransferases. In this case, antiporters present in the Golgi membranes import nucleotide sugars in exchange for the corresponding nucleoside monophosphates (Hirschberg and Snider, 1987). Biochemical and morphological studies described a nucleoside diphosphatase in the *trans*-Golgi compartment that hydrolyzes the nucleoside diphosphates generated as products of glycosylation reactions into nucleoside monophosphates. It prevents the accumulation of diphosphonucleosides that would otherwise cause product inhibition of the glycosyltransferases, and at the same time generates the nucleoside monophosphates needed for import of new sugar nucleotides (Allen and Slater, 1961; Novikoff and Goldfischer, 1961; Allen, 1963a,b; Friend, 1969; Cheetham *et al*., 1971; Kuhn and White, 1977; Abeijon *et al*., 1993).

We report here the identification and further characterization of a ubiquitous nucleoside diphosphatase from the ER of mammalian cells. It hydrolyzes UDP and GDP to UMP and GMP. We propose that by preventing accumulation of UDP in the ER lumen it may promote reglucosylation reactions and thus support the calnexin/calreticulin chaperone cycle.

Results

ER and Golgi complexes contain distinct UDPase activities

To determine whether the ER possesses an enzyme capable of cleaving UDP to UMP different from the one present in the Golgi complex, we prepared ER- and Golgi-enriched

Table I. Solubility of ER and Golgi UDPases

Substrate		Phosphatase activity					
		No detergent		1% Triton		0.2% OG	
		ER	Golgi	ER	Golgi	ER	Golgi
Man-6-P	Tot.	42	5	38	6	44	5
	SN	1	0	3	1	1	0
TPP	Tot.	18	96	25	130	28	119
	SN	5	16	25	102	22	46
UDP	Tot.	810	1507	693	1390	705	1427
	SN	31	88	418	1199	409	263
GDP	Tot.	749	1531	642	1154	687	1307
	SN	52	84	405	949	413	223

ER and Golgi complex derived organelle fractions were pre-treated with or without detergents as indicated. An aliquot was preserved (Tot.) and the remainder was ultracentrifugated to separate the solubilized material (SN). Phosphatase activity was meassured on total (Tot.) and solubilized (SN) fractions using the following substrates: mannose-6-phosphate (Man-6-P), thiamine pyrophosphate (TPP), uridine diphosphate (UDP) and guanosine diphosphate (GDP). Incubations were done in a total volume of 50 µl containing 2 mM $CaCl_2$, 50 mM NaCl, 20 mM HEPES buffer pH 7.5, 0.1% Triton X-100, 2 mM substrate and 5 µl of membrane fraction to be assayed. Activity is expressed as nanomoles of inorganic phosphate released in 10 min. OG, octylglucoside.

Table II. Triton X-114 partition of ER and Golgi UDPase activities

	Phosphatase activity			
	ER		Golgi	
Fraction	UDP	TPP	UDP	TPP
Total	422	47	794	103
SN	284	3	141	39
Pellet	31	36	235	54

Triton X-114 partition of ER and Golgi derived organelle fractions was performed as described in the Materials and methods. For measurement of phosphatase activities, incubations were done in a total volume of 50 µl containing 2 mM $CaCl_2$, 50 mM NaCl, 20 mM HEPES buffer pH 7.5, 0.1% Triton X-100, 2 mM substrate and 5 µl of membrane fraction to be assayed. Activity is expressed as nanomoles of inorganic phosphate released in 5 min.

Table III. Purification of ER-UDPase from bovine liver

Fraction	Volume (ml)	Activity (U)	Protein (mg)	Specific activity (U/mg)	Yield (%)
ER memb.	1600	8000	6400	1.25	100
Soluble	1500	10480	2760	3.8	110
DEAE	60	5600	240	23	70
Con A	45	3800	9	420	47
Mono Q	1	1700	0.7	2428	21
S-75	1	560	0.1	5600	7
Alkyl S	2	82	0.015	5460	1

organelle fractions from rat liver. As expected, efficient hydrolysis of UDP and thiamine pyrophosphate (TPP) was detected in the Golgi fractions (see total values 'Tot.' in Table I). The ER fractions also contained potent nucleoside diphosphatase activity capable of degrading UDP and GDP, although TPP was degraded much more efficiently by Golgi fractions. This suggested that the ER contained one or more nucleoside diphosphatases with substrate specificities distinct from those present in the Golgi complex.

To compare further the activities detected in ER and Golgi, we compared their solubilization with detergents. As shown in Table I, both Golgi- and ER-associated nucleoside diphosphatase activities sedimented with the organelles in the absence of added detergent. When solubilizing amounts of a nonionic detergent were added (1% Triton X-100), both activities were recovered in the supernatant. However, the activity present in the ER could also be efficiently extracted in soluble form by low amounts of detergent (0.2% octylglucoside) under conditions that did not solubilize the activity from the Golgi membranes. As illustrated by the persistent membrane association of glucose-6-phosphatase, this mild detergent treatment caused membrane lysis without solubilization of integral membrane proteins. The ER nucleoside diphosphatase thus behaved as a soluble lumenal protein while the Golgi enzyme behaved like an integral membrane protein.

To pursue the apparent solubility difference, the ER and Golgi fractions were submitted to Triton X-114 partition, which allows distinction between soluble/peripheral and integral membrane proteins (Bordier, 1981). The UDPase activity associated with the ER partitioned almost exclusively in the aqueous phase, indicating that the ER-nucleoside diphosphatase was hydrophilic and did not bind detergent (Table II). In contrast, the UDP- and TPP-hydrolyzing activities associated with the Golgi complex partitioned preferentially into the detergent phase, consistent with the nucleoside diphosphatase behaving as an integral membrane protein. The low TPPase activity detected in the ER partitioned like the one associated with the Golgi complex, suggesting that it represented a contamination of the ER fractions with Golgi-derived fractions.

The subcellular fractionation, solubility and Triton X-114 partitioning data indicated that the ER of mammalian cells contains a soluble UDP activity different from the membrane bound enzyme present in the *trans*-Golgi. We will hereafter refer to it as the ER-UDPase.

Purification of ER-UDPase

Isolated ER fractions were used as the starting material for the purification of the ER-UDPase. Initial attempts were made with ER fractions prepared from rat liver, but very little protein was left at later stages of the purification. In order to scale up, we switched to bovine liver, where the behavior of the ER-UDPase activity was indistinguishable from that detected in rat liver (not shown). The use of bovine tissue provided sufficient material to purify the ER-UDPase to homogeneity (see Table III; Figure 1B).

The ER-UDPase was first released from the membranes by lysis with low concentrations of detergent. Upon DEAE–cellulose chromatography of the soluble extract, the major UDPase activity present in ER membranes was recovered in a single peak (Figure 1A) which was pooled and applied to a Concanavalin A (Con A)–Sepharose column. The α-methyl-mannopyranoside eluate from the Con A–Sepharose column was subjected to anion exchange, gel filtration and hydrophobic chromatography. When analyzed by SDS–PAGE, the eluate from the last

step showed a band of 45 kDa that accompanied the UDPase activity and could thus be identified as the ER-UDPase (Figure 1B).

Characterization of the ER-UDPase

The purified ER-UDPase eluted from gel filtration columns as a symmetric peak comigrating with a 45 kDa protein standard, indicating it was a monomer (Figure 1C). All the UDPase activity bound to the Con A–Sepharose column and was eluted with α-methyl-mannopyranoside, suggesting the presence of high mannose type oligosaccharides. Furthermore, digestion by Endo H reduced its molecular weight by 2–3 kDa (Figure 1D), a shift compatible with the loss of one or two high mannose oligosaccharides. The homogeneous ER-UDPase had a neutral optimum pH (not shown). Since no activity could be detected in the absence of divalent cations and since the enzyme was almost irreversibly inactivated by EDTA, the activity was routinely measured in the presence of 10 mM $CaCl_2$ (Figure 1A). However, the purified enzyme was also active in the presence of $MgCl_2$ and $MnCl_2$, with half maximal activity reached at 0.3–0.5 mM $CaCl_2$ or $MgCl_2$ (Figure 2B).

The availability of homogenous ER-UDPase enabled us to analyze its substrate specificity. As shown in Table IV, it hydrolyzed nucleoside diphosphates specifically, i.e. it did not act on the mono- or triphosphates tested (Table IV). It was also inactive on TPP, indicating that the low levels of TPPase detected in ER fractions (Table I) reflected contamination with Golgi. Of the pyrimidine diphosphates tested, only UDP was hydrolyzed, while thymidine or cytidine diphosphate were not. Among the purine diphosphates, the enzyme hydrolyzed guanine and inosine diphosphates but not adenosine or xantosine diphosphates. The enzyme was thus dependent on the base for activity, and required a terminal α–β pyrophosphate linkage.

High performance liquid chromatography (HPLC) analysis of the products formed by the ER-UDPase demonstrated that the enzyme converted the nucleoside diphosphates to nucleoside monophosphates (Figure 3). The low activity detected on UTP and GTP (Table IV) was clearly due to contaminating nucleoside diphosphate in the corresponding triphosphate preparations (Figure 3). The peaks corresponding to UTP and GTP were not affected by the ER-UDPase, whereas the UDP and GDP peaks were converted to UMP and GMP.

ER-UDPase activity on UDP and GDP in the presence of saturating concentrations of $CaCl_2$ or $MgCl_2$ was similar, characterized by a hyperbolic behavior with a nearly identical V_{max} and an apparent K_m of 0.2–0.5 mM for both nucleoside diphosphates (Figure 2C and D). In the presence of 1 mM $MgCl_2$ the activity on UDP and GDP was essentially the same. In the presence of 1 mM $CaCl_2$, however, the enzyme seemed to hydrolyze UDP more rapidly than GDP.

UDP inhibits GT

A possible reason why cells have a nucleoside diphosphatase in the ER is that UDP accumulated as a product of reglucosylation reactions might inhibit GT, similar to the

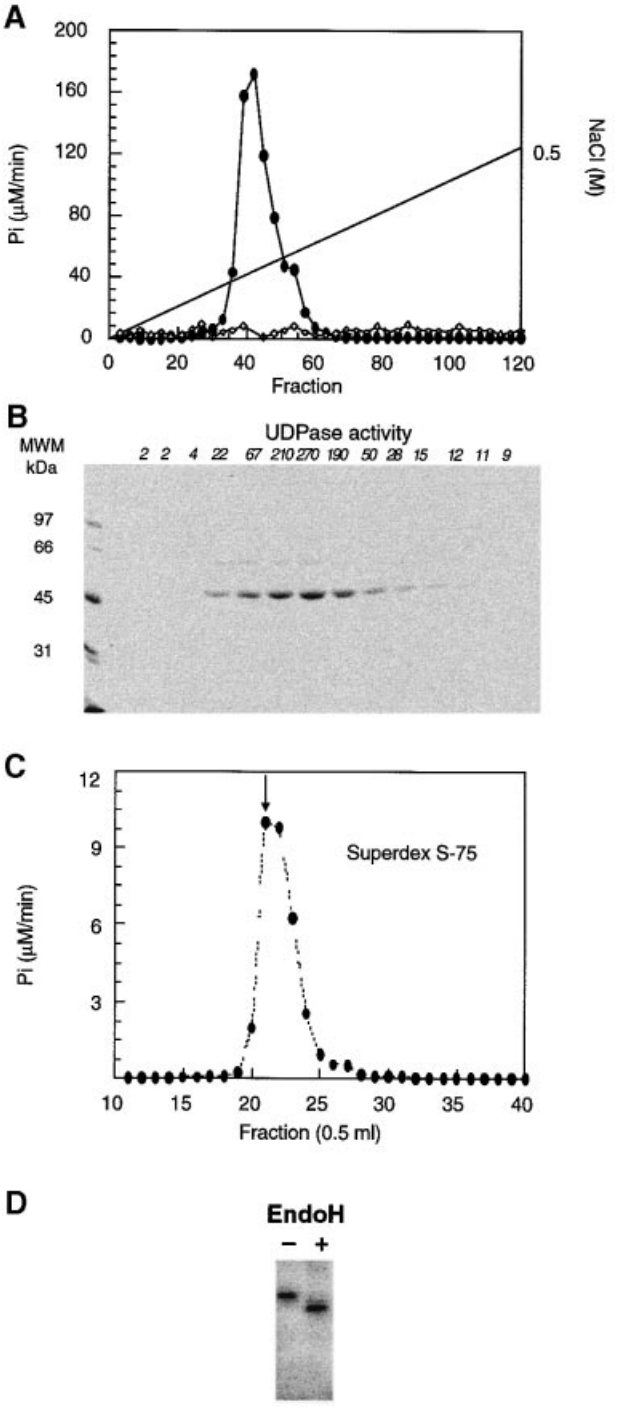

Fig. 1. Purification of the ER-UDPase. (**A**) DEAE–cellulose chromatography. The soluble extract from bovine liver ER-derived organelle fractions was applied to a DEAE–cellulose column as described in the Materials and methods. Fractions of 20 ml were collected and 5 μl aliquots were assayed for UDPase activity in the presence (●) or absence (○) of 10 mM $CaCl_2$. (**B**) SDS–PAGE analysis of the ER-UDPase containing fractions from the eluate of the hydrophobic column. Aliquots containing 15 μl from each fraction were run on a 10% SDS–PAGE and protein was detected with Coomassie Blue R-250. Numbers on top of each lane indicate the phosphatase activity detected using UDP as substrate. (**C**) Purified ER-UDPase (0.5 ml) was run on a Superdex S-75 column equilibrated and developed at 0.5 ml/min in 5 mM 2-mercaptoethanol, 150 mM NaCl, 10 mM HEPES pH 7.5. The arrow denotes the elution volume of the 45 kDa standard ovoalbumin. (**D**) Purified ER-UDPase was digested overnight at 37°C with Endo H (lane +) or mock-treated under the same conditions (lane –). Samples were run on a 10% SDS–PAGE and protein detected with Coomassie Blue R-250.

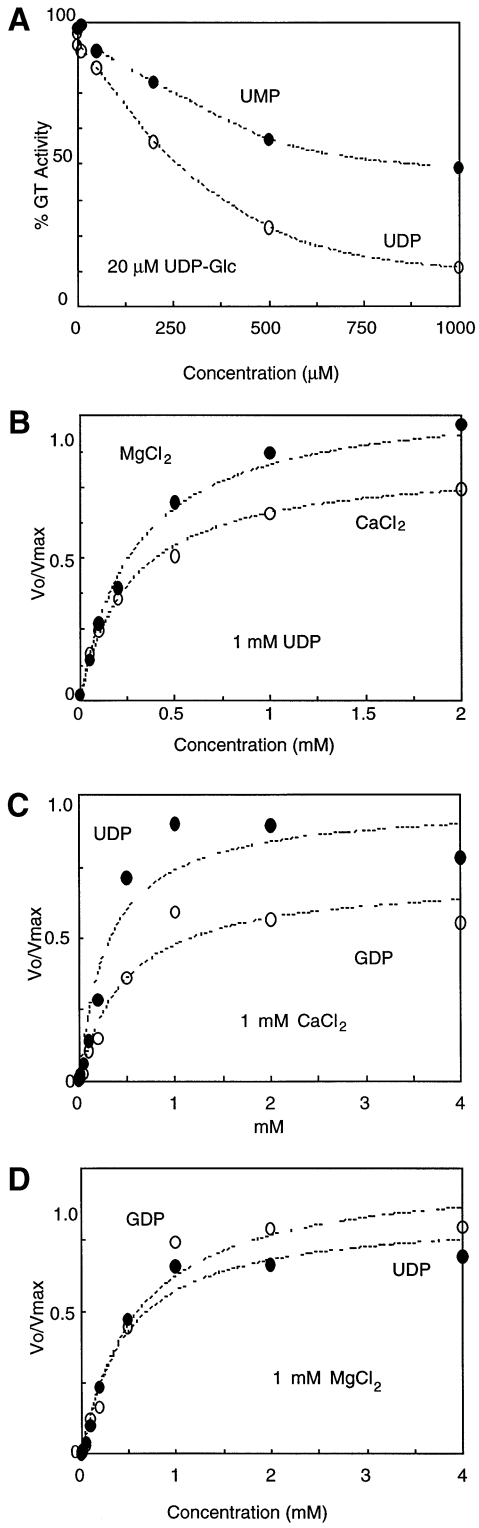

Fig. 2. Characterization of ER-UDPase. (**A**) Inhibition of glycoprotein reglucosylation by UDP and UMP. Glycoprotein reglucosylation using homogeneous UDP-Glc:glycoprotein glucosyltransferase was assayed as described in Trombetta *et al*. (1992). Reactions contained 20 μM UDP [^{14}C]-Glc and the indicated concentrations of UDP (○) or UMP (●). (B–D) Characterization of ER-UDPase activity. Homogeneous ER-UDPase from the hydrophobic chromatography step was used for the UDPase activity measurements and incubations were performed as described in the Materials and methods. (**B**) The effect of $CaCl_2$ (○) or $MgCl_2$ (●) on the activity of homogeneous ER-UDPase in the presence of 1 mM UDP. (**C**) ER-UDPase activity as a function of UDP (●) or GDP (○) in the presence of 1 mM $CaCl_2$ (C) or $MgCl_2$ (**D**).

Table IV. Specificity of homogeneous ER-UDPase

Base	Phosphatase activity		
	Monophosphate	Diphosphate	Triphosphate
Adenosine	<1	<1	<1
Guanosine	2	127	34[a]
Xanthosine	<1	1	1
Uridine	2	153	31[a]
Thymidine	2	1	1
Cytosine	<1	1	1
Inosine	1	131	21
Thiamine	<1	<1	N.D.

Phosphatase activity was measured as described in the Materials and methods using the indicated substrates at 1 mM concentration in the presence of 2 mM $CaCl_2$.
[a]In these cases, the phosphate released comes from contaminant nucleoside diphosphates, as detected by HPLC analysis of the substrates and the reaction and products (see Figure 3).
N.D., not determined.

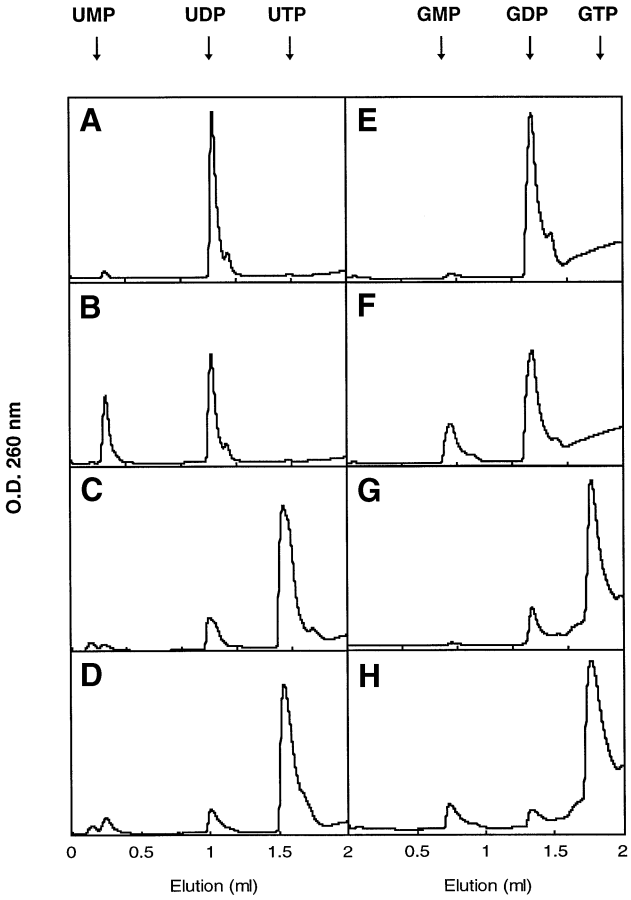

Fig. 3. HPLC analysis of the reaction products formed by homogeneous ER-UDPase. Incubations and HPLC analysis were performed as described in the Materials and methods. All samples contained 1 mM $CaCl_2$ and homogeneous ER-UDPase. The substrates used were as follows: UDP (**A**, **B**), UTP (**C**, **D**), GDP (**E**, **F**) and GTP (**G**, **H**). For inactivated samples (A, C, E and G), 2 mM EDTA was present before the addition of ER-UDPase. In the digested samples (B, D, F and H), EDTA was added at the end of the incubations.

product inhibition of glycosyltransferases in the Golgi complex. Glycoprotein reglucosylation was measured *in vitro* using homogeneous GT and acceptor glycoproteins, in the presence or absence of UDP and UMP.

A

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1 ATG GCC ACT TCC TGG GGG GCT GTC TTC ATG CTG ATC ATA GCC TGC GTT GGC AGC ACT GTC TTC TAC AGA GAA
  M   A   T   S   W   G   A   V   F   M   L   I   I   A   C   V   G   S   T   V   F   Y   R   E>

73 CAG CAG ACC TGG TTT GAA GGT GTC TTC TTG TCT TCC ATG TGC CCC ATT AAT GTC AGT GCC GGC ACC TTT TAT
   Q   Q   T   W   F   E   G   V   F   L   S   S   M   C   P   I   N   V   S   A   G   T   F   Y>
                                                   M   C   P   V   W   V   S   A   G   T   F

145 GGA ATT ATG TTT GAT GCG GGC AGC ACT GGA ACT CGG ATT CAT GTT TAC ACT TTT GTG CAG AAA ACA GCA GGA
    G   I   M   F   D   A   G   S   T   G   T   R   I   H   V   Y   T   F   V   Q   K   T   A   G>
                                                    I   H   V   Y   T   F   V   Q       T   A   G

217 CAG CTC CCC TTT CTG GAA GGT GAA ATT TTT GAT TCT GTG AAG CCG GGA CTT TCT GCT TTT GTG GAT CAG CCC
    Q   L   P   F   L   E   G   E   I   F   D   S   V   K   P   G   L   S   A   F   V   D   Q   P>
    Q   L   P   V   L   E   G   E   I   F   D   S   V   K   I   G   L   S   A   F   V   x   Q

289 AAA CAG GGT GCT GAG ACT GTC CAG GAG CTC TTG GAG GTG GCC AAA GAC TCG ATC CCC AGA AGC CAC TGG GAA
    K   Q   G   A   E   T   V   Q   E   L   L   E   V   A   K   D   S   I   P   R   S   H   W   E>

368 AGG ACC CCG GTG GTT CTG AAA GCA ACG GCC GGA CTC CGT TTG CTG CCT GAG CAG AAA GCC CAG GCT CTG CTC
    R   T   P   V   V   L   K   A   T   A   G   L   R   L   L   P   E   Q   K   A   Q   A   L   L>

433 TTG GAG GTA GAG GAG ATC TTC AAG AAT TCA CCT TTC CTG GTC CCA GAT GGC AGC GTT AGC ATC ATG GAT GGG
    L   E   V   E   E   I   F   K   N   S   P   F   L   V   P   D   G   S   V   S   I   M   D   G>

505 TCC TAT GAA GGC ATA CTA GCC TGG GTT ACC GTG AAC TTT CTA ACA GGT CAG CTG CAT GGT CGT GGC CAG GAG
    S   Y   E   G   I   L   A   W   V   T   V   N   F   L   T   G   Q   L   H   G   R   G   Q   E>

577 ACT GTG GGG ACC CTT GAC CTG GGG GGT GCC TCC ACC CAA ATC ACG TTT CTA CCC CAG TTT GAG AAA ACC CTG
    T   V   G   T   L   D   L   G   G   A   S   T   Q   I   T   F   L   P   Q   F   E   K   T   L>

649 GAA CAA ACA CCT AGG GGC TAC CTC ACT TCC TTT GAG ATG TTT AAC AGC ACT TTT AAG CTC TAT ACA CAT AGT
    E   Q   T   P   R   G   Y   L   T   S   F   E   M   F   N   S   T   F   K   L   Y   T   H   S>

721 TAC TTG GGA TTT GGA CTG AAA GCT GCA AGA CTG GCA ACT CTG GGA GCC CTG GAA GCA AAA GGG ACT GAT GGA
    Y   L   G   F   G   L   K   A   A   R   L   A   T   L   G   A   L   E   A   K   G   T   D   G>
                                            L   A   T   L   G   A   L   E   T   A   G   I   D   G

793 CAT ACG TTT CGA AGT GCC TGT TTA CCA AGA TGG TTG GAA GCA GAG TGG ATC TTT GGG GGT GTG AAA TAC CAG
    H   T   F   R   S   A   C   L   P   R   W   L   E   A   E   W   I   F   G   G   V   K   Y   Q>
    Y   T   F   R

865 TAT GGT GGT AAC CAA GAA GGG GAG ATG GGC TTT GAA CCC TGC TAT GCG GAA GTG CTG AGG GTA GTA CAG GGG
    Y   G   G   N   Q   E   G   E   M   G   F   E   P   C   Y   A   E   V   L   R   V   V   Q   G>

937 AAA CTT CAC CAG CCA GAA GAA GTC CGA GGA AGC GCC TTC TAC GCT TTC TCT TAC TAC TAC GAT CGA GCC GCT
    K   L   H   Q   P   E   E   V   R   G   S   A   F   Y   A   F   S   Y   Y   Y   D   R   A   A>
                                                            A   F   S   Y   Y   Y   D   R

1009 GAC ACA CAC TTG ATC GAT TAT GAA AAG GGC GGG GTT TTA AAA GTT GAA GAT TTT GAA AGA AAA GCC AGA GAA
     D   T   H   L   I   D   Y   E   K   G   G   V   L   K   V   E   D   F   E   R   K   A   R   E>

1081 GTG TGT GAC AAC TTG GGG AGC TTC TCC TCG GGC AGT CCT TTC CTC TGC ATG GAC CTC ACT TAC ATC ACA GCC
     V   C   D   N   L   G   S   F   S   S   G   S   P   F   L   C   M   D   L   T   Y   I   T   A>

1153 CTG TTG AAA GAT GGT TTT GGC TTT GCC GAC GGC ACC CTC TTA CAG CTC ACA AAG AAA GTG AAC AAC ATA GAG
     L   L   K   D   G   F   G   F   A   D   G   T   L   L   Q   L   T   K   K   V   N   N   I   E>
                 D   G   F   G   F   A   S   S   T   V   L   Q   L   T   K

1225 ACT GGT TGG GCC TTG GGG GCC ACC TTT CAC CTG CTC CAG TCT CTG GGC ATC ACC AGC TGA
     T   G   W   A   L   G   A   T   F   H   L   L   Q   S   L   G   I   T   S   *
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B

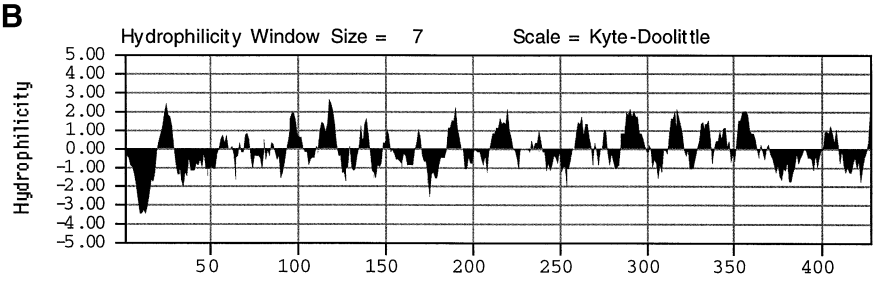

Fig. 4. Mouse liver ER-UDPase cDNA sequence. (**A**) Predicted ORF for mouse liver ER-UDPase (DDBJ/EMBL/GenBank accession No. AJ238636). Underlined portions correspond to peptides sequenced from the purified bovine ER-UDPase. The actual amino acid sequence of peptides obtained from the bovine enzyme are shown under the corresponding positions in the mouse sequence. The peptide MCPVWVSAGT corresponds to the N-terminus of the purified ER-UDPase. (**B**) Hydrophobicity plot of mouse liver ER-UDPase.

Glycoprotein reglucosylation was more strongly inhibited by UDP than by similar concentrations of UMP (Figure 2A), indicating that conversion of UDP into UMP may serve to alleviate product inhibition of GT.

Characterization of ER-UDPase cDNA

Amino acid sequence information was obtained from the N-terminus and from internal peptides of the purified bovine ER-UDPase (Figure 4). Based on codon usage preferences in mammals, and amino acid sequence of peptide 27 (vpgqlpvlegeifdsvkpglsafv) and peptide 9 (ihvytfvqk), pools of degenerate primers were used to amplify a first strand cDNA library from mouse liver. A combination of primers S92 (5′-athcaygtntayacntty-3′) and A273 (5′-gartcraanatytcnccytc-3′) yielded a short PCR product that revealed an amino acid sequence (ihvytfvqktagqlpflegeifd) comprised of peptide 9 immediately followed by that of peptide 27, and containing an amino acid sequence corresponding to peptides 9 and 27 not included in the primers, indicating that it corresponded to a portion of the ER-UDPase. Comparison of the peptide sequence with EST databases matched a fragment in entries aa116990 and aa120757. Complete sequencing on both strands of these two cDNAs showed that the two clones contained the same open reading frame (ORF) shown in Figure 4. Differences were only found in the 5′ and 3′ flanking regions: Clone aa116990 had an insertion of 70 bp in the 5′ end, while clone aa120757 had

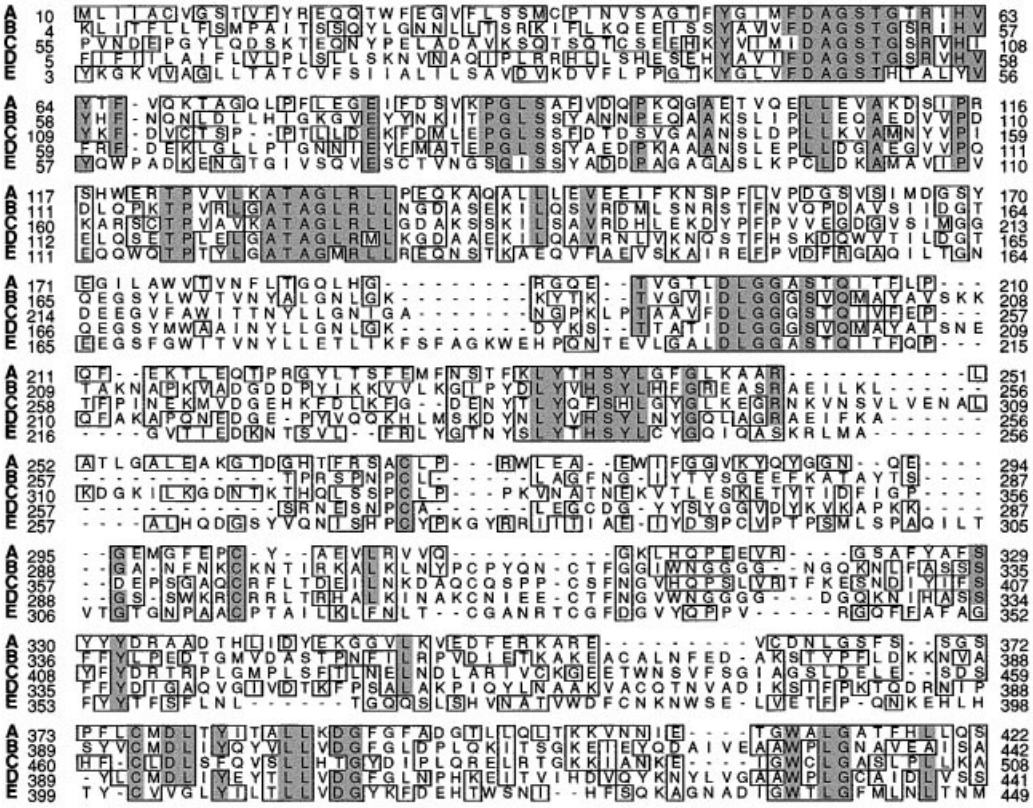

Fig. 5. Sequence comparison of mouse liver ER-UDPase with related pyrophosphatases. Pyrophosphatases homologous to mammalian ER-UDPase were aligned using a CLUSTAL_W algorithm, and conserved residues boxed. Numbering on left and right denotes amino acid position in the corresponding sequence. Letters on the left refer to the cDNA compared: A, mouse ER-UDPase; B, Pea NTPase (DDBJ/EMBL/GenBank accession No. Z32743) (Hsieh *et al*., 1996); C, *S.cerevisiae* GDPase (L19560) (Abeijon *et al*., 1993); D, potato ATP-diphosphohydrolase (U18778) (Handa and Guidotti, 1996); E, chicken ecto-ATP-diphosphohydrolase (AF041355) (Nagy *et al*., 1998).

an insertion of 231 bp at the 3′ end. The following characteristics indicated that the cDNA identified indeed coded for the ER-UDPase: (i) all six peptide sequences obtained from the purified enzyme could be found in the cDNA; (ii) the cDNA sequence showed a putative hydrophobic N-terminal signal sequence, followed by the sequence found in the N-terminus of the purified enzyme; (iii) the overall sequence coded for a hydrophilic protein of 43 kDa, in agreement with the biochemical properties of the isolated enzyme; (iv) the presence of two consensus triplets for *N*-glycosylation was consistent with the presence of glycans in the purified enzyme (Figure 1D); and (v) the sequence showed significant homology with enzymes that cleave α–β pyrophosphate linkages in nucleoside di- and triphosphates (Figure 5). A mouse cDNA sequence described as homologous to NTPases (Chadwick *et al*., 1998; DDBJ/EMBL/GenBank accession No. AF006482) is identical to most of the ER-UDPase presented here but differs from it at the C-terminus. We are confident the cDNA sequence presented in Figure 4 belongs to the purified ER-UDPase since we found a peptide in that region that precisely matches our cDNA sequence (see Figure 4) and does not appear in the mouse cDNA (Chadwick *et al*., 1998).

It is noteworthy that we found the ER-UDPase to be broadly expressed in mouse tissues. Using RT–PCR, we detected the mRNA in every tissue tested (Figure 6). Furthermore, numerous EST clones from diverse tissues were identified in databases.

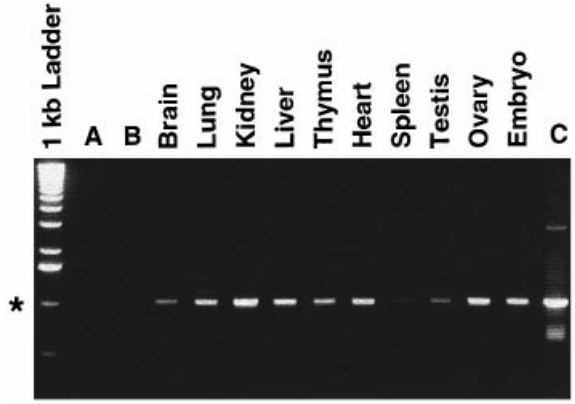

Fig. 6. Tissue distribution of ER-UDPase. First strand cDNA synthesis was primed with oligo(dT) using total RNA from the indicated tissues. The mRNA for ER-UDPase was amplified as described in the Materials and methods. Lanes A, B and C contain controls: in lane A, the PCR template was a first strand synthesis devoid of RNA. In lane B, mouse liver RNA was subjected to a mock first strand cDNA synthesis lacking reverse transcriptase. In lane C, 10 ng of a plasmid containing ER-UDPase cDNA was used as template. The asterisk denotes the position of the 1 kb standard.

Intracellular localization

To analyze in more detail the subcellular localization of the enzyme, antibodies were raised against a peptide corresponding to amino acids 108–131 (EVAKDSIPR-SHWERTPVVLKATAG) of the mouse ER-UDPase. By immunoblotting, the rabbit antibodies detected a single

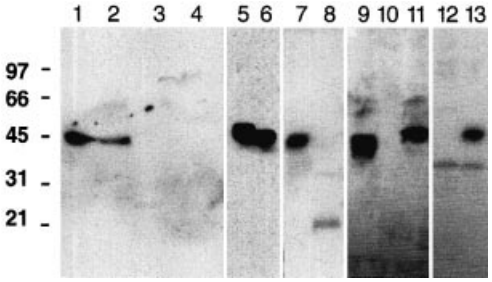

Fig. 7. Characterization anti ER-UDPase antibodies. Protein samples were run on 10% SDS–PAGE, transferred to nitrocellulose and probed with anti-peptide antibody against mouse ER-UDPase (lanes 1–11) or anti-myc monoclonal antibody 9E10 (lanes 12 and 13). Lanes 1–4 and 9 contain rat liver subcellular fractions: lanes 1 and 9, partially purified ER-UDPase (1 μg of protein, DEAE step); lane 2, ER-derived membranes (10 μg); lane 3, Golgi derived membranes (10 μg); lane 4, cytosol (20 μg). Lanes 5 and 6 contain 2 μg of the same partially purified ER-UDPase sample used in lane 1, mock-digested (lane 5) or digested (lane 6) with Endo H. Lane 7 contained 10 μg of microsomal protein, and lane 8 contained 20 μg of cytosolic protein extracted from the A20 mouse lymphoma cell line. Lanes 10–13 contain 30 μg of a lysate from control transfected CHO cells (lanes 10 and 12) or myc-tagged ER-UDPase cDNA transfected CHO cells (lanes 11 and 13).

45 kDa band that comigrated with the purified enzyme (Figure 7). The same 45 kDa band was detected in rat (Figure 7, lanes 1, 2, 5 and 6) and mouse (not shown) liver microsomes, and in lysates from mouse lymphocyte cell line A20, where the antigen was present in the organelle fraction (lane 7) but not in the cytosol (lane 8). That the polypeptide recognized by the antibody was indeed the ER-UDPase was confirmed by the shift of the reactive band upon Endo H digestion (Figure 7, lanes 5 and 6), consistent with the effect seen on the purified enzyme.

Antibodies were also used to investigate the distribution of the ER-UDPase in subcellular fractionations of rat liver prepared by isopycnic density-gradient centrifugation. The ER-UDPase was detected in fractions corresponding to the ER, but not in low density fractions containing the Golgi complex marker mannosidase II (Figure 8).

Since attempts to study the intracellular localization of the ER-UDPase by immunocytochemical techniques using the above-described anti-peptide antibodies failed, we introduced an epitope tag at the C-terminus of the ER-UDPase and transiently expressed the epitope-tagged ER-UDPase cDNA in COS, HeLa, Chinese hamster ovary (CHO) and normal rat kidney (NRK) cells. Indirect immunofluorescence showed that the c-myc tagged ER-UDPase accumulated in intracellular compartments that also stained for the ER marker calnexin in all four cell lines (Figure 9A–H). Moreover, the distribution observed in NRK cells clearly differed from that of the Golgi apparatus marker mannosidase II (Figure 9I and J). The c-myc-tagged ER-UDPase expressed in CHO cells had the expected molecular weight and organelle association as detected by Western blot using anti-mouse ER-UDPase (Figure 7, lanes 10 and 11) or anti myc antibodies (Figure 7, lanes 12 and 13). Moreover, it appeared to be biologically active, since microsomal fractions from transfected cells showed a 3.5-fold increase in UDPase activity over control-transfected cells (not shown). Together with the presence of an N-terminal signal sequence and Endo H-sensitive oligosaccharides, these

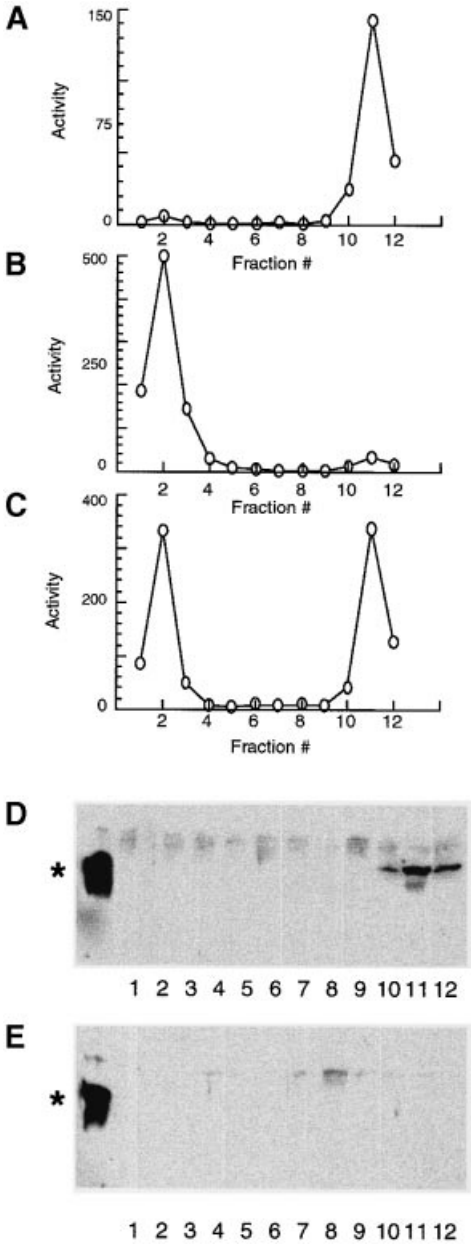

Fig. 8. Subcellular distribution of ER-UDPase. Rat liver post nuclear supernatant (400 μl) was prepared and analyzed on Optiprep gradients as described in the Materials and methods. Twelve fractions (350 μl each) were collected and analyzed for (**A**) the ER marker glucose-6-phosphatase; (**B**) the Golgi marker mannosidase II; or (**C**) UDPase activity. (**D**) ER-UDPase was detected in the same fractions by Western blotting using anti-peptide antibodies. (**E**) As a control to compensate for the low abundance of Golgi membranes in the post-nuclear supernatant, Golgi-enriched membranes (400 μl at 0.5 mg/ml) were loaded at the top of a gradient, which was run, fractionated and analyzed as in (D). The asterisks show the band detected in a lane containing purified ER-UDPase.

observations indicated that the ER-UDPase is a resident protein of the ER.

Discussion

We report the identification, isolation, characterization and subcellular localization of a nucleoside diphosphatase ubiquitously expressed in the ER of mammalian cells. In

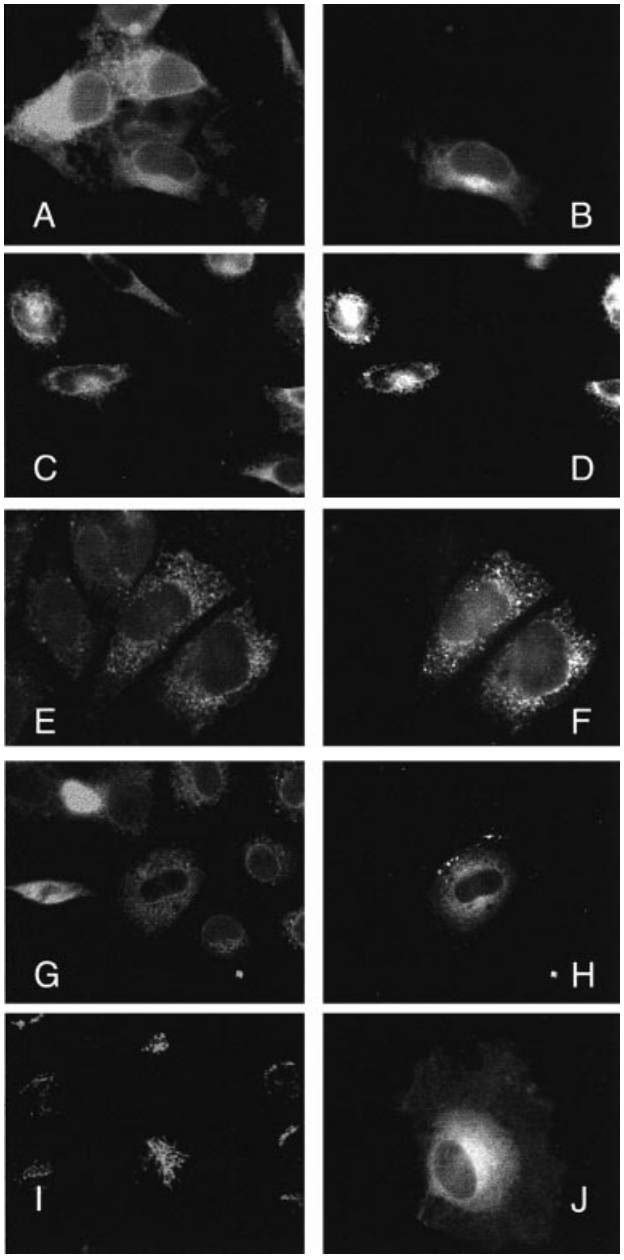

Fig. 9. Intracellular localization of c-myc-tagged ER-UDPase. COS (**A**, **B**), HeLa (**C**, **D**), CHO (**E**, **F**) and NRK (**G**–**J**) cells were transiently transfected with ER-UDPase cDNA containing a c-myc epitope at the C-terminus. Twenty-four hours after transfection, cells were fixed, permeabilized and processed for immunofluorescence. Calnexin was detected with polyclonal rabbit antiserum (A, C, E and I) and the c-myc epitope was detected with monoclonal antibody 9E10 (B, D, F, H and J). Golgi mannosidase II is shown in (G).

isolated form, the protein is a soluble monomer of 45 kDa. It is synthesized with a cleaved signal sequence and carries one or two high mannose N-linked glycans. While of low abundance, it is easily detected in purified microsomes and isolated ER fractions by its enzymatic activity, and may represent the so called 'microsomal' nucleoside diphosphatase (Kuriyama, 1972; Ohkubo *et al*., 1980; RayChaudhuri *et al*., 1985; Sano *et al*., 1988). We found that homogeneous ER-UDPase is able to cleave UDP, GDP and IDP to their corresponding nucleoside monophosphates. It is inactive on nucleoside mono- or triphosphates as well as ADP, CDP and TDP. While the Golgi complex associated nucleoside diphosphatase efficiently cleaves TPP, the ER-UDPase is inactive on this compound. The enzyme seems to require a terminal α–β pyrophosphate group and some specific base for activity. It also requires divalent cations with half maximal activation occurring at 0.3–0.5 mM for $MgCl_2$ and $CaCl_2$. Thus the enzyme should be fully active in its proposed location within the ER lumen.

The cDNA sequence revealed that the ER-UDPase belongs to a family of enzymes that cleave α–β pyrophosphate bonds in nucleoside di- and triphosphates. Members of this family include type I and II membrane proteins, as well as soluble proteins of ~50–60 kDa present in diverse organelles and species. They all share a central core (~30–35 kDa) that seems to provide the catalytic functions.

The ER-UDPase has an N-terminal signal sequence and is glycosylated with high mannonse *N*-glycans. The following observations indicate that it is a lumenal enzyme of the ER: (i) the enzymatic activity and the protein were exclusively localized to ER fractions of rat and bovine liver after subcellular fractionation; (ii) detergent solubilization showed that it was trapped in the lumen of the membrane bound organelles; (iii) when a C-terminal myc-tagged recombinant ER-UDPase cDNA was expressed in COS, HeLa, CHO and NRK cells, indirect immunofluorescence showed a typical ER distribution, colocalizing with the ER marker calnexin; and (iv) the presence of an Endo H-sensitive oligosaccharide(s) was consistent with a pre-Golgi localization of the protein.

Whilst clearly a lumenal ER protein according to our present data, the ER-UDPase showed no obvious transmembrane domains and it possessed no C-terminal KDEL-related sequence, which in many soluble ER proteins serves as a signal for retention in the ER. However, a number of other soluble resident ER proteins are known to lack such signals. Some of them form a complex with other subunits that do carry KDEL-related C-terminal sequences. This is the case for prolyl 4-hydroxylase (Pihlajaniemi *et al*., 1987) and triglyceride transfer protein (Gordon *et al*., 1995), which associate with protein disulfide isomerase, or glucosidase II α-subunit, which in turn associates with a β-subunit containing a KDEL-related motif (Trombetta *et al*., 1996). For other proteins, such as s-cyclophilin, no additional subunits are known (Arber *et al*., 1992). Although the ER-UDPase is a monomer after isolation, we cannot exclude the possibility that it associates with other proteins *in situ*. Alternatively, it may possess an ER-localization signal that has not yet been identified.

The ER-UDPase is clearly distinct in its solubility properties from the nucleoside diphosphatase activity of the Golgi complex. In agreement with previous data (Brandan and Fleischer, 1982), we found that the activity in fractions enriched in Golgi complex markers behaved like an integral membrane protein. Moreover, unlike the Golgi enzyme, the ER-UDPase did not hydrolyze TPP. After this study was completed, a cDNA with homology to ecto (E)-ATPases was shown to localize to the Golgi complex (Wang and Guidotti, 1998). Since membrane fractions prepared from cells overexpressing the cDNA

displayed increased UDPase activity, it was proposed that the gene encoded the Golgi UDPase (Wang and Guidotti, 1998). The proposed Golgi enzyme is not related to the ER-UDPase presented here. It appears that cells utilize two entirely different enzymes to hydrolyze nucleoside diphosphates in consecutive compartments of the secretory pathway.

The broad tissue distribution suggests that the ER-UDPase has a conserved, ubiquitous function. Of the three substrates identified, only UDP is so far known to be present in the ER lumen. UDP-Glc is translocated from the cytosol into the lumen of the ER (Pérez and Hirschberg, 1986; Vanstapel *et al*., 1988; Abeijon and Hirschberg, 1992), where it serves as substrate for reglucosylation of incompletely folded glycoproteins (Trombetta *et al*., 1991). UDP is formed as a product of this reaction. It is likely that the ability to cleave UDP into UMP prevents the accumulation of inhibitory levels of UDP that would affect reglucosylation in the ER. The ER-UDPase may also play a role in the transport and utilization of UDP derived from other sugar nucleotides such as UDP-*N*-acetylglucosamine and UDP-glucuronic acid imported from the cytosol into the lumen of the ER (Hirschberg and Snider, 1987; Abeijon and Hirschberg, 1992). The latter is used in some tissues as the sugar donor for glucuronidation of xenobiotics (Hauser *et al*., 1988).

The GDPase activity of the ER-UDPase is puzzling since GDP is not known to be present in the lumen of the ER (Pérez and Hirschberg, 1986). All the GDP-Man-dependent mannosyltransferases in the ER are known to face the cytosol (Abeijon and Hirschberg, 1992). Therefore, if GDP is indeed present in the lumen of the ER it is unlikely to arise as a product of mannosylation reactions. The demonstration of GDPase activity in the ER raises the interesting possibility that guanosine nucleosides could be present within the secretory pathway and serve as substrates for lumenally oriented GTP/GDP-binding proteins. The structural similarity between guanosine and inosine may, on the other hand, explain the ability of the ER-UDPase to hydrolyze IDP, which may not be a substrate for the enzyme *in vivo*.

The main function of the ER-UDPase is probably similar to that of the nucleoside diphosphatase(s) present in the Golgi apparatus (Kuhn and White, 1977; Hirschberg and Snider, 1987). Nucleoside diphosphates generated by lumenally oriented glycosyltransferases are product inhibitors and therefore must be eliminated. Moreover, the nucleoside monophosphates in the Golgi are exchanged for new sugar nucleotides from the cytosol. Such a transport mechanism has been elegantly demonstrated in *Saccharomyces cerevisiae*, where a GDPase was shown to modulate mannosylation reactions by reducing the import of GDP-Man into the Golgi (Abeijon *et al*., 1993; Berninsone *et al*., 1994). The ER-UDPase is also likely to be indirectly involved in sugar nucleotide transport, producing UMP that exits the ER in a coupled antiporter reaction allowing entry of UDP monosaccharides into the ER lumen. The identification of the mammalian ER-UDPase described here opens the possibility of performing further functional and genetic studies required to test this hypothesis, and of establishing the role of nucleoside hydrolysis in the calnexin/calreticulin pathway.

Materials and methods

Materials

Chromatography media and columns were from Pharmacia (Piscataway, NJ). All chemicals were from Sigma (St Louis, MO), except where otherwise indicated. Homogeneous UDP-Glc:glycoprotein glucosyltransferase was prepared and assayed as described previously (Trombetta *et al*., 1991). Anti-calnexin C-terminal tail antibodies have been described previously (Hammond and Helenius, 1994). c-myc epitope-tagged ER-UDPase was detected with monoclonal antibody 9E10. Anti-mannosidase II antibodies were generously provided by Dr M.G.Farquhar.

Assays

Protein concentration was determined by the method of Lowry. Mannosidase II activity was measured as described in Ayad *et al*. (1997). Glucose-6-phosphatase was assayed as described in Trombetta *et al*. (1991). Phosphatase activity was detected by measuring the inorganic phosphate released as described in Chen *et al*. (1956). Incubations were done in a total volume of 50 µl containing 2–10 mM $CaCl_2$, 50 mM NaCl, 20 mM HEPES buffer pH 7.5, 0.1% Triton X-100 and 2 mM substrate. Reactions were initiated with the addition of enzyme (5–10 µl). After the indicated time at 37°C (typically 5 min), reactions were stopped by adding 150 µl of 10% trichloroacetic acid (TCA) and incubated on ice for 10 min. After centrifugation, 100 µl of the supernatant were mixed with 200 µl of color development reagent and incubated at 37°C for 30 min, and Abs 820 nm was measured. Assays employing purified or homogeneous ER-UDPase were carried out as described above, but at the end of the incubations, 150 µl of color development reagent were added directly to the reactions. After 30 min at 37°C, Abs 820 nm was measured. All the nucleoside phosphates analyzed (Table IV) were degraded to the same extent by calf alkaline phosphatase, discounting the presence of non-specific inhibitors in the substrates that were not degraded by the ER-UDPase.

For chromatographic analysis of the reaction products generated by purified ER-UDPase, HPLC separation of nucleoside phosphates was performed as described in Kremmer *et al*. (1989) with minor variations. A Mini Q PC 3.2/3 (Pharmacia) was used instead of a Mono Q 5/5. A linear gradient was run from solvent A (20 mM ammonium phosphate, pH 7.0) to varying concentrations solvent B (500 mM ammonium phosphate, pH 7.0). For separation of UMP, UDP and UTP (monitored at 260 nm), a gradient from 0–60% solvent B in 4 ml at 0.4 ml/min was used. For separation of GMP, GDP and GTP (monitored at 253 nm), a gradient from 0 to 100% solvent B was used under the same conditions. To maintain the UV profiles similarly, all the samples contained homogeneous ER-UDPase and EDTA, but differed in the order of additions. All reactions were carried in a final volume of 50 µl, incubated at 37°C, and contained 1 mM $CaCl_2$ and 0.05 mU of homogeneous ER-UDPase. Inactivated samples contained 2 mM EDTA before the addition of ER-UDPase. Incubations with enzyme were performed in the absence of EDTA, which was added at the end of the reactions. All samples were centrifuged for 10 min at 15 000 *g* before injecting in the HPLC.

Membrane fractions

All steps were carried out on ice or at 4°C. Protease inhibitors were included in all the buffers for membrane preparations and extractions. The inhibitors used were: PMSF, TPCK, TLCK (50 µg/ml each), aprotinin, leupeptin, pepstatin and E64 (1 µg/ml each). One or two Albinus Wistar rats were used each time for preparation of purified ER and Golgi membranes. Livers were excised and rinsed with solution B (0.5 M sucrose, 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 50 mM potassium phosphate pH 6.5, including protease inhibitors). They were then chopped into pieces with a razor blade and immediately homogenized in 3 vol. solution B using a glass-Teflon potter. The homogenate was centrifuged for 10 min at 10 000 *g* and the supernatant mixed with one volume of solution E (2.0 M sucrose, 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 50 mM potassium phosphate pH 6.5). Ten milliliters of the diluted homogenate were layered on top of 5 ml of solution D (1.2 M sucrose, 5 mM $MgCl_2$) at the bottom of a SW28 tube, overlaid with 18 ml of solution B (0.6 M sucrose, 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 50 mM potassium phosphate pH 6.5) and topped with solution A (0.25 M sucrose, 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 50 mM potassium phosphate pH 6.5). The samples were centrifuged in a SW28 rotor at 28 000 r.p.m. for 1 h. The fraction enriched in Golgi membranes floated at the interface between solutions A and B and was collected with a Pasteur pipette. ER membranes pelleted through the cushion of solution C. Membrane fractions thus obtained were used immediately without freezing.

Detergent solubilization and Triton X-114 partition

Golgi and ER membrane fractions obtained as described above were brought to 0.5 mg protein/ml in solution A including protease inhibitors. Triton X-100 or octylglucoside were added from 10% stocks to the final concentrations required. After incubating for 15 min on ice, 100 µl aliquots were removed as controls and the remaining 900 µl were centrifuged for 45 min at 100 000 *g*. The supernatants were kept for further analysis (solubilized fraction).

For Triton X-114 partition, 1 ml of Golgi or ER membrane fractions at 0.5 mg/ml in solution A were made with 1% Triton X-114 (Calbiochem) and incubated on ice for 10 min. Aliquots (100 µl) were removed and kept on ice as controls. The remaining 900 µl were then incubated at 30°C for 10 min, followed by centrifugation for 5 min at 12 000 *g*, at 22°C. The aqueous phase (supernatant) was removed and the hydrophobic phase (pellet) resuspended in 0.9 ml of solution A on ice. Total, aqueous and hydrophobic phases were tested for UDPase and T-PPase activities (Table II).

Density gradients

To analyze the sub cellular distribution of marker enzymes and ER-UDPase by continuous gradient centrifugation, samples of rat liver post nuclear supernatant (400 µl) prepared as described above were applied on top of a 10–30% Optiprep® gradient and run for 16 h at 22 000 r.p.m. in a SW55 rotor. Fractions (350 µl) were collected and analyzed for mannosidase II, glucose-6-phosphatase and UDPase activity. For detection of ER-UDPase by Western blotting, 200 µl of each fraction were made 10% in TCA and incubated on ice for 30 min. After centrifugation, the pellets were dissolved in 15 µl of sample buffer, run on 10% SDS–PAGE and transferred to nitrocellulose. ER-UDPase was detected using the anti-peptide antibody against amino acids 108–131 (EVAKDSIPRSHWERTPVVLKATAG) of the mouse ER-UDPase. To compensate for the low abundance of Golgi membranes in the post nuclear supernatant, the same analysis was performed but loading samples (400 µl) of Golgi enriched membranes instead of post nuclear supernatant (Figure 8).

Purification of ER-UDPase

The same procedure described for the preparation of rat liver membrane fractions was scaled up using a fresh calf liver (~450 g) with minor variations. All the fat and hard tissue was removed. The crude homogenate was passed through five layers of cheese cloth and then centrifuged twice for 10 min at 10 000 *g*. The supernatant of the second spin was mixed with 1 vol. solution E. Forty-five milliliters of the diluted samples were layered at the bottom of a 95 ml ultracentrifuge bottle, overlaid with 40 ml of solution D and the tube filled with solution A. The samples were centrifuged at 150 000 *g* for 3 h in a 45Ti rotor. The pellet at the bottom of the tubes were collected, diluted with solution A and spun at 100 000 *g* for 1 h. The pellets were resuspended in solution A containing protease inhibitors, flash-frozen and stored at –80°C.

Extraction. ER membranes were resuspended at a final protein concentration of 5 mg/ml in buffer A (20 mM Tris–HCl, 5 mM 2-mercaptoethanol, pH 7.5), containing the protease inhibitors mentioned above. Triton X-100 was added from a 20% stock solution to a final concentration of 0.05%. After 10 min on ice, the sample was centrifuged for 60 min at 100 000 *g*.

DEAE–cellulose. The soluble microsomal extract was loaded onto a column of DEAE Sephacell (5×10 cm) equilibrated in buffer B (buffer A plus 0.1% Triton X-100). After washing with 200 ml of buffer B, the column was eluted with a linear gradient of 1200 ml from 0 to 500 mM NaCl in buffer B at a flow rate of 10 ml/min. Fractions of 20 ml were collected and assayed for UDPase activity, with or without 10 mM $CaCl_2$ in the assay.

Con A–Sepharose. Peak fractions from the DEAE column containing UDPase activity were pooled. $CaCl_2$, $MnCl_2$ and $MgCl_2$, were added to 1 mM and loaded onto a Con A–Sepharose column (2.5×5 cm) equilibrated in buffer C (20 mM Tris–HCl, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$, 1 mM $MgCl_2$, pH 7.5). After washing with 100 ml of buffer C, 20 ml of buffer D (buffer C containing 1 M α-methyl-mannoside) were added to the column. After this addition the column was warmed to 37°C for 30 min, and eluted with more buffer D at 37°C. UDPase activity was measured in the eluate.

Mono Q. All the ER-UDPase activity eluted from the Con A–Sepharose was loaded onto a Mono Q HR 5/5 column (Pharmacia) equilibrated in buffer A. Elution was performed by a linear gradient of 30 ml from 0 to 0.5 M NaCl in buffer A at a flow rate of 1 ml/min. Fractions (0.5 ml) were collected and assayed for UDPase activity.

Gel filtration. A Superdex 75 HR 10/30 column (Pharmacia) was equilibrated and run at 0.5 ml/min in 5 mM 2-mercaptoethanol, 150 mM NaCl, 10 mM HEPES pH 7.5. Fractions of 0.4 ml were collected. The fractions most active in ER-UDPase activity were pooled, concentrated and re-run under the same conditions.

Hydrophobic interaction. A Poros ET10 column (4.6×50 mm, Perseptive Biosystems) was equilibrated in 10 mM HEPES, 5 mM 2-mercapto-ethanol, 0.5 M ammonium sulfate pH 7.4 (buffer E). The UDPase sample was diluted 10-fold with buffer E, centrifuged for 5 min at 15 000 *g* and loaded at 0.5 ml/min onto the column. Activity was eluted with a linear gradient from 100% buffer E to 100% buffer F (10 mM HEPES, 5 mM 2-mercaptoethanol, 10% sucrose, pH 7.4) over 20 ml at 0.5 ml/min. Fractions (0.5 ml) were collected.

Peptide sequencing

For N-terminal sequence analysis, purified ER-UDPase was alkylated with iodoacetamide, run on SDS–PAGE gels and transferred to immobilon-P membranes (Millipore). For internal sequence analysis, purified ER-UDPase was run on SDS–PAGE, gels were Coomassie-stained, and the band corresponding to the ER-UDPase was digested *in situ* with trypsin, peptides eluted, alkylated and separated by reverse phase HPLC as described in Hellman *et al*. (1995).

Recombinant DNA procedures

Recombinant DNA procedures were carried out according to standard protocols. For cDNA cloning, a first strand cDNA library was synthesized using total mouse liver RNA as template, primed with oligo(dT) and using superscript II reverse transcriptase (Gibco-BRL). After RNase H treatment, the cDNA was used as template in PCRs employing degenerate primers S92 (5′-athcaygtntayacntty-3′) and A273 (5′-gartcraana-tytcnccytc-3′). PCR products were cloned into pT7-Blue T-Vector (Novagen) and sequenced. Clones aa116990 and aa120757 were obtained from Genome Systems (St Louis, MO). The sequence from clone aa116990, described in Figure 4, was assigned DDBJ/EMBL/GenBank accession No. AJ238636. For detection of the mRNA corresponding to the ER-UDPase, total RNA from different tissues was prepared using Trizol Reagent (Gibco-BRL). First strand cDNA synthesis was primed with oligo dT using superscript II reverse transcriptase (Gibco-BRL). ER-UDPase was amplified using sense primer 372 (5′-aaacagggtgctga-gactg-3′) and antisense primer 133 (5′-tctctgagtccacgcc-3′). Both primers are located within the ORF and yield a 1063 bp product.

C-terminal epitope tagged ER-UDPase cDNA was constructed by PCR, using a C-terminal primer containing the codons for the last seven amino acids for ER-UDPase followed by the codons for the c-myc epitope ending in a stop codon. The sense primer contained the N-terminus of the ER-UDPase including the starting ATG. The final constructs were sequenced and cloned into vector pCDNA3.1 (Invitrogen). Transfected cells were incubated at 30°C for 24 h, after which cells were washed, fixed in 4% paraformaldehide and processed for immunofluorescence.

Acknowledgements

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