Cloning, expression and characterization of a mammalian Nudix hydrolase-like enzyme that cleaves the pyrophosphate bond of UDP-glucose

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A distinct UDP-glucose (UDPG) pyrophosphatase (UGPPase, EC 3.6.1.45) has been characterized using pig kidney (*Sus scrofa*). This enzyme hydrolyses UDPG, the precursor molecule of numerous glycosylation reactions in animals, to produce glucose 1-phosphate (G1P) and UMP. Sequence analyses of the purified enzyme revealed that, similar to the case of a nucleotide-sugar hydrolase controlling the intracellular levels of ADP-glucose linked to glycogen biosynthesis in *Escherichia coli* [Moreno-Bruna, Baroja-Fernández, Muñoz, Bastarrica-Berasategui, Zandueta-Criado, Rodríguez-López, Lasa, Akazawa and Pozueta-Romero (2001) Proc. Natl. Acad. Sci. U.S.A. **98**, 8128–8132], UGPPase appears to be a member of the ubiquitously distributed group of nucleotide pyrophosphatases designated Nudix hydrolases. A complete cDNA of the UGPPase-encoding gene, designated *UGPP*, was isolated from a human thyroid cDNA library and expressed in *E. coli*. The resulting cells accumulated a protein that showed kinetic properties identical to those of pig UGPPase.

Key words: gluconeogenesis, glycogen, nucleotide-sugar.

INTRODUCTION

Being a predominant nucleotide-sugar in mammalian cells, UDP-glucose (UDPG) acts as the sugar donor in numerous glycosylation reactions, including those involved in the production of glycogen [1–3]. In spite of the fact that UDPG is synthesized by UDPG pyrophosphorylase (UGPase) [2], it is widely accepted that glycogen biosynthesis is subject to multiple controls such as reversible phosphorylation of some gluconeogenic enzymes, and allosteric regulation by the gluconeogenic intermediate glucose 6-phosphate [4,5]. Furthermore, there is an increasing body of experimental evidence indicating the operation of a regulatory gluconeogenic cycling process wherein synthesis and breakdown of glycogen take place concurrently [5–7].

Simultaneous synthesis and degradation of glycogen and starch have also been reported to occur in bacteria and plants, respectively [8–11], thus indicating that gluconeogenic cycling may entail advantages such as channelling of excess metabolic intermediates towards various pathways in response to physiological and biochemical needs. In this context, it should be emphasized that enzymes catalysing the breakdown of gluconeogenic intermediates such as nucleotide-sugars have been implicated to play a key role in connecting gluconeogenesis with other metabolic pathways [12–16].

Enzymes catalysing the hydrolytic breakdown of UDPG have been reported to occur in mammalian cells [17–19]. Having suggested roles such as control of the levels of nucleotide-sugars linked to glycosylation reactions [14,20,21], interruption of the insulin signal-transduction pathway [22–24] and generation of PP_i necessary for soft-tissue calcification and bone mineralization [25,26], these enzymes are nucleotide pyrophosphatases/phosphodiesterases that show broad substrate specificity and are associated with membrane particles. Some of them have been denoted 'ectoenzymes' because their active sites face the external medium rather than the cytosol [26–31].

Cells having high activity of membrane-bound nucleotide pyrophosphatases/phosphodiesterases have normal levels of UDPG, suggesting that these enzymes do not have access to the major cytoplasmic UDPG pool. Because glycogen biosynthesis takes place in the cytosol, the possible involvement of enzymic breakdown of UDPG in controlling carbon flow towards glycogen in mammalian cells has prompted us to identify a soluble protein, designated UDPG pyrophosphatase (UGPPase), that hydrolyses UDPG. The possible involvement of UGPPase in regulating gluconeogenesis is discussed.

EXPERIMENTAL

UGPPase purification

For purification of UGPPase, 3.6 kg of pig kidney were homogenized with 12 l of extraction buffer (50 mM Hepes, pH 7.0/2 mM dithiothreitol/2 mM EDTA) using a Waring blender. The homogenate was filtered through four layers of Miracloth and centrifuged at 30000 ***g*** for 30 min. The supernatant was dialysed against 1 mM dithiothreitol/2 mM β-mercaptoethanol and centrifuged at 100000 ***g*** for 30 min. The supernatant was then applied to a Q-Sepharose Fast Flow column (Amersham Biosciences) and UGPPase eluted with a stepped gradient of 50 mM Tris/HCl (pH 8.0) plus 0–0.5 M NaCl. Active fractions were mixed with a hydroxyapatite resin (Seikagaku Corporation) and UGPPase eluted with 400 mM sodium hydrogen phosphate buffer (pH 7.0). Active fractions were loaded on to a Q-Sepharose HP HiLoad 26/10 column (Amersham Biosciences) and UGPPase eluted with a linear

Abbreviations used: G1P, glucose 1-phosphate; UDPG, UDP-glucose; UGPase, UDPG pyrophosphorylase; UGPPase, UDPG pyrophosphatase.

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The nucleotide sequence of the human *UGPP* cDNA will appear in the DDBJ Nucleotide Sequence Database under accession number AB087802.

Table 1 Purification of UGPPase from pig kidney

Step numbers correspond to lane numbering in Figure 1.

Step no.	Purification step	Total volume (ml)	Total protein (mg)	Total activity (units) (m-units/mg of protein)	Specific activity	Yield (%)	Purification (fold)
1	Supernatant, 30000 ***g***	10000	131000	187.22	1.4	100	–
2	Q-Sepharose	1600	2352	19	8.2	11	6
3	Hydroxyapatite	1800	1062	18	17.4	10	12
4	Q-Sepharose	110	219	13	61	7	43
5	Superdex 200	250	90	16	178	8	125
6	MonoQ	22	42	12	284	6	199
7	MonoP	3	7	6	862	3	603
8	Native PAGE	2.5	0.07	2	31286	1	21900

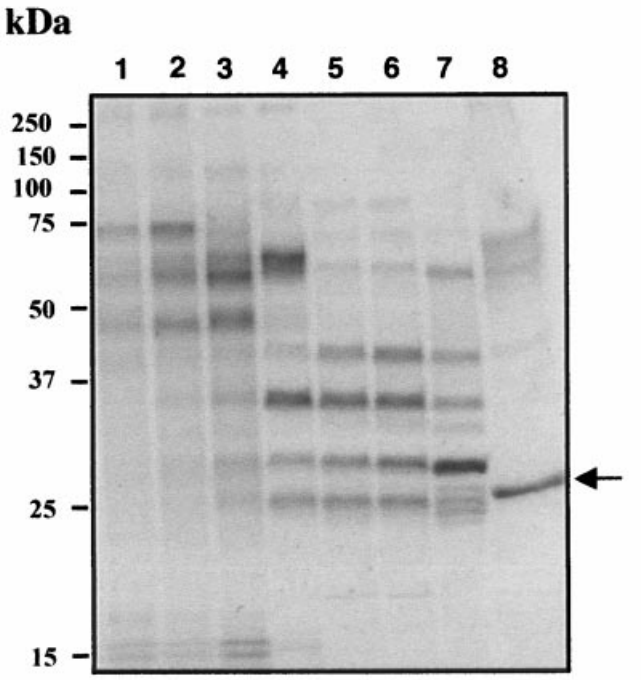

Figure 1 SDS/PAGE (10% gels) and Coomassie Blue staining of protein samples after various purification steps corresponding to those in Table 1

In each lane 3 μg of protein was loaded on to the gel. A precision protein standard kit from Bio-Rad containing 10 highly purified recombinant proteins (Bio-Rad catalogue no. 161-0362) was used as a reference.

gradient of 40 mM Tris/HCl (pH 8.0) plus 0–0.5 M NaCl. Fractions showing UDPG hydrolytic activity were subjected to gel filtration in a Superdex 200 column (Amersham Biosciences) equilibrated with 50 mM Hepes, pH 7.5/0.2 M NaCl. Fractions containing the partially purified enzyme were loaded on to a HR5/5 MonoQ column (Amersham Biosciences) equilibrated with 50 mM Tris/HCl (pH 8.0) and eluted with a 30 ml linear gradient of 0–1 M KCl in 40 mM Tris/HCl (pH 8.0). Fractions showing UGPPase activity were loaded on to a MonoP HR5/20 column (Amersham Biosciences) and UGPPase eluted with a linear gradient of 50 mM Tris/HCl (pH 9.0) plus 0–0.5 M NaCl. Enzymically active fractions were then subjected to electrophoretic separation in a polyacrylamide/Tris/glycine native system. After electrophoresis, the gel was cut into pieces that were immersed for 12 h in 10 mM Tris/HCl, pH 7.4/10 mM β-mercaptoethanol/500 mM NaCl.

The molecular mass of the native UGPPase was determined from a plot of K_{av} (partition coefficient) versus log(molecular mass) of protein standards subjected to gel filtration in a Superdex 200 column. Protein content was measured by the Bradford method using a Bio-Rad prepared reagent.

Isoelectrofocusing

Purified UGPPase was examined by isoelectrofocusing using Amersham Biosciences narrow-range (pH 4.5–6.0) polyacrylamide plates in a Multiphor II system. As standards, a Bio-Rad protein mixture was utilized. After electrophoresis, the gel was cut and UGPPase eluted at 4 °C in 10 mM Tris/HCl, pH 7.4/10 mM β-mercaptoethanol/500 mM NaCl.

Enzyme assays

Measurements of UGPPase activities on UDPG were performed by using the two-step spectrophotometric determination of glucose 1-phosphate (G1P) described by Rodríguez-López et al. [15] but in 'step one' the reaction mixture contained 50 mM Tris/HCl, pH 9.0, 10 mM $MgCl_2$, 5 mM UDPG and protein extract. Measurements of UGPPase activities on other nucleotides was performed chromatographically using a Waters Associates HPLC system fitted with a Partisil-10-SAX column (4.6 mm × 25 cm), as described elsewhere [15]. UGPase was determined as described by Ciereszko et al. [32].

One unit was defined as the amount of enzyme that catalysed the production of 1 μmol of product/min. Kinetic parameters such as K_m and V_{max} were evaluated by Lineweaver–Burk plots.

Protein sequence analyses

Purified UGPPase was separated electrophoretically by SDS/PAGE (10% gels) and subsequently subjected to internal amino acid sequencing by MS on Micromass Q-TOF at the Australian Proteome Analysis Facility, Macquarie University, Sydney, Australia.

Cloning and expression of a human *UGPP* cDNA

A complete UGPPase-encoding cDNA was amplified by PCR by using a human thyroid cDNA library and two primers specific for the human UGPPase-encoding gene, *UGPP* (NCBI accession number LOC122643): 5′-CATATGGAGCGCATCGAGGGG-

Table 2 Substrate specificity of UGPPase

All substrates were present at a concentration of 5 mM. b.d.l., below detection limit; Ap_nA, diadenosine 5′,5‴-P^1,P^n-oligophosphate, where $n = 2$, 3 or 4; PNPP, *p*-nitrophenyl phosphate.

	Substrate specificity (% activity relative to UDPG)	
Substrate	Pig UGPPase	Recombinant human UGPPase
UDPG	100	100
ADP-ribose	95	130
ADP-glucose	20	15
GDP-glucose	10	10
CDP-glucose	2	3
UDP-glucuronic acid	b.d.l.	b.d.l.
UDP-galactose	b.d.l.	b.d.l.
ATP	b.d.l.	b.d.l.
UTP	b.d.l.	b.d.l.
AMP	b.d.l.	b.d.l.
UTP	b.d.l.	b.d.l.
ADP	b.d.l.	b.d.l.
NAD	b.d.l.	b.d.l.
Ap_2A	b.d.l.	b.d.l.
Ap_3A	b.d.l.	b.d.l.
Ap_4A	b.d.l.	b.d.l.
CoA	b.d.l.	b.d.l.
Bis-PNPP	b.d.l.	b.d.l.
PNPP	b.d.l.	b.d.l.

GCGTCCGT-3′ (forward) and 5′-GGATCCTCACTGGAGA-TCCAGGTTGGGGGCCA-3′ (reverse).

The cDNA was then cloned into the pT7Blue T-vector (Novagen) to create pUGPP, and introduced into *Escherichia coli* (JM109).

For *UGPP* overexpression experiments, the *Nde*I/*Bam*HI fragment of pUGPP was ligated with the corresponding restriction sites of the pET11a expression vector (Novagen) to create pET-UGPP. *E. coli* AD494(DE3) (Novagen) transformed with this plasmid were grown at 37 °C in 1 l of LB medium with 50 μg/ml ampicillin to an attenuance at 600 nm of about 0.6, and then 1 mM isopropyl β-D-thiogalactoside was added to the culture medium. After 3 h of growth the induced bacteria were harvested, washed and resuspended in 100 ml of 50 mM Tris/HCl (pH 8.5)/1 mM dithiothreitol/2 mM β-mercaptoethanol, and lysed by sonication on ice. After centrifugation at 10000 ***g***, the supernatant was recovered and subjected to the same purification protocol of pig UGPPase.

RESULTS AND DISCUSSION

Identification of a soluble UDPG hydrolytic activity

The spectrophotometric measurement of the UGPase in the pyrophosphorolysis direction is a commonly used method that is

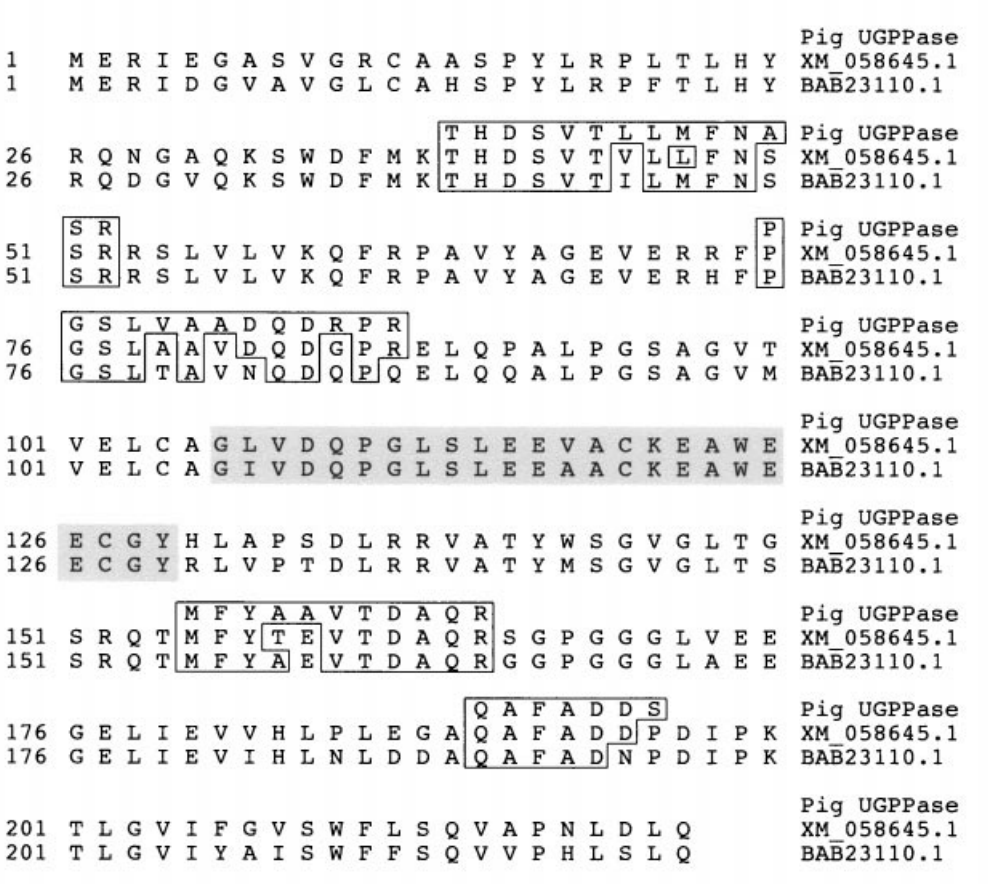

Figure 2 Alignments of internal pig UGPPase amino acid sequences with human and mouse 'putative proteins' that contain a Nudix box (NCBI accession no. XM_058645.1 and EMBL accession no. BAB23110.1, respectively)

The numbers to the left are the amino acid positions in each complete sequence. Amino acid identities with the pig UGPPase are boxed and the Nudix sequence signature is shaded.

based on the coupling of G1P production with an accompanying reduction of NAD^+ [32,33]. Surprisingly, while monitoring UGPase activities using the 100000 ***g*** supernatant of a kidney homogenate, we were able to detect the UDPG-dependent production of G1P when PP_i was not included in the reaction mixture (results not shown). These results thus strongly indicated the occurrence of a soluble enzyme catalysing the hydrolytic breakdown of UDPG.

Enzyme purification

We then attempted to purify the enzyme molecule responsible for this activity. As presented in Table 1, it was purified by over 20000-fold. Its isoelectric point was shown to be 4.7. SDS/PAGE and subsequent staining of the purified protein revealed a single band of about 28 kDa (Figure 1). Its apparent molecular mass measured by gel filtration was estimated to be 50–60 kDa, indicating that the enzyme responsible for UDPG breakdown is a homodimer.

Substrate specificity

Substrate specificity was tested using a wide range of compounds at a concentration of 5 mM. These analyses showed that the enzyme is a pyrophosphatase that hydrolyses UDPG and ADP-ribose to produce G1P plus UMP, and ribose 5-phosphate plus AMP, respectively (Table 2). The enzyme poorly hydrolyses other nucleotide-sugars such as ADP-glucose, CDP-glucose, GDP-glucose and GDP-mannose. It does not recognize PP_i, synthetic phosphodiester-bond-containing compounds such as bis-*p*-nitrophenyl phosphate, diadenosine polyphosphates, CoA or phosphomonoester-bond-containing compounds such as *p*-nitrophenyl phosphate, sugar-phosphates and nucleotide mono-, di- and triphosphates.

Mg^{2+} was shown to be absolutely required for optimal activity and could not be replaced with 0.2–5 mM Mn^{2+} (results not shown). No activity was observed in the absence of Mg^{2+} or in the presence of 1 mM EDTA in the reaction mixture. With UDPG as a substrate, the enzyme was shown to display optimal activity at alkaline pH (8.0–9.5).

Kinetic parameters and enzyme designation

Kinetic parameters were determined with UDPG and ADP-ribose as substrates. K_m and V_{max} values for UDPG were 0.6 mM and 8 units/mg of protein, respectively, whereas K_m and V_{max} values for ADP-ribose were 1.7 mM and 5.7 units/mg of protein, respectively. Taking into account these kinetic parameters and considering that (i) the V_{max}/K_m for UDPG is 13.3 units $\cdot$ $mg^{-1} \cdot mM^{-1}$ whereas that for ADP-ribose is 3.3 units $\cdot$ $mg^{-1} \cdot mM^{-1}$, (ii) the intracellular concentrations of UDPG in mammalian cells range between 0.1 and 1 mM [3,4], (iii) the intracellular ADP-ribose concentration in mammalian cells is in the micromolar range [34] and (iv) mammalian cells possess various enzymes that actively and specifically cleave ADP-ribose [35,36], it is highly conceivable that the physiological substrate of the enzyme described in this study is UDPG rather than ADP-ribose. Therefore we propose that the enzyme should be designated UGPPase.

UGPPase is a member of the Nudix hydrolases

Purified UGPPase was subjected to peptide sequencing. Multiple alignments against sequences existing in data banks showed that UGPPase shares high sequence similarity to 'hypothetical' or 'unknown' proteins from human and mouse (NCBI accession number XM_058645.1 and EMBL accession number BAB23110.1, respectively), which have been classified as members of a previously characterized group of proteins called 'Nudix' (nucleoside diphosphate linked to some other moiety, X) hydrolases that exist in organisms ranging from viruses to mammals [37] (Figure 2).

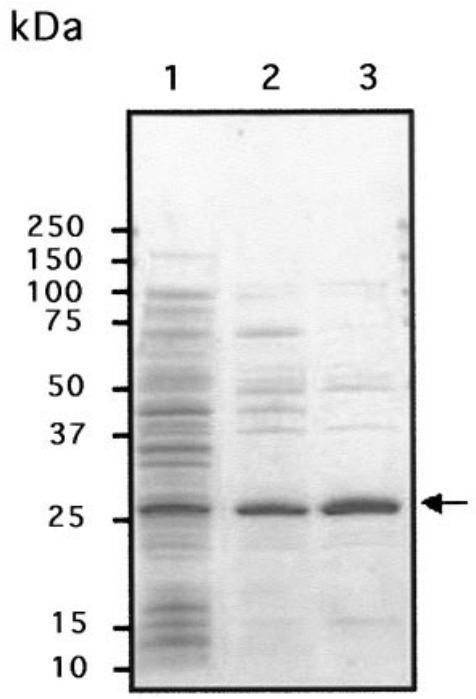

Figure 3 SDS/PAGE and Coomassie Blue staining of protein samples after various purification steps of a recombinant UGPPase

Lane 1, crude extract of *E. coli* overexpressing a complete human *UGPP* cDNA (1.8 m-units); lane 2, Q-Sepharose step (6.2 m-units); lane 3, MonoP purification step (13.5 m-units).

cDNA cloning and gene designation

By using primers specific for the human genomic sequence encoding the 'unknown protein' (NCBI accession number LOC122643) a complete cDNA was amplified from a thyroid cDNA library and cloned into the pET11a expression vector of *E. coli*. The resulting plasmid (pET-UGPP) was used to transform *E. coli* AD494(DE3). These cells were found to accumulate an approx. 28 kDa protein that, once purified (Figure 3), showed properties identical to those of pig UGPPase, i.e. it hydrolyses UDPG (Table 2), has optimal activity at alkaline pH and has an absolute Mg^{2+} requirement (results not shown). These results thus demonstrate that the LOC122643 genomic sequence is indeed an UGPPase-encoding gene which, following recent guidelines for human gene nomenclature [38], will be designated as *UGPP* in this and following work.

Structural divergences of the Nudix signature sequence

The Nudix hydrolases comprise a large family of proteins that are characterized by the following highly conserved array of 23 amino acids, Gly-Xaa_5-Glu-Xaa_7-Arg-Glu-U-Xaa-Glu_2-Xaa-Gly-U, where U represents a bulky, hydrophobic amino acid (usually Ile, Leu or Val). Although this highly conserved Nudix motif has been shown to be essential for metal binding and

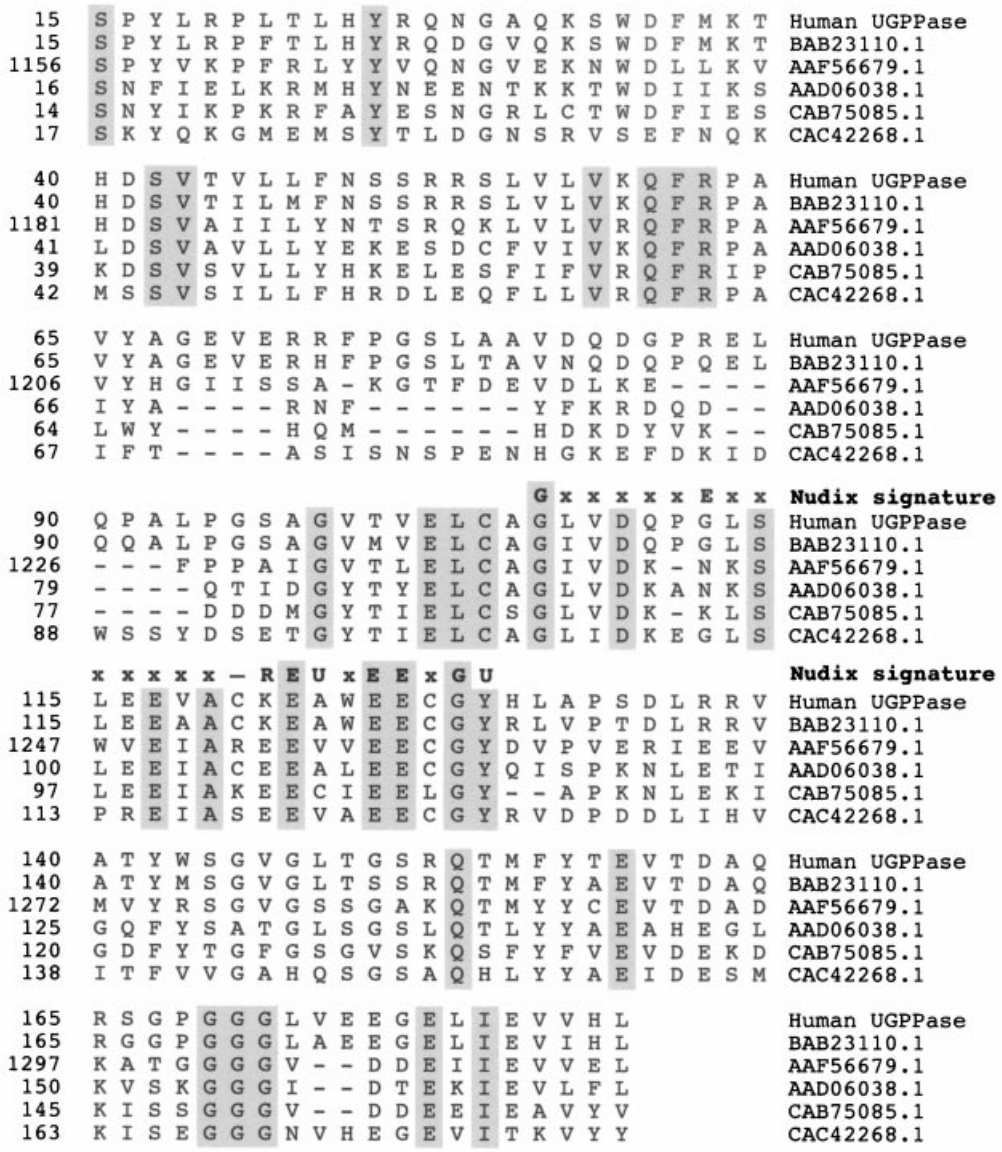

Figure 4 Amino acid sequence alignments

Amino acid sequence alignments are shown of the human UGPPase (NCBI accession number XM_058645.1) with other possible homologues containing Nudix-like sequence signatures from *Mus musculus* (EMBL accession number BAB23110.1), *Drosophila melanogaster* (AAF56679.1), *Helicobacter pylori* (AAD06038.1), *Campylobacter jejuni* (CAB75085.1) and *Caenorhabditis elegans* (CAC42268.1), and in comparison with the Nudix signature sequence (where x denotes a non-conserved residue). The numbers to the left are the amino acid positions in each complete sequence. Conserved amino acids are shaded.

pyrophosphatase activity [39], there are various examples of non-consensus motifs among the Nudix hydrolases, including extra amino acids and missing glutamate or glycine residues ([40], cf. Figure 3). As presented in Figure 4, both human and mouse UGPPases contain remarkably important modifications in the Nudix box, including the absence of the conserved Glu at position 7. Moreover, it is particularly noteworthy that Arg at position 15 is conservatively replaced by Lys. This is, to our knowledge, the first such case described in the literature. Whether or not the function of this basic residue is linked to the recognition of UDPG should be a matter for future research.

Computer searches of the data banks revealed the occurrence of other 'hypothetical proteins' from various species with Nudix-like motifs that were structurally similar to those of both human and mouse UGPPases, characterized by the following array of amino acids: Gly-Xaa$_2$-Asp-Xaa$_{3/4}$-Ser-Xaa$_2$-Glu-Xaa-Ala-Xaa$_2$-Glu-Xaa$_2$-Glu$_2$-Xaa-Gly-Tyr (Figure 4). These analyses also revealed additional conserved amino acid residues, distal from the signature.

The overall structural characteristics of UGPPase and other Nudix hydrolase-like nucleotide hydrolases thus indicate that the typical Nudix sequence is not a *sine qua non* requirement for both metal binding and pyrophosphatase activity.

Additional remarks

This is, to our knowledge, the first report of a soluble hydrolase showing notable specificity towards UDPG. Hydrolytic enzymes

cleaving UDPG have been shown to occur in bacteria that act as important factors affecting bacterial invasion of host cells [41–45]. However, these bacterial enzymes are known to be surface localized, possess a 5′-nucleotidase activity, and their amino acid sequences differ from that of the UGPPase reported in the present paper. Other bacterial enzymes catalysing the hydrolytic breakdown of UDPG are the products of *DR004* and *DR0329*, two open reading frames from *Deinococcus radiodurans* [46]. However, it is still unknown whether or not these enzymes can recognize other substrates.

Our computer-assisted analyses using the PSORT algorithm [47] have revealed that UGPPase has characteristics of a typical cytosolic protein of mammalian cells. It is therefore highly conceivable that this enzyme is in the same compartment of the cell as the established processes of UDPG synthesis and utilization. Taking into account that the intracellular concentration of UDPG in mammalian cells ranges between 0.1 and 1 mM [3,4] and that the K_m value of UGPPase for UDPG is 0.6 mM, it is tempting to speculate that UGPPase has a physiological relevance in preventing UDPG accumulation. Furthermore, considering that UGPPase competes with glycogen synthase for the same substrate, and that glycogen synthase has a K_m for UDPG in the millimolar range [48] it can be readily predicted that, unless UGPPase is highly regulated, it will prevent glycogen biosynthesis.

The occurrence of a Nudix hydrolase controlling the intracellular levels of ADPG linked to glycogen biosynthesis in *E. coli* [16] strongly evokes the idea that UGPPase may equally play a crucial role in both preventing the gluconeogenic process and in connecting it with other metabolic pathways in response to physiological and biochemical needs of the mammalian cell. However, a possibility cannot be ruled out that UGPPase may block the metabolic flux to other gluconeogenic processess such as glycoprotein and glycolipid biosynthesis wherein UDPG plays a rate-limiting role [1,2]. Needless to say, further research testing possible mechanisms regulating *UGPP* expression and/or UGPPase activity will be essential to elucidate the possible role of this enzyme in controlling gluconeogenesis in mammalian cells.

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