Molecular cloning of ERp29, a novel and widely expressed resident of the endoplasmic reticulum

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Abstract We have isolated a full-length cDNA clone for a novel 29 kDa protein that is highly expressed in rat enamel cells. The clone encodes a 259-residue protein, here named ERp29, with structural features (signal peptide and a variant endoplasmic reticulum-retention motif, KEEL) that indicate it is a reticuloplasmin. ERp29 has limited homology with protein disulfide isomerase and its cognates, but lacks their characteristic thioredoxin-like catalytic moiety and calcium-binding motifs. ERp29 mRNA was expressed in all rat tissues tested, and a homologous transcript was detected in other animal livers (primate, ruminant, marsupial). In human hepatoma cells, ERp29 mRNA expression was not increased by stresses (tunicamycin, calcium ionophore) that induced other reticuloplasmins. We conclude that ERp29 is a new, highly conserved member of the reticuloplasmin family which is widely expressed. The apparent lack of both calcium binding properties and stress responsiveness distinguish ERp29 from all major reticuloplasmins characterised to date.

Key words: Endoplasmic reticulum; Reticuloplasmin; Protein disulfide isomerase; Stress response; Enamel cell

1. Introduction

The endoplasmic reticulum (ER) has a well characterised role in biomolecular synthesis (e.g. assembly and modification of proteins, lipid synthesis), and more recently was recognised as a site of protein degradation [1]. Several of these functions depend on the high (mM) levels of calcium normally found in the ER lumen [2,3]. The ER also functions as a mobilisable calcium store that sequesters excess cytosolic calcium and acts as a reservoir for calcium signalling [2,4].

Proteins that reside in the ER lumen, termed reticuloplasmins [2], are characterised by a four-residue ER-retention motif (usually KDEL) at the carboxyl-terminus. Interaction of KDEL with a specific receptor is thought to prevent export of reticuloplasmins through the secretory pathway [5,6]. An amino-terminal signal peptide enables entry of reticuloplasmin preproteins into the ER. Reticuloplasmins that are highly expressed in many tissues include calreticulin, endoplasmin (grp94, ERp99), protein disulfide isomerase, and BiP (grp78), all of which are calcium-binding proteins [2,7–9]. Multiple roles have been ascribed to these major reticuloplasmins, but growing evidence suggests that they might share common functions as molecular chaperones during protein assembly and degradation [10–12], and as calcium buffers [8,9,13]. Supporting this notion, several reticuloplasmins are coordinately up-regulated in response to cellular stresses that disrupt protein secretion or ER calcium levels [8,14–16].

Enamel cells first secrete then hypermineralize dental enamel, the most highly calcified tissue (≈60% calcium by mass). Recently, we found that rat enamel cells express unusually high amounts of several ER proteins, namely reticuloplasmins (calreticulin, endoplasmin, protein disulfide isomerase and BiP), the inositol trisphosphate-gated calcium channel and a SERCA-class calcium pump ([13,17] and unpublished results). The present study arose from our detection of a novel 29 kDa protein in enamel cell extracts, using internal microsequencing of a two-dimensional gel spot as before [13,17,18]. The 29 kDa protein was abundant (≈0.2% of soluble protein) and appeared to reside in the lumen of an organelle, based on its behaviour during subcellular fractionation and cell permeabilisation experiments (M.J.H. et al., unpublished results). Here, we have isolated and characterised a cDNA clone for this protein, which we now term ERp29. Our findings indicate that ERp29 is a novel and highly conserved member of the reticuloplasmin family, that is widely expressed in animal tissues.

2. Materials and methods

2.1. RNA isolation

Enamel epithelium was microdissected from rat mandibular first molars at secretion and maturation phases (5- and 10-day-old pups, respectively) and immediately frozen [18]. RNA was isolated by the guanidinium-acid-phenol-chloroform method [19], yielding 30–40 μg total RNA from 10 epithelia. The same method was used for the other tissues and, where indicated, poly(A)$^+$ RNA was prepared by chromatography on oligo(dT)-cellulose [20]. For the stress response experiments, RNA was isolated following direct addition of guanidinium solution to the washed, attached cells.

2.2. RT-PCR

Random primed cDNA was synthesised from total RNA (2 μg) using Superscript II reverse transcriptase (Gibco/BRL), and a portion (typically 1%) was used as the template for RT-PCR. The degenerate primers used to isolate the 270 bp ERp29 PCR product were: forward, 5′-TTGGATCCGARAAYCC(G/A/T)GTNCCNTA-3′; reverse, 5′-TTGAATTCGAARTCYTCNCCYTGRTC-3′. PCR products were amplified using HotTub polymerase and buffers (Amersham) and the following amplification profile for 35 cycles: 20 s/94°C, 30 s/45°C, 60 s/72°C. PCR products were cloned in the pBluescript-ks$^+$ vector (Stratagene) and both strands sequenced manually with a T7 DNA polymerase sequencing kit (Pharmacia) or with an Applied Biosystems Model 373 sequencer (Centre for Gene Research, Otago University).

2.3. cDNA cloning

An oligo(dT)-primed cDNA library was made using enamel cell total RNA (10 μg, secretion phase) and the ZAP-Express cDNA Synthesis and Cloning kit (Stratagene), except that the reverse transcriptase used was Superscript II (Gibco/BRL). The enamel cell cDNA library was amplified and screened with the ^{32}P-labelled (Rediprime,

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Abbreviations: ER, endoplasmic reticulum; RT, reverse transcription; PCR, polymerase chain reaction; kb, kilobase(s); bp, base pair(s).

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from Amersham) 270 bp ERp29 PCR product, using standard procedures [21]. Recombinants were isolated as single plaques, and the internal pBK-CMV plasmids excised with the ExAssist (Stratagene) helper phage. Automated DNA sequencing was done in both directions, as above.

2.4. Northern blot analysis

RNA was resolved by gel electrophoresis (1% agarose, 0.6 M formaldehyde) and transferred to Hybond N membrane (Amersham). Blots were hybridised overnight at 60°C with ^{32}P-labelled DNA probes in either Heparin or Denhardt's solution, following prehybridisation under the same conditions for 1 h [20]. Blots were washed twice with 2×SSPE (360 mM NaCl, 20 mM sodium phosphate, 2 mM EDTA, pH 7.7) and 0.5% SDS (60°C, 15 min), once with 1×SSPE and 0.1% SDS (60°C, 15 min) and then visualised by autoradiography at −80°C (X-OMAT AR film and double intensifying screens, from Kodak) or with a phosphor-imager (Fuji BAS-1500).

2.5. Reticuloplasmin cDNA probes

PCR products (427, 624, and 577 bp for BiP, ERp72 and protein disulfide isomerase, respectively) were amplified from HepG2 cDNA using the following primers: BiP, forward 5′-CAGATTGAGAAGTCACCTTTGAG-3′, reverse 5′-CACTTCCATAGAGTTTGCTGAT-3′; ERp72, forward 5′-GTGGACTTCAGCTTTGATTAC-3′, reverse 5′-GCTCAAATGCTCCAGATCTCT-3′; protein disulfide isomerase, forward 5′-AAGATCCTGTTCATCTTCATCG-3′, reverse 5′-ATCCTGGCCACCGCTCTC-3′.

2.6. Other methods and materials

Wistar-derived rats were kept as before [18]. The ovine liver sample was from a Coopworth, and the marsupial liver was from a feral Australian brush-tailed possum (*Trichosurus vulpecula*). HepG2 cells (ATCC HB 8065), a gift from Dr Michael Eccles of this Department, were maintained conventionally in DMEM supplemented with 10% fetal calf serum and 10 μg ml^{-1} transferrin (Gibco/BRL). Tunicamycin was from Boehringer Mannheim, and calcium ionophore A23187 from Sigma. Rat liver microsomes were prepared with osmotic stabiliser (250 mM sucrose) [10] and subjected to denaturing two-dimensional gel electrophoresis [18]. Following electroblotting of ERp29 on polyvinylidinedifluoride membrane (ProBlott, from Applied Biosystems), the amino-terminus was microsequenced as described [13].

Structural and homology analyses were carried out using standard programs available locally or accessed through the Internet, as follows: secondary structure – Chou and Fasman, and Garnier methods; signal peptide analyses – PSORT (NIBB, Japan), Signalp (CBSA, Denmark); functional motifs – PROSITE, NetOglyc (CBSA, Denmark); homology – BLAST, FASTA; alignments and identity scores – ALIGN; statistical significance of relatedness – RDF2 Monte Carlo shuffle analysis. Additional comparative analyses of ERp29 and protein disulfide family members (data not shown) were done with DIAGON and ARGOS.

3. Results and discussion

3.1. Cloning of ERp29

We obtained the partial amino acid sequence of a major soluble protein (29 kDa, p*I* 6.4) from rat enamel cells, now referred to as ERp29, following denaturing two-dimensional gel electrophoresis, in-gel tryptic digestion and reverse-phase high-performance liquid chromatography (M.J.H., N.J. McHugh and D.L. Carne, unpublished results). Two tryptic peptide sequences were used to design highly degenerate PCR primers for both the sense and antisense DNA strands. Using cDNA from secretion phase enamel cells, one primer combination gave a 270 bp PCR product that contained these peptide sequences and that of a third ERp29 tryptic peptide, in frame (Fig. 1A). The PCR product was used to screen an enamel cell cDNA library (200 000 plaques) and five putative ERp29 clones were isolated. One of these clones (pRnERp29, 1084 bp insert) had a single open reading frame that encoded a protein of the approximate size expected, that is 260 residues including the initiator methionine. The predicted protein structure (Fig. 1A) was identical to those regions (74% of mature protein) determined by microsequencing and mass spectrometry of ERp29 tryptic peptides (M.J.H. et al., unpublished results). We conclude that clone pRnERp29 contains a full coding cDNA for ERp29. The four other clones appeared to contain truncated forms of ERp29, based on partial sequence analysis (data not shown).

3.2. Structure of ERp29

Secondary structure analyses indicated that ERp29 is generally hydrophilic, but with a strongly hydrophobic amino-terminus (Fig. 1B). The hydrophobic region (i.e. first 32 residues) was identified as a probable signal peptide, using both the neural network and weighted matrix computational approaches. Furthermore, the predicted mature ERp29 sequence from residue 33 coincided exactly with the amino-terminal 20 residues of an unknown 24.5 kDa protein (spot 35) isolated from human liver [22]. When rat liver microsomes were subjected to two-dimensional gel electrophoresis, we found a major protein spot with the same electrophoretic mobility as enamel cell ERp29 and with the amino-terminal sequence, LHTKGALPLD.... (data not shown). Together, these findings indicate that ERp29 is translated as a preprotein, and are consistent with the observed solubility of mature ERp29 which lacks the 32-residue signal peptide. Mature ERp29 (Fig. 1A) comprises 228 residues with a theoretical mass of 25 609 Da and a mildly acidic net charge, p*I* 6.0, similar to the values (29 kDa, p*I* 6.4) observed in denaturing two-dimensional gel electrophoresis.

A known variant ER-retention motif, KEEL [5,23], is present at the carboxyl-terminus of ERp29 (Fig. 1A), which suggests that ERp29 is a reticuloplasmin. In other studies, we have found that ERp29 partitions with established reticuloplasmins (calreticulin, endoplasmin, protein disulfide isomerase, BiP) during a variety of subcellular fractionation procedures, both in enamel cells and in liver. For example, following mechanical disruption of liver, ERp29 and the reticuloplasmins were largely insoluble (microsomal fraction) when the homogenisation buffer contained an osmotic stabiliser but soluble when osmotic stabiliser was omitted. In unbroken enamel cells permeabilised with the detergent digitonin, ERp29 was retained (i.e. insoluble) following selective release of cytosolic proteins at a low digitonin concentration. At moderate levels of digitonin, ERp29 was solubilised in parallel with the reticuloplasmins whereas solubilisation of a mitochondrial matrix protein required more digitonin (M.J.H., N.J. McHugh and D. Verhamme, unpublished re-

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Fig. 1. ERp29 structure. Clone pRnERp29 was isolated from a rat enamel cell cDNA library and sequenced, as described in Section 2. (A) Nucleotide and deduced amino acid sequences, numbered from the Met that conforms to the consensus translation initiation site [36] and with the poly(A) tail omitted. The predicted signal peptide cleavage site (arrowhead), 270 bp PCR product (box) and microsequenced tryptic peptides (underlined) referred to in the text, and a consensus polyadenylation signal (bold), are indicated. These sequence data have been submitted to the EMBL/Genbank/DDBJ (accession Y10264) and SWISS-PROT (accession P80749) databases. (B) Secondary structure analyses of ERp29, evaluating the propensity for hydrophobicity, alpha helix and random coil, as indicated.

A

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cacaatccggcgtg ATG GCT GCC GCC GTT CCT GGC GCC GTT TCG CTC TCC CCA CTG CTG TCC   48
               M   A   A   A   V   P   G   A   V   S   L   S   P   L   L   S   16

GTT CTC CTT GGC CTG CTG CTC CTC TCT GCT CCT CAT GGC GCC AGC GGC CTG CAC ACG AAG  108
V   L   L   G   L   L   L   L   S   A   P   H   G   A   S   G   L   H   T   K   36

GGC GCC CTT CCC TTG GAC ACA GTC ACT TTC TAC AAG GTC ATT CCC AAA AGC AAG TTC GTC  168
G   A   L   P   L   D   T   V   T   F   Y   K   V   I   P   K   S   K   F   V   56

TTG GTG AAG TTC GAC ACC CAG TAC CCC TAT GGA GAG AAG CAA GAT GAG TTT AAG CGT CTG  228
L   V   K   F   D   T   Q   Y   P   Y   G   E   K   Q   D   E   F   K   R   L   76

GCT GAG AAC TCA GCC TCC AGC GAT GAT CTC TTG GTG GCA GAG GTG GGG ATC TCA GAC TAT  288
A   E   N   S   A   S   S   D   D   L   L   V   A   E   V   G   I   S   D   Y   96

GGT GAC AAG CTG AAC ATG GAG CTG AGT GAG AAG TAC AAG CTG GAC AAA GAG AGC TAC CCA  348
G   D   K   L   N   M   E   L   S   E   K   Y   K   L   D   K   E   S   Y   P  116

GTC TTC TAC CTC TTC CGG GAT GGG GAC TTT GAG AAT CCT GTC CCA TAC AGC GGG GCA GTT  408
V   F   Y   L   F   R   D   G   D   F   E   N   P   V   P   Y   S   G   A   V  136

AAA GTT GGA GCC ATC CAG CGC TGG CTC AAG GGG CAG GGA GTC TAT CTG GGC ATG CCT GGA  468
K   V   G   A   I   Q   R   W   L   K   G   Q   G   V   Y   L   G   M   P   G  156

TGT CTG CCT GCG TAC GAT GCC CTG GCG GGC CAG TTC ATC GAG GCC TCC AGC AGA GAG GCC  528
C   L   P   A   Y   D   A   L   A   G   Q   F   I   E   A   S   S   R   E   A  176

CGC CAG GCC ATC CTG AAA CAG GGG CAG GAT GGC CTC TCA GGT GTG AAG GAG ACA GAC AAG  588
R   Q   A   I   L   K   Q   G   Q   D   G   L   S   G   V   K   E   T   D   K  196

AAG TGG GCC AGT CAG TAC CTG AAG ATC ATG GGG AAG ATC TTG GAC CAA GGC GAA GAC TTC  648
K   W   A   S   Q   Y   L   K   I   M   G   K   I   L   D   Q   G   E   D   F  216

CCG GCC TCC GAG CTG GCC CGG ATC AGT AAG CTC ATT GAG AAC AAG ATG AGT GAG GGT AAG  708
P   A   S   E   L   A   R   I   S   K   L   I   E   N   K   M   S   E   G   K  236

AAG GAA GAG CTG CAG AGG AGC CTC AAC ATC CTC ACC GCC TTC CGC AAG AAA GGC GCC GAG  768
K   E   E   L   Q   R   S   L   N   I   L   T   A   F   R   K   K   G   A   E  256

AAG GAG GAG CTG TGA gggcaccaggctgctagggtttgtgagggcggggagggagagccgcctgctgactgtgg  842
K   E   E   L   *                                                              260

ggccctgtgggtggaggggcagtggagcaaagcagtcctgagccagagacctgtgccccgagtgcctggacactgagg  921
ctgctgagaccataccctggacatccttggagcagtttgtggggagcctgtgtgccaagacaagatggctgtgccccca 1000
caggagctggtactatagaggaaggtcctgacaggtatagttctgatcaataaaaagtctgttttggtt           1070
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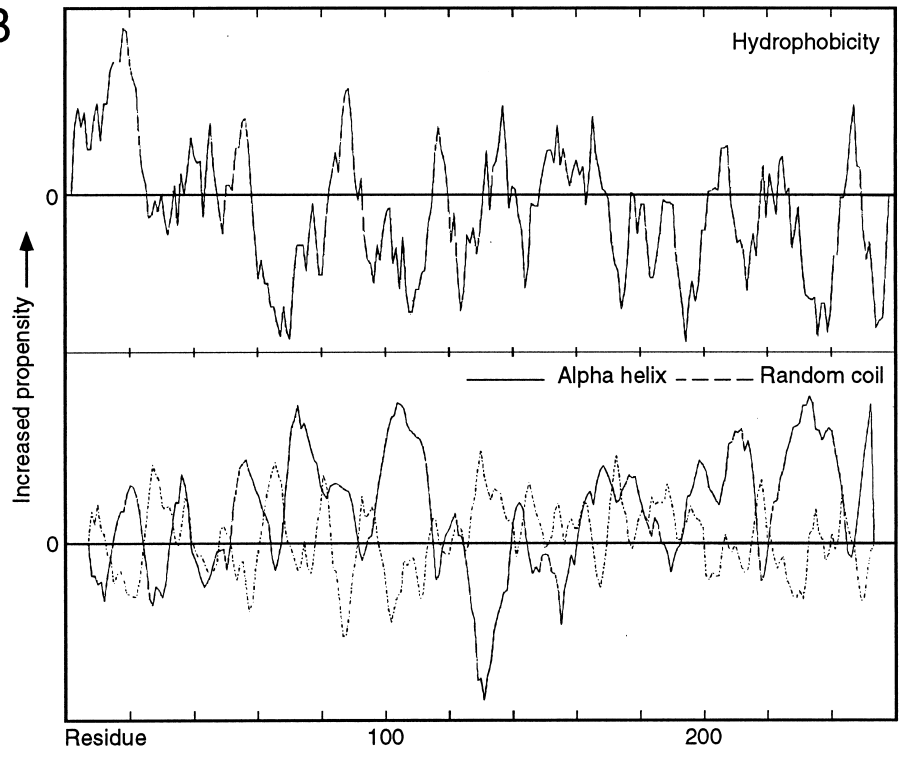
B



sults). Together, the structural features (KEEL motif, signal peptide) and the subcellular fractionation properties strongly support the identification of ERp29 as a reticuloplasmin. However, it remains to demonstrate ERp29 localisation within the ER directly (e.g. by immunocytochemistry). Two established rat reticuloplasmins also do not have the classical KDEL motif (KEEL in ERp72, QEDL in ERp60 [24,25]). Interestingly, the KEEL sequence is repeated 20 residues from the carboxyl-terminus of ERp29 (Fig. 1A), and similar sequences exist in reticulocalbin and HSP-47 (i.e. KEEI and REEL, 25 and 42 residues from the carboxyl-terminus, respectively [26,27]). It remains to be established whether these additional motifs serve as back-up ER-retention signals, for example after being exposed by carboxyl-terminal proteolysis within the ER.

ERp29 lacks the calcium-binding motifs (i.e. low affinity acidic residue clusters and high affinity EF-hands) that are present in many reticuloplasmins [8,9,24,26,28], which is consistent with our failure to detect ERp29 as a calcium-binding protein in ^{45}Ca-overlays [13,17]. ERp29 does not contain predicted glycosylation sites, unlike some reticuloplasmins (e.g. endoplasmin, HSP-47, ERp72 and calreticulin [2,24,27,29]). Secondary structure analyses indicated a high alpha-helical potential throughout mature ERp29, except in a central region where a random coil was predicted (Fig. 1B). This pattern suggests that ERp29 might be folded as two globular halves, which we refer to hereafter as the N- and C-domains (Fig. 2), and is supported by the following homology data.

3.3. Homologues of ERp29

ERp29 appears to be the product encoded by two unpublished cDNA sequences submitted recently to the Genbank database. A human cDNA derived from adult liver (accession no. X94910) exhibits ≈90% deduced amino acid identity with ERp29 from rat enamel cells (Fig. 2). Interestingly, the structural differences are limited to the signal peptide and to the C-domain, where the putative human product also contains one extra residue. We infer that strict conservation of the N-domain between rat and human ERp29 (Fig. 2) reflects evolutionary pressure to maintain an important function. A rat

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Rat ERp29     MAAAVPGAVSLSPLLSVLLGLLLLSAPHGASGLHTKGALPLDTVIFYKVIPKS   53
Human X94910  ......R.AF.....PL...F........G.....................   53
Domain        Signal ──────────────────────┘ N──────────────────────

Rat ERp29     KFVLVKFDTQYPYGEKQDEFKRLAENSASSDDLLVAEVGISDYGDKLNMELSE  106
Human X94910  ........................................................  106
Domain        N──────────────────────────────────────────────────────

Rat ERp29     KYKLDKESYPVFYLFRDGDFENPVPYSGAVKVGAIQRWLKQQGVYLGMPGCLP  159
Human X94910  ..........................T.............................  159
Domain        N──────────────────────┘ C──────────────────────────────

Rat ERp29     AYDALAQQFTRASSREARQAILKQGQDGLSGVKETDKKWASQYLKIMGKILDQ  212
Human X94910  V......E..R..GV.....L......N..S....Q....E.............  212
Domain        C──────────────────────────────────────────────────────

Rat ERp29     GEDFPASELARISKLIE-NKMSEGKKEELQRSLNILTAFRKKGAEKEEL     260
Human X94910  ..H.....MT..AR...K....D.......K.........Q.............  261
Domain        C──────────────────────────────────────────┘ER─
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Fig. 2. Inferred domain structure of ERp29 and homology across species. Alignment of rat ERp29 (Fig. 1) with the putative human homologue, derived from an unpublished cDNA sequence (Genbank accession no. X94910). The human sequence is identical to rat ERp29 (dots) except for the indicated residues and insertion (residue 230). Positions of the inferred signal peptide (signal), N-domain (N), C-domain (C) and ER-retention motif (ER) are underlined. For simplicity, the boundary between the putative N- and C-domains is placed at the position of maximal random coil propensity (cf. Fig. 1B).

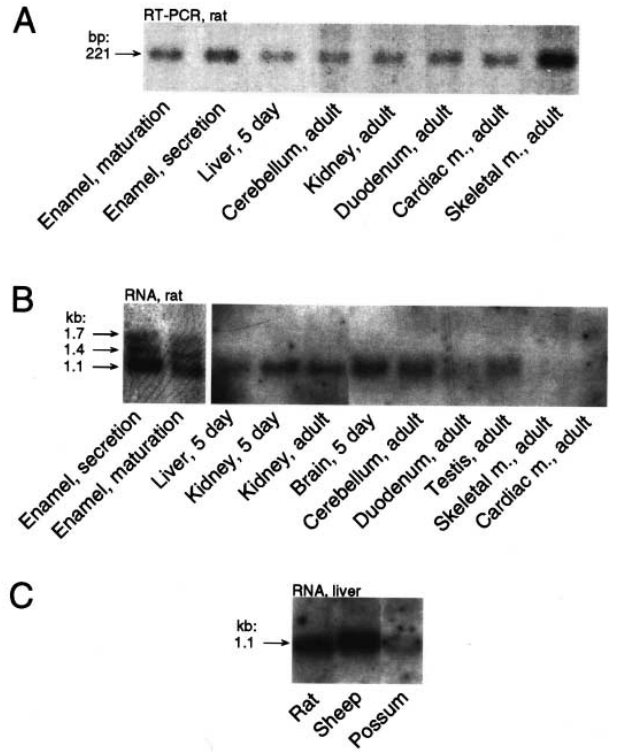

Fig. 3. Expression of ERp29 in animal tissues. RNA was isolated and analysed by RT-PCR, and by Northern blotting with the ERp29 cDNA insert, as described in Section 2. Rat tissues were from 5-day-old (secretion phase enamel), 10-day-old (maturation phase enamel) or 10–15-week-old animals (adult), as indicated. (A) RT-PCR products amplified (20 s/94°C, 30 s/60°C, 20 s/72°C, for 35 cycles) from the indicated rat tissues, using ERp29-specific primers (forward 5′-CAGCGGGGCAGTTAAAGTTGG-3′, reverse 5′-ATGATCTTCAGGTACTGACTGG-3′) and resolved by gel electrophoresis (1×TAE, 2% agarose). The 221 bp ERp29 product (arrow) was the only major band detected with ethidium bromide staining (image contrast is reversed), and was absent from controls lacking RT or template (not shown). (B) Northern blot of total RNA (10 μg) from the indicated rat tissues, washed to 0.7×SSPE and 0.1% SDS at 60°C and autoradiographed for 7 days. The major 1.1 kb transcript evident in most tissues, and the enamel cell-specific transcripts (1.4 kb, 1.7 kb) are arrowed. No major bands were detected outside the region shown. (C) Northern blot of adult liver samples from rat (0.5 μg poly(A)$^+$ RNA), sheep (1.0 μg poly(A)$^+$ RNA) and possum (10 μg total RNA), as indicated. The blot was hybridised overnight with ERp29 cDNA at 55°C, stringency washed to 2×SSPE and 0.5% SDS at 55°C and autoradiographed for 4 days.

cDNA derived from 'liver ER membrane' (accession no. U36482) encodes a putative protein that is ≈96% identical to ERp29, with the divergence limited to one region (residues 176–186, not shown). To date, our investigations of rat liver microsomal ERp29 (two-dimensional gels, tryptic peptide sequence and mass analyses, immunoblotting) have failed to reveal any differences from enamel cell ERp29 (M.J.H. et al., unpublished results). Given the strong similarities of primary structure, mass and p*I*, it is likely that ERp29 and its apparent homologues (U36482, X94910) correspond to spot 35, the unknown human liver protein originally characterised by Hughes et al. [22].

Protein database analyses revealed a significant overall homology between ERp29 and members of the protein disulfide isomerase family [30,31]. The strongest homology ($P = 10^{-11}$ overall) was in the carboxyl-terminus of P5, a putative protein

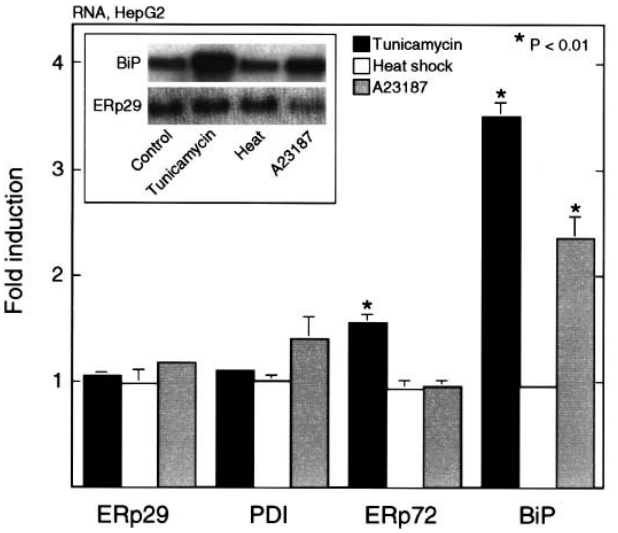

Fig. 4. Expression of ERp29 in response to cellular stress. Northern blot analysis of HepG2 cells (70% confluent) grown at 37°C for 6 h in fresh culture medium containing tunicamycin (10 μg ml^{-1}), ionophore A23187 (7 μM) or no addition (control). For heat shock, cells were incubated at 43°C for 30 min and otherwise treated as for the control. Northern blots (10 μg total RNA) were hybridised with the ERp29 cDNA insert or with cDNA probes to other reticuloplasmins (PDI is protein disulfide isomerase), as indicated. The inset shows representative blots for BiP and ERp29. Relative intensities of the reticuloplasmin bands were quantified following phosphorimaging and normalised on the basis of 28S rRNA content following reprobing with an oligonucleotide probe, as described [37]. Data (mean ± S.E.M.) are from three separate experiments. Statistical comparison of paired mean values (i.e. stress condition vs. control) was performed with Student's *t*-test (two-tailed, homoscedastic).

disulfide isomerase cognate from alfalfa [32], where the ERp29 C-domain shares 25% identity over 103 residues. Mammalian protein disulfide isomerase also has substantial homology with ERp29 ($P = 10^{-5}$ overall), particularly in the N-domain (24% identity over 122 residues). Lesser homology ($P \geq 10^{-3}$ overall) was apparent between ERp29 and mammalian ERp72, ERp60, and P5 (data not shown).

While exhibiting significant homology with protein disulfide isomerase and its cognates, ERp29 lacks the thioredoxin-like (Cys-X-X-Cys) catalytic moieties that distinguish this class of reticuloplasmins [30,31,33] and is therefore unlikely to be a functional disulfide isomerase. The possibility remains that the single Cys in ERp29 (residue 157) is part of an atypical catalytic site [33]. Protein disulfide isomerase is a multifunctional protein with a modular domain structure [30,31]. The domain of protein disulfide isomerase most related to ERp29 (i.e. the b domain, with 31% identity over 55 residues) currently has no assigned function [31,34].

3.4. Expression of ERp29

To investigate the tissue distribution of ERp29, ERp29-specific primers were synthesised for RT-PCR. ERp29 expression was detected in all rat tissues tested (Fig. 3A), and the identity of the PCR products was confirmed by Southern analysis (data not shown). Northern blot analysis revealed low levels of a 1.1 kb ERp29 transcript in most rat tissues examined (enamel cells, liver, kidney, brain, duodenum and testis), but none was detected in skeletal and cardiac muscle under these conditions (Fig. 3B). It is likely that the failure to detect ERp29 mRNA in muscle reflects a lower specific abundance of transcript, since an immunoblot investigation now underway has confirmed the presence of an ERp29-like protein (≈28 kDa) in skeletal muscle (M.J.H. et al., unpublished results). No evidence of major developmental regulation was found for ERp29 mRNA in enamel cells, kidney, liver and brain (Fig. 3B, and data not shown). Two larger transcripts (1.4 and 1.7 kb) were detected weakly in enamel cells, but not in other tissues or in liver poly(A)$^+$ RNA (Fig. 3B,C). Further work is required to establish whether the enamel cell-specific transcripts encode ERp29 (e.g. splice variants) or homologous proteins. A single transcript of a similar size to that in rat tissues was detected in ovine and marsupial liver (Fig. 3C) and in the human hepatoma cell line, HepG2 (Fig. 4). Together, the apparently high conservation of ERp29 sequences across different animal phyla, and the broad tissue distribution in rat, suggest that ERp29 has a function of general importance.

3.5. Stress responsiveness of ERp29

Several reticuloplasmins are stress proteins that undergo increased expression in response to disrupted protein synthesis or calcium homeostasis in the ER [14,15,35]. Experimental stimuli commonly used to investigate such stress responses include the glycosylation inhibitor tunicamycin, and the calcium ionophore A23187 [8,14,16,24,32]. In contrast, the major reticuloplasmins are generally unresponsive to heat stress, unlike the closely-related cytosolic stress ('heat shock') proteins [14,15]. Having characterised ERp29 as a reticuloplasmin, we questioned whether it functions in the cellular stress response.

An initial investigation with HepG2 cells (Fig. 4) showed that ERp29 mRNA expression was unaffected by tunicamycin and ionophore A23187. In contrast, two known stress-responsive reticuloplasmins, BiP and ERp72 [8,16,24,35], were significantly up-regulated by these stimuli. Protein disulfide isomerase was less responsive, as expected [8,16]. A moderate heat treatment, as conventionally used to induce heat shock proteins [14], was without effect on ERp29 and the established reticuloplasmins (Fig. 4). These findings indicate that ERp29 is relatively unresponsive to stress, at least for the stimuli and 6 h exposure tested, in HepG2 cells. We infer that ERp29 is unlikely to have a pivotal function in the cellular stress response, although more extensive investigations are warranted to establish the generality of this behaviour.

In conclusion, molecular cloning of ERp29 has revealed a novel primary structure with features (signal peptide, ER-retention motif) that identify it as a probable resident of the ER lumen (i.e. a reticuloplasmin). While having structural similarities with protein disulfide isomerase and its cognates (P5, ERp72, ERp60), ERp29 is unlikely to function as a disulfide isomerase since it lacks the characteristic thioredoxin-like catalytic motif. In addition, ERp29 is unlikely to be a calcium-binding protein, in contrast to many established reticuloplasmins. It appears that ERp29 is of general functional importance, given its broad tissue distribution and high conservation across animal phyla, but not pivotal to the cellular stress response. The combined lack of calcium binding properties and stress responsiveness distinguish ERp29 from the major reticuloplasmins characterised to date, suggesting that it might have a unique role. We are now particularly interested to establish the functional importance of ERp29 to enamel cells, where it is expressed in relatively high abundance.

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