ERp60 does not substitute for protein disulphide isomerase as the β -subunit of prolyl 4-hydroxylase

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Prolyl 4-hydroxylase (EC 1.14.11.2) catalyses the formation of 4-hydroxyproline in collagens. The vertebrate enzymes are $\alpha_2\beta_2$ tetramers while the *Caenorhabditis elegans* enzyme is an $\alpha\beta$ dimer. The β -subunit is identical to protein disulphide isomerase (PDI), a multifunctional endoplasmic reticulum luminal polypeptide. ERp60 is a PDI isoform that was initially misidentified as a phosphatidylinositol-specific phospholipase C. We report here on the cloning and expression of the human and *Drosophila* ERp60 polypeptides. The overall amino acid sequence identity and similarity between the processed human ERp60 and PDI polypeptides are 29 % and 56 % respectively, and those between the *Drosophila* ERp60 and human PDI polypeptides 29 % and

55 %. The two ERp60 polypeptides were found to be similar to human PDI within almost all their domains, the only exception being the extreme C-terminal region. Nevertheless, when the human or *Drosophila* ERp60 was expressed in insect cells together with an α -subunit of human prolyl 4-hydroxylase, no tetramer was formed and no prolyl 4-hydroxylase activity was generated in the cells. Additional experiments with hybrid polypeptides in which the C-terminal regions had been exchanged between the human ERp60 and PDI polypeptides demonstrated that the differences in the C-terminal region are not the only reason for the lack of prolyl 4-hydroxylase tetramer formation by ERp60.

INTRODUCTION

Protein disulphide-isomerase (PDI, EC 5.3.4.1), an abundant component of the lumen of the endoplasmic reticulum, is regarded as the *in vivo* catalyst of disulphide bond formation in the biosynthesis of various secretory and cell surface proteins. It catalyses thiol–disulphide interchange *in vitro*, leading to net protein disulphide formation, reduction or isomerization, depending on the reaction conditions (for reviews, see [1,2]). The polypeptide appears to have a modular structure, consisting of domains *a*, *e*, *b*, *b'*, *a'* and *c* [2–5]. The *a* and *a'* domains are similar to thioredoxin [3], and they both contain the sequence -CGHC- which represent two independently acting catalytic sites for the isomerase activity [6,7]. PDI also serves as the β -subunit of the prolyl 4-hydroxylases [4,8,9] and the microsomal triglyceride transfer protein [10,11]. Additional suggested functions are: as a chaperone-like polypeptide that non-specifically binds various peptides within the lumen of the endoplasmic reticulum [1,12–17]; a major cellular thyroid hormone-binding protein [18]; a dehydroascorbate reductase [19]; and a developmentally regulated retina protein termed r-cognin [20].

Prolyl 4-hydroxylase (EC 1.14.11.2) catalyses the formation of 4-hydroxyproline in collagens and other proteins with collagen-like amino acid sequences [21] by the hydroxylation of proline residues in -XPG- sequences. The 4-hydroxyproline residues are essential for the folding of the newly synthesized collagen polypeptide chains into triple-helical molecules, and hence this enzyme plays a central role in the biosynthesis of all collagens

(for reviews, see [5,22,23]). The vertebrate type I and type II [24] enzymes are $[\alpha(I)]_2\beta_2$ and $[\alpha(II)]_2\beta_2$ tetramers respectively, while the *Caenorhabditis elegans* enzyme is an $\alpha\beta$ dimer [25]. The function of the PDI/ β -subunit in all these enzymes appears to be to keep the α -subunits in a catalytically active, non-aggregated conformation [24–26]. Site-directed mutagenesis studies have demonstrated that PDI activity at the two -CGHC- catalytic sites is not required for the formation of these protein tetramers or dimers [7,27].

ERp60 is a PDI isoform that resembles PDI in size and has thioredoxin-like domains in positions corresponding to the *a* and *a'* domains of PDI, each containing the -CGHC- sequence [2,28,29]. This protein was initially misidentified as a phosphatidylinositol-specific phospholipase C [28], but subsequent studies have shown that it represents a PDI isoform [2,30,31] and is devoid of phospholipase activity [29–31]. The similarity between ERp60 and PDI has been assumed to include essentially only the two thioredoxin-like domains (e.g. [28,29,32]), but, as reported here, the two proteins are in fact similar within almost all of their domains.

Studies on the specificity of the prolyl 4-hydroxylase α -subunit–PDI interaction have demonstrated that the human PDI polypeptide can form an active $\alpha\beta$ dimer with the *C. elegans* α -subunit [25], and the *C. elegans* PDI polypeptide can form an active $\alpha_2\beta_2$ tetramer with the human α -subunit (J. Veijola, P. Annunen, P. Koivunen, T. Pihlajaniemi and K. I. Kivirikko, unpublished work). In order to study the specificity of this interaction further, we cloned the human ERp60 polypeptide and expressed it in insect cells together with an α -subunit of

Abbreviations used: PDI, protein disulphide isomerase; ERp60, a polypeptide initially called phosphatidylinositol-specific phospholipase C, also known as ERp61; human ERp60-PDI, a hybrid polypeptide containing 421 amino acids of the processed ERp60 polypeptide followed by the last 72 amino acids of PDI; human PDI-ERp60, a hybrid polypeptide containing 378 amino acids of the processed PDI polypeptide followed by the last 101 amino acids of ERp60.

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human prolyl 4-hydroxylase. In order to identify sequences of the ERp60 polypeptide that are conserved during evolution and might thus be functionally important, we also cloned and expressed ERp60 from *Drosophila*.

EXPERIMENTAL

Isolation of cDNA clones

To isolate cDNA clones for human ERp60, two rat sequence-specific cDNA probes covering nucleotides 235–1091 and 1279–1584 in the sequence reported by Bennett et al. [28] were produced by PCR from rat liver RNA converted into cDNA with the primers ERP1 (5'-TGGTGT/CGGC/ACAT/CTGC/TAA-3') and ERP3 (5'-AAC/TTCC/TTCC/TTGCAT-3') and ERP1 and ERP5 (5'-TTAGAGGTCCTCTGTGCCTTCTT-3') respectively. Primers ERP1 and ERP3 include alternative nucleotides as indicated. The reactions were cycled 12 times as follows: denaturation for 1 min at 94 °C, annealing for 1 min at 50 °C and extension for 2 min at 72 °C. This was followed by 18 cycles of annealing at 48 °C for 1 min with the denaturing and extension temperatures and times kept the same. The resulting PCR products were ³²P-labelled using a random-primed DNA labelling kit (Boehringer Mannheim) and used to screen a human heart cDNA library in the λ ZAP II vector (Stratagene #936208) under stringent conditions [33]. Three positive clones were obtained, two of which were found to be identical, starting at nucleotide 785 in the rat sequence [28] and extending towards the 3'-end for about 900 nucleotides. The third clone, HERP1, began at nucleotide 281 in the rat sequence and continued to the poly(A)⁺ tail at the 3'-end. This clone was sequenced for both strands. The missing 5'-end of the human cDNA was synthesized from human fibroblast RNA converted into cDNA by PCR using primers 5'-CCGCAGTCCCAGCCGAGCCGC-3' and 5'-TGCTTCTTC-ACCATCTCTAAA-3' for the first round and nested primers 5'-GCCGCGACCCTTCCGCGCCGTCC-3' and 5'-CTTCAGGG-TTGGATATCCACTG-3' for the second round. The sequences of these primers correspond to those submitted to the GenBank Nucleotide Sequence Database by N. Hirano, F. Shibasaki, H. Katoh, R. Sakai, T. Tanaka, J. Nishida, Y. Yazakai, Y. Takenawa and H. Hirai (accession no. P30101).

To isolate cDNA clones for *Drosophila* ERp60, a 500-nucleotide PCR fragment was synthesized from a *Drosophila melanogaster* cDNA library in the λ Uni-ZAP vector (Stratagene #937602) with the primers 5'-GGAATTCTAATACGACTCACTATAGG-3', containing an artificial *EcoRI* site, and 5'-GCGGATCCCC/ATGGTGTGGT/CCACTGCAA-3', containing an artificial *BamHI* site. The former was the vector-specific primer of the cDNA library and the latter was based on the catalytic-site sequences of human [4] and rat [3] PDI. The reaction was cycled 27 times as follows: denaturation for 1 min at 94 °C, annealing for 1 min at 65 °C and extension for 2 min at 72 °C. The resulting fragment was used to screen the *Drosophila melanogaster* cDNA library under stringent conditions [33]. Two positive clones were obtained, one of which, DERP1, was found to cover essentially all of the corresponding mRNA and was sequenced for both strands.

Nucleotide sequencing and sequence analysis

The nucleotide sequences were obtained by the Sanger dideoxy-nucleotide chain-termination method [34] using T7 DNA polymerase (Pharmacia) and vector-specific or sequence-specific 17-mer primers synthesized in an Applied Biosystems DNA synthesizer (Department of Biochemistry, University of Oulu, Oulu, Finland). DNASIS and PROSIS version 6.00 sequence

analysis software (Pharmacia), Antheprot [35], the Wisconsin GCG package version 8 (September 1994) and BOXHADE were used to compile the sequence data.

Construction of baculovirus transfer vectors and generation of recombinant baculoviruses

A full-length cDNA construct for human ERp60 was obtained by digesting the 5' PCR product with *EagI* and *EcoRV* and the HERP1 clone with *EcoRV* and *EcoRI*, these fragments being ligated to the *EagI*–*EcoRI* site of the baculovirus transfer vector pVL1392 [36]. To obtain an expression construct for *Drosophila* ERp60, the DERP1 clone was digested with *EcoRV* and *XhoI*, giving a fragment covering nucleotides 74–1736. The *XhoI* site was blunt-ended and the fragment was ligated to the *SmaI* site of pVL1392 [36]. The hybrid cDNA construct, human ERp60-PDI, was built by digesting the full-length cDNA for human ERp60 with *EagI* and *NdeI*, giving a fragment of 1358 bp which was then ligated to a 422 bp 3'-fragment covering nucleotides 1353–1772 of human PDI cDNA [4]. The latter fragment was produced by PCR and contained an artificial *NdeI* site at the 5'-end and the internal *BamHI* site at the 3'-end. This construct, coding for the first 421 amino acids of human ERp60 and the last 72 amino acids of human PDI, was ligated to the *EagI*–*BamHI* site of pVL1392 [36]. The hybrid cDNA construct, human PDI-ERp60, was created by digesting a cDNA for human PDI [4] with *EcoRI* and *NcoI* to obtain a 1233 bp fragment, which was ligated to a 337 bp PCR fragment from the 3'-region of the cDNA for human ERp60 containing an artificial *NcoI* site at the 5'-end and a *BamHI* site at the 3'-end. The fragment coding for the first 378 amino acids of human PDI and the last 101 amino acids of human ERp60 was then ligated to the *EcoRI*–*BamHI* site of pVL1392 [36].

The recombinant baculoviruses were cotransfected into *Spodoptera frugiperda* Sf9 cells with a modified *Autographa californica* nuclear polyhedrosis virus DNA (PharMingen) by calcium phosphate transfection [36,37]. The resultant viral pools were collected 4 days later and amplified [37].

Analysis of recombinant proteins in insect cells

The Sf9 cells were cultured in TNM-FH medium (Sigma) supplemented with 10% (v/v) fetal bovine serum (BioClear) at 27 °C. Recombinant proteins were produced by infecting insect cells seeded at a density of 10⁶/ml with any of the following recombinant viruses human ERp60, *Drosophila* ERp60, human ERp60-PDI, human PDI-ERp60, human PDI [26] or human prolyl 4-hydroxylase α59 [26] alone at a multiplicity of 5. In double-infection experiments the two viruses were used in a 1:1 ratio. The cells were harvested 72 h after infection, washed once with a solution of 0.15 M NaCl and 0.02 M phosphate, pH 7.4, homogenized in 0.1 M NaCl, 0.1 M glycine, 10 μM dithiothreitol, 0.1% Triton X-100 and 0.01 M Tris buffer, pH 7.8, and centrifuged at 10000 g for 20 min. Aliquots of the supernatants were analysed by denaturing SDS/8%-PAGE or non-denaturing 8%-PAGE and assayed for prolyl 4-hydroxylase activity. The remaining cell pellets were washed once with the homogenizing buffer and solubilized in 1% (w/v) SDS at 1/20 of the original culture volume and analysed by denaturing SDS/8%-PAGE.

Other assays

Prolyl 4-hydroxylase activity was measured by a method based on the hydroxylation-coupled decarboxylation of 2-oxo[1-¹⁴C]glutarate [38]. PDI activity was determined by an assay based on catalysis of the oxidative refolding of reduced, denatured

RNase [39]. Before measuring the PDI activity, the polypeptides expressed were partially purified by gel filtration in an S-200 high-resolution gel-filtration column of size 1.5×90 cm (Pharmacia), equilibrated and eluted with a solution of 0.3 M NaCl and 0.1 M Tris/acetate, pH 7.8. The amounts of recombinant polypeptides were compared by densitometry of Coomassie Brilliant Blue-stained bands in SDS/PAGE using a bioimage (BioImage, Millipore). Protein concentrations were measured with a Bio-Rad protein assay kit (Bio-Rad).

RESULTS

cDNA-derived amino acid sequences of the human and *Drosophila* ERp60 polypeptides

The cDNA clone HERP1, isolated here for the human ERp60 polypeptide, covers most of the coding sequences and 319 nt of the 3'-untranslated sequence. The latter contains the poly(A)⁺ signal AATAAA, which is accompanied 12 nt downstream by a poly(A)⁺ tail of 65 nt. Comparison of the cDNA-derived amino acid sequences with those of rat ERp60 [30] suggested that the cDNA clone does not cover the 5'-untranslated region or the sequences corresponding to the signal peptide and 46 extreme N-terminal amino acids of the processed polypeptide. The missing 5'-end was synthesized by PCR using human sequences submitted to the GenBank Database by N. Hirano, F. Shibasaki, H. Katoh, R. Sakai, T. Tanaka, J. Nishida, Y. Yazakai, Y. Takenawa and H. Hirai (accession no. P30101) for construction of the primers.

The cDNA clone DERP1 for the *Drosophila* ERp60 polypeptide isolated here covers the whole coding region, 95 nt of the 5'-untranslated sequences and 174 nt of the 3'-untranslated sequences. The 3'-untranslated sequences contain the poly(A)⁺ signal AATAAA, and end 15 nt downstream of this signal.

The human ERp60 polypeptide is 505 amino acids in length. A putative signal peptide is present at the N-terminal end of the deduced polypeptide, the most likely first amino acid of the processed polypeptide being serine, based on the parameters of von Heijne [40]. This means that the size of the signal peptide must be 24 amino acids and that of the processed human ERp60 polypeptide 481 amino acids (Figure 1). In agreement with the suggested cleavage site, the N-terminal sequence of the rat [30] and murine [29] ERp60 polypeptides is SDVLEL.

The *Drosophila* ERp60 polypeptide is shorter, being only 489 amino acids in length. The most likely first amino acid of the processed polypeptide is the latter of the two consecutive serines [40], and thus the size of the signal peptide must be 17 amino acids and that of the processed *Drosophila* ERp60 polypeptide 472 amino acids (Figure 1).

The calculated molecular mass of the processed human ERp60 polypeptide is 54260 Da and the pI 5.61, the corresponding values for the processed *Drosophila* ERp60 polypeptide being 53500 Da and 5.53.

The overall amino acid sequence identity between the human and *Drosophila* ERp60 polypeptides is 46 %, the similarity being 70 %. The highest degree of sequence identity between the ERp60 polypeptides from the two species is found within domains corresponding to the two thioredoxin-like domains *a* and *a'* of human PDI (Table 1), but a high degree of similarity is also found within the N-terminal region of the C-terminal domain *c*, a region termed *c*-1 here in both species (Table 1). The two thioredoxin-like regions contain the PDI catalytic site sequences -CGHC- in both species (Figure 1). The lowest degree of amino acid sequence identity between the human and *Drosophila* ERp60 polypeptides is found within the C-terminal region of domain *c* (*c*-2 in Table 1). The C-terminus of human ERp60 has the

H-PDI	-17	MIRRALCLAVAAAL-VRADAPFEEDHVLVLRKSNF
H-ERp60	-24	MRLRRLLALFPVALL-LAAARLAAASDVLELTDNDF
D-ERp60	-17	MMWRLLA---GVLLLGTAISSCADEDVLELGDDDF
H-PDI	18	AEALAAH---KYLVEFYAPWCGHCKKALAPYAKAA
H-ERp60	12	ESRISDTGSAGLLVEFAPWCGHCKKRLAPYEAFAA
D-ERp60	16	ATTLKQHETT---LVMFYAPWCGHCKKRLAPYAKAA
H-PDI	51	GKLKAESEIRLAKVDAT-ESDLAQYGVGRGYPTI
H-ERp60	48	TRLKGIIVP---LAKVDCI-ANTNTCNKYGVSGYPTL
D-ERp60	49	EIVKDDDEPIKRLAKVDCTEAGKTCISKYVSGYPTL
H-PDI	86	KFRNGDTASPKRYTAGREADDIVNWLKKRTGPAIT
H-ERp60	80	KIFRDGEAG--AYDGPRTADGIVSHLKKQAGPASV
D-ERp60	85	KIFRQDEVSQ--DYNGPRDSSGIATKYMRAQVGPASK
H-PDI	122	TIPDGAASLESSEVAIVGFFKDVESDSAKOFLQ
H-ERp60	114	PLRTEEFKKFISDKDAIVGFFDDSFSAHSEFLK
D-ERp60	119	TVRTVAELKKFLDTKDTLLFCVPSDSKSLAKIFLK
H-PDI	158	AAEAT-DDIPFCITNSDVSFKYQLDKDCVVLK--
H-ERp60	150	AASNIRDNRYRFAHTNVSFLVNEVDNNGEITLFRPS
D-ERp60	155	FADKNREKRYRFGHSSEKVELDK-OGETKIVLIRAP
H-PDI	191	---KFDGRNNEFEGE-VTKENLDFEIKHNQPLWVI
H-ERp60	186	HLTNKFEDKTVAYTEQKMTSGKIKKFIQENIFGICP
D-ERp60	190	HLNKFESSSIFEGS--SESDISTFVKENEHGLVG
H-PDI	222	EFTQTAAPKIFGCEIKTHILLFLPKSVSDYDGKLSN
H-ERp60	222	EMTEDNKDLIQKDL-----LIAYVDVYBK
D-ERp60	224	ERTQDSVKDEQ-NPL-----ITAYYSVDYQK
H-PDI	258	FKTAARESFKGKILFI-----FIDSDHTDN
H-ERp60	248	NAKGSNYWRNRVMMVAKFELDAGHKLNFAVASRKT
D-ERp60	249	NPKGNYWRNRVRLKVAKEFVG---QINFATASKDTF
H-PDI	282	QRILEFFGLKKECPAVRLITLLEEMTKYKPESE-E
H-ERp60	284	SHELSDFGLESTAGEIPVVAIRAKGEFVVMQEFES
D-ERp60	282	QHELNEFGYD-FVGDKPVVILARLEKNLYALKDEFS
H-PDI	317	LTAERITFECHRFLECKKPKHLMSELPEDWDKQPV
H-ERp60	320	RDGKALERFLDYDFDGNLKRVLKSEPIPS-NDGPV
D-ERp60	317	VE--NLQDFEVEKLLANLEPYIKSEPIPS-NDAPV
H-PDI	353	KVLVCKNFEDVAFDEKKNVVEFYAPWCGHCKOLAP
H-ERp60	355	KVVVAENFDEIVNNENKDVLIIFYAPWCGHCKNLAP
D-ERp60	350	KVAVAKNFDDLVNNKGDILIFYAPWCGHCKKLETP
H-PDI	389	ITWKLGETYKDHENIVIAKMDSTANEV-EAVKVSF
H-ERp60	391	KYKELGEKLSKDPNIVIAKMDATANDVSPYEVGRGF
D-ERp60	386	IYEELAQKL-QDEDVAIVKMDATANDVPEFNVGRGF
H-PDI	424	PTLKFFPASADRTVIDYNGERTLDGFKKLESGQD
H-ERp60	427	PTLYFSPANKKLNPKKYGGRELSDFESYLQREATN
D-ERp60	421	PTLFWLEKDAKNKPVSYNGGREVDPLKYLAKEAT
H-PDI	460	GAGDDDLLEDLEAEPEPMEEDDDQKAVKDEL*
H-ERp60	463	PPVIQEEKPKKKKKAQEDL*
D-ERp60	457	ELKGFDRSGKPKKTEL*

Figure 1 Alignment of the amino acid residues of the human PDI (H-PDI), human ERp60 (H-ERp60) and *Drosophila* ERp60 (D-ERp60) polypeptides

The amino acid sequence is shown using the one-letter code. Gaps (—) are introduced for maximal alignment of the polypeptides; *, stop codon of translation. Numbering of amino acids begins with the first residue in the processed polypeptide. Negative numbers indicate signal peptides. Identity between amino acids is indicated by a black box and similarity by a grey box. Similar amino acids: Gly = Ala = Ser; Ala = Val; Val = Ile = Leu = Met; Ile = Leu = Met = Phe = Tyr = Trp; Lys = Arg = His; Asp = Glu = Gln = Asn; Ser = Thr = Glu = Asn.

sequence QEDL and of *Drosophila* ERp60, KTEL, both these sequences being functional variants of the KDEL retention signal [41].

Comparison of amino acid sequences of the human and *Drosophila* ERp60 polypeptides with those of human PDI

The overall amino acid sequence identity and similarity between the processed human ERp60 and human PDI polypeptides are 29 % and 56 % respectively, and those between *Drosophila* ERp60 and human PDI 29 % and 55 % (Figure 1). As reported previously [28,29,32], the highest degree of identity is found within domains corresponding to the two thioredoxin-like domains *a* and *a'* of

Table 1 Comparison of amino acid sequence identities and similarities between various domains of the ERp60 and PDI polypeptides

The region numbers correspond to those of the human PDI polypeptide, starting at the first amino acid of the processed polypeptide. The exact start and end points of the various regions and the corresponding inter-domain regions are uncertain [2,3], and the present limits of some of the regions differ slightly from those reported previously. Similar amino acids are as given in the legend to Figure 1. The statistical significance of the comparison data was determined by using 100 randomized alignments of each region as an estimate of the probability of obtaining the same result by chance. All the similarities, except those for the *c*-2 domains, were found to be statistically significant ($P < 0.001$).

Region	Human PDI versus human ERp60		Human PDI versus <i>Drosophila</i> ERp60		Human ERp60 versus <i>Drosophila</i> ERp60	
	Identity (%)	Similarity (%)	Identity (%)	Similarity (%)	Identity (%)	Similarity (%)
<i>a</i> (9–91)	49.4	66.7	50.6	63.2	57.5	71.3
<i>e</i> (99–146)	35.4	62.5	22.9	62.5	45.8	77.1
<i>b</i> (152–225)	23.1	58.5	32.1	58.0	29.3	64.6
<i>b'</i> (262–345)	14.4	46.4	17.0	47.9	43.3	70.1
<i>a'</i> (351–433)	53.6	77.4	48.8	82.1	61.9	82.1
<i>c</i> -1 (440–453)	42.9	64.3	50.0	78.6	57.1	85.7
<i>c</i> -2 (454–491)	2.6	23.7	5.3	10.5	24.0	36.0

human PDI (Table 1). A 23-amino acid sequence around the -CGHC- sequence in region *a* (residues 28–50 in human PDI) is 83 % identical between human ERp60 and human PDI and 87 % identical between *Drosophila* ERp60 and human PDI (Figure 1). Correspondingly, a 16-amino acid sequence around the -CGHC- sequence in region *a'* (residues 374–389 in human PDI) is 81 % identical between human ERp60 and human PDI and 88 % identical between *Drosophila* ERp60 and human PDI (Figure 1).

Nevertheless, the amino acid sequence similarity between the ERp60 polypeptides and PDI is clearly not limited to the two thioredoxin-like domains, but a significant ($P < 0.001$) degree of similarity is found within almost all the domains (Figure 1 and Table 1). The only exception is the C-terminal region of domain *c* (*c*-2 in Table 1), in which the identity between human ERp60 and human PDI is only 3 %, and that between *Drosophila* ERp60 and human PDI only 5 %. The only other domain that shows an identity to the corresponding domain of human PDI of less than 20 % is domain *b'*, with an identity of 14 % in human ERp60 and 17 % in *Drosophila* ERp60, but the similarities of these domains to human PDI are statistically significant (Table 1). The N-terminal region of domain *c* (*c*-1 in Table 1) shows a particularly high degree of identity between the two ERp60s and PDI (Table 1).

Expression of the human and *Drosophila* ERp60 polypeptides in insect cells

Recombinant baculoviruses coding for the human and *Drosophila* ERp60 polypeptides were generated and used to infect *S. frugiperda* cells. The cells were harvested 72 h after the infection, homogenized in a buffer containing 0.1 % (v/v) Triton X-100, and centrifuged. The cell pellets were further solubilized in 1 % (w/v) SDS and the 0.1 % (v/v) Triton X-100- and 1 % (w/v) SDS-soluble proteins were analysed by SDS/PAGE under reducing conditions. Essentially, all of the recombinant ERp60 polypeptides were found in the Triton X-100-soluble fraction (Figure 2), there being little if any of these polypeptides in the 1 % (w/v) SDS-soluble fraction (details not shown).

The 0.1 % (v/v) Triton X-100-soluble fractions of the various cell homogenates were also studied by PAGE performed under non-denaturing conditions (Figure 3). As reported previously [25], essentially all of the human PDI polypeptide is found under these conditions as a monomer seen close to the bottom of the gel (Figure 3A and 3B, lanes 2). The mobility of the human ERp60 polypeptide (Figure 3A, lane 3) is markedly lower than that of

Figure 2 Analysis of expression of the human and *Drosophila* ERp60 polypeptides and the human PDI-ERp60 and human ERp60-PDI hybrid polypeptides in insect cells by SDS/PAGE under reducing conditions

Lanes 1, 2 and 3 show 0.1 % (v/v) Triton X-100-soluble proteins from cells infected with viruses coding for the human ERp60, *Drosophila* ERp60 and human PDI polypeptides respectively. Lanes 4 and 5 show expression of the human PDI-ERp60 and human ERp60-PDI hybrid polypeptides in cells infected with the corresponding viruses. The gel was analysed by Coomassie Blue staining. VP indicates a viral protein expressed in all samples.

human PDI, while the *Drosophila* ERp60 polypeptide (Figure 3A, lane 4) shows intermediate mobility. These differences may be, at least in part, due to the differences in their calculated pI values, 4.69 for PDI versus 5.61 and 5.53 for the two ERp60s (see above).

In order to study whether the expressed ERp60 polypeptides have PDI activity, 0.1 % (v/v) Triton X-100 extracts of cell homogenates were partially purified by gel filtration, which removed their RNase activity. The PDI activity of the partially purified extracts was then measured by a method based on catalysis of renaturation of reduced, denatured RNase [39], and the proportions of the PDI and ERp60 polypeptides were estimated by densitometry of the corresponding bands in SDS/PAGE. The human ERp60 polypeptide was found to be an active PDI but its isomerase activity was lower than that of human PDI (details not shown).

Co-expression of ERp60 and an α -subunit of human prolyl 4-hydroxylase in insect cells

In order to study whether it is possible to achieve association between an ERp60 and an α -subunit of human prolyl 4-

Figure 3 Analysis of co-expression of the human prolyl 4-hydroxylase α -subunit with the human PDI, human ERp60 or *Drosophila* ERp60 polypeptide in insect cells by PAGE under non-denaturing conditions

The Triton X-100-soluble proteins from cells infected with viruses coding for the human α -subunit, human PDI, human ERp60 or *Drosophila* ERp60 alone are shown in lanes 1–4 respectively. Lanes 5–7 show extracts from cells co-infected with a virus for the human α -subunit and a virus for the human PDI, human ERp60 or *Drosophila* ERp60 polypeptide respectively. The samples were analysed by Coomassie Blue staining in (A) and by Western blotting using an anti-PDI human antibody in (B). Abbreviations: 4-PH, Human prolyl 4-hydroxylase; H-ERp60, human ERp60; D-ERp60, *Drosophila* ERp60; PDI, human PDI; VP, viral protein. The samples were electrophoresed on an 8%-polyacrylamide gel in a running buffer of 0.192 M glycine and 0.025 M Tris, pH 8.3, at 15 V for 20 h at 4 °C.

Table 2 Prolyl 4-hydroxylase activity of Triton X-100 extracts from cells expressing various recombinant polypeptides

The values are given in d.p.m./10 μ g of extractable cell protein, means $\pm$ S.D. for four experiments, and as percentages of the value obtained by co-expression of the human prolyl 4-hydroxylase α -subunit with human PDI.

Recombinant polypeptide expressed	Prolyl 4-hydroxylase activity	
	(d.p.m./10 μ g)	(%)
None	6 $\pm$ 5	< 1
α -Subunit alone	138 $\pm$ 46	8
PDI alone	17 $\pm$ 16	1
Human ERp60 alone	14 $\pm$ 12	< 1
α -Subunit and PDI	1630 $\pm$ 66	100
α -Subunit and human ERp60	59 $\pm$ 18	4
α -Subunit and <i>Drosophila</i> ERp60	44 $\pm$ 17	3
α -Subunit and human PDI-ERp60	125 $\pm$ 66	8
α -Subunit and human ERp60-PDI	71 $\pm$ 40	4

hydroxylase, insect cells were co-infected with two recombinant baculoviruses, one coding for human PDI, human ERp60 or *Drosophila* ERp60, and the other for the α -subunit of human type I prolyl 4-hydroxylase. The Triton X-100-soluble fractions of the cell homogenates were then analysed by PAGE performed under non-denaturing conditions (Figure 3). As reported previously [24–26], the human PDI polypeptide formed an $\alpha_2\beta_2$ prolyl 4-hydroxylase tetramer with the human α -subunit (Figure 3A and 3B, lanes 5). A weak band corresponding to the non-associated PDI monomer is also seen in these lanes, whereas the non-associated α -subunit is insoluble in 0.1 % (v/v) Triton X-100 [24–26] and is not seen (Figure 3A, lane 1). No association of the human or *Drosophila* ERp60 polypeptide with the α -subunit was found in any experiment (Figure 3A, lanes 6 and 7).

In order to exclude the possibility that the ERp60 polypeptides might form an enzyme tetramer with the α -subunit that is unstable under conditions of non-denaturing PAGE, the 0.1 % (v/v) Triton X-100 extracts from cell homogenates were also

analysed for prolyl 4-hydroxylase activity with an assay based on the hydroxylation-coupled decarboxylation of 2-oxo[1- 14 C]glutarate [38]. As reported previously, the α -subunit expressed alone, without PDI, gives a small amount of activity [25], possibly due to prolyl 4-hydroxylase formation with the endogenous *S. frugiperda* PDI polypeptide (Table 2). Co-expression of the α -subunit with human PDI generated a high level of enzyme activity, whereas co-expression with human or *Drosophila* ERp60 generated no activity (Table 2).

Expression of hybrid ERp60-PDI and PDI-ERp60 polypeptides in insect cells with and without the α -subunit of human prolyl 4-hydroxylase

As described above, the C-terminal region *c*-2 of domain *c* differs distinctly from all other domains of the ERp60 polypeptides in that no amino acid sequence identity is found within this region between the two ERp60s and PDI (Table 1). The chaperone-like non-specific peptide-binding property of the bovine PDI polypeptide has been localized to a single 26-amino acid sequence in domain *c* [12]. The first two amino acids of this sequence are located at the C-terminal end of domain *c*-1 and the rest in domain *c*-2.

In order to study whether the lack of prolyl 4-hydroxylase formation in the presence of the ERp60 polypeptides is due to differences in sequences present in the C-terminal region, two hybrid constructs were prepared. One of them (ERp60-PDI) codes for human ERp60 until amino acid 421 of the processed polypeptide (Figure 1), which is followed by the last 72 amino acids of human PDI. The other construct (PDI-ERp60) codes for human PDI until amino acid 378 of the processed polypeptide, which is followed by the last 101 amino acids of human ERp60.

When these two hybrid polypeptides were expressed in insect cells alone, essentially all of the recombinant polypeptides were found in the 0.1 % (v/v) Triton X-100-soluble fraction upon SDS/PAGE performed under reducing conditions, as shown in Figure 2 (lanes 4 and 5). When the samples were studied by PAGE performed under non-denaturing conditions, the mobility of the human ERp60-PDI hybrid (Figure 4A, lane 3) was similar to that of human ERp60 (not shown), while that of human PDI-

Figure 4 Analysis of co-expression of the human prolyl 4-hydroxylase α -subunit with the human PDI or a hybrid human PDI-ERp60 or ERp60-PDI polypeptide in insect cells by PAGE under non-denaturing conditions

Lanes 1 to 3 show the Triton X-100-soluble proteins from cells infected with a virus coding for human PDI, human PDI-ERp60 or human ERp60-PDI alone, respectively. Lanes 4–6 show co-expression of the human α -subunit with human PDI, human PDI-ERp60 or human ERp60-PDI. The samples were analysed by Coomassie Blue staining in (A) and by Western blotting in (B). Abbreviations: 4-PH, human prolyl 4-hydroxylase; ERp60-PDI, human ERp60-PDI; PDI-ERp60, human PDI-ERp60; PDI, human PDI; VP, viral protein. The faint lower band seen in (B) in lanes 2 and 5 may represent a degradation product of human PDI-ERp60. The electrophoresis was performed as in Figure 3.

ERp60 (Figures 4A and 4B, lanes 2) was distinctly less than that of human PDI (Figure 4A and B, lanes 1).

When human ERp60-PDI was co-expressed in insect cells with the α -subunit of human prolyl 4-hydroxylase, no tetramer formation was detected (Figure 4A, lane 6) and no prolyl 4-hydroxylase activity was generated in the cells (Table 2). Interestingly, when human PDI-ERp60 was expressed together with the prolyl 4-hydroxylase α -subunit, no prolyl 4-hydroxylase tetramer was formed either (Figures 4A and 4B, lanes 5) and no prolyl 4-hydroxylase activity was generated in the insect cells (Table 2).

DISCUSSION

The data reported here indicate that the human and *Drosophila* ERp60 polypeptides are similar to human PDI within almost all their domains. The highest degree of similarity is found within the two thioredoxin-like domains *a* and *a'*, and ERp60 is in fact an active disulphide isomerase [2,30], as confirmed here. Nevertheless, the human and *Drosophila* ERp60 polypeptides were found to differ distinctly from PDI in that they did not act as the β -subunit of prolyl 4-hydroxylase.

Many recent studies suggest that PDI may catalyse protein folding by being both a disulphide isomerase and a chaperone [1,2,12–17]. The role of PDI as the β -subunit of prolyl 4-hydroxylases may be related to this chaperone function of the polypeptide, as its role appears to be to keep the α -subunits in a catalytically active, non-aggregated conformation [24–26]. This role is similar to the function of the PDI subunit in the microsomal triglyceride transfer protein $\alpha\beta$ dimer [11,27] and to that of certain molecular chaperones such as heat-shock protein 90 in some other proteins [2,26].

The chaperone-like non-specific binding of various peptides by PDI has been localized to a single 26-amino acid sequence in the C-terminal domain *c* of the rat PDI polypeptide [12,16]. The corresponding 27-amino acid sequence in the human PDI polypeptide extends from residue 452 to 478. Accordingly, the first two amino acids of this sequence are located at the end of region *c*-1, while the other 25 are in region *c*-2. Recent expression studies in insect cells performed in our laboratory have demonstrated that deletion of the three N-terminal amino acids of this 27-amino acid sequence from the human PDI polypeptide

prevents prolyl 4-hydroxylase tetramer formation (P. Koivunen, T. Helaakoski, A.-P. Kvist, T. Pihlajaniemi and K. I. Kivirikko, unpublished work). It therefore seemed possible that the difference between the ERp60 polypeptides and PDI with respect to prolyl 4-hydroxylase tetramer formation might be due to differences in the C-terminal regions of these polypeptides.

The data obtained with the hybrid polypeptides support the above suggestion, in that the human PDI-ERp60 polypeptide, in which the C-terminal region of PDI had been replaced by that of ERp60, formed no prolyl 4-hydroxylase tetramer and generated no prolyl 4-hydroxylase activity in the insect cells in double expression experiments with a prolyl 4-hydroxylase α -subunit. On the other hand, neither did the human ERp60-PDI polypeptide, which contained the C-terminal region of PDI. It is thus evident that differences in the sequences within the C-terminal region are not the only reason for the lack of prolyl 4-hydroxylase tetramer formation by ERp60.

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