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Molecular cloning of the cDNA encoding a novel protein disulfide isomerase-related protein (PDIR)

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Abstract **We isolated the cDNA of a novel protein disulfide isomerase (PDI)-related protein, designated PDIR, from a human placental cDNA library. Deduced from its nucleotide sequence, PDIR has the three CXXC-like motifs (Cys-Ser-Met-Cys, Cys-Gly-His-Cys and Cys-Pro-His-Cys), which are found in proteins within the PDI superfamily and are responsible for oxidoreductase activity. PDIR has a hydrophobic stretch at its amino terminus, which may serve as a signal sequence, and the putative endoplasmic reticulum (ER) retention signal 'Lys-Glu-Glu-Leu' at its carboxy terminus, indicating that PDIR is an ER resident protein. Northern blots showed that PDIR is preferentially expressed in cells actively secreting proteins and that the expression of PDIR is stress-inducible. These results suggested that PDIR has oxidoreductase activity of disulfide bonds against polypeptides and that it acts as a catalyst of protein folding in the lumen of the ER.**

Key words: Protein disulfide isomerase; CXXC motif; Endoplasmic reticulum; Stress response; Protein folding

1. Introduction

Protein disulfide isomerase (PDI) catalyzes the oxidation, reduction, and isomerization of disulfide bonds in proteins in vitro. It is believed to accelerate the folding of disulfide-bonded proteins by catalyzing the disulfide interchange reaction, a rate-limiting step during protein folding, within the luminal space of the endoplasmic reticulum (ER) [1–3]. PDI has two distinct regions with similarity to that of thioredoxin, an electron transporter in ribonucleotide and disulfide reduction [4]. Thioredoxin contains the sequence Cys-Gly-Pro-Cys and each of the thioredoxin-like domains of PDI contains the sequence Cys-Gly-His-Cys. The CXXC motif is responsible for the thiol-disulfide bond exchange activity in these proteins [5]. The presence of the CGHC sequence in a variety of proteins, such as P5, ERp60 and ERp72, in mammalian tissues led to their inclusion in the PDI superfamily [6]. While P5 and ERp60 have two CGHC sequences like PDI, ERp72 has three. P5 and ERp72 catalyze the reduction, oxidation and isomerization of disulfide-bonded proteins in vitro [7,8]. However, they are less active in these reactions than PDI, indicating that P5 and ERp72 have functions other than those involved in disulfide bond formation. In addition to catalyzing protein folding, PDI functions as a β-subunit of prolyl-4-hydroxylase [9], a component of a triglyceride transfer protein [10] and a triiodothyronine (T3)-binding protein [11]. The relationship between the motif and the function of these members of the PDI superfamily remains unclear. Identifying other members of this superfamily should lead to further understanding of the precise functions and the molecular evolution of proteins with the CXXC motif.

Here we report the isolation of the cDNA clone encoding a novel human PDI-related protein (PDIR) with three CXXC motifs and discuss the possible cellular role of this member of the PDI superfamily.

2. Experimental

2.1. Molecular cloning and DNA sequencing analysis of the human PDIR cDNA

Human PDIR cDNA was cloned by screening a placental λgt11 cDNA library (Clontech) with a human PDI cDNA fragment [9,11], radiolabeled with [α-^{32}P]dCTP (~220 TBq/mmol) (Amersham) using a random primer DNA labeling kit (Takara Shuzo). Hybridization proceeded at 37°C overnight in hybridization buffer [6× SSC (1× SSC: 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0) containing 0.5% SDSa, 0.2% polyvinylpyrrolidone, 0.2% Ficoll 400, 0.2% BSA, and 20 μg/ml of sheared salmon sperm DNA], followed by a wash in 2× SSC containing 0.1% SDSa at 37°C for 30 min. After three rounds of screening, positive clones encoding human PDI cDNA were subtracted by hybridization with the same probe under stringent conditions, in which the probe was washed out in 0.1× SSC containing 0.1% SDSa at 65°C for 30 min. *Eco*RI-DNA inserts of the clones were sequenced after subcloning their restriction fragments into pUC118 and pUC119 (Takara Shuzo). Single-strand templates were prepared according to standard procedures, and both strands were sequenced by means of dideoxy chain termination [12] using the 7-deaza sequencing kit (Takara Shuzo) and the universal primer. One of the clones encoded a novel PDI-related protein and the plasmid, in which the entire cDNA insert was cloned into the *Eco*RI site of pUC118, was designated phPDIR. Routine recombinant DNA manipulations proceeded as described [13].

2.2. Primer extension

The transcriptional initiation site of the human PDIR gene was determined by primer extension as described [13]. The primer (5′-CCGGCCCGGCCCGCGCCATCCCAGCAGCGCTGCCC-3′), radiolabeled with [γ-^{32}P]ATP (~220 TBq/mmol) (Amersham) using T4 polynucleotide kinase (Takara Shuzo), was hybridized with 0.5 μg of poly(A$^+$)RNA isolated from human placenta (Clontech) in 40 mM PIPES buffer, pH 6.4, containing 1 mM EDTA, 0.4 M NaCl, and 80% formamide at 37°C overnight. The DNA-RNA hybrid was subjected

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The nucleotide sequence reported in this paper appears in the GenBank database under the accession number D49490.

Abbreviations: BSA, bovine serum albumin; PDI, protein disulfide isomerase; PDIR, protein disulfide isomerase-related protein; PIPES, piperazine-*N*,*N*′-bis[2-ethane-sulfonic acid]; PI-PLC I, form-I phosphoinositide-specific phospholipase C; SDSa, sodium dodecyl sarcosinate.

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    gaattcgggggcgccggagtggagaaaggagccagcggtgggcagcgctgctggg    54
atggcgcgggccgggccggcgtggctgctgctggcaatctgggtggtcctgccatcatgg   114
M  A  R  A  G  P  A  W  L  L  L  A  I  W  V  V  L  P  S  W      20
ctgtcctctgcaaaggtctcctcgctcattgagagaatctctgaccccaaggacttgaaa   174
L  S  S  A  K  V  S  S  L  I  E  R  I  S  D  P  K  D  L  K      40
aaactgctcagaacccggaataatgtactggtgctttactccaaatctgaggtggcagct   234
K  L  L  R  T  R  N  N  V  L  V  L  Y  S  K  S  E  V  A  A      60
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E  N  H  L  R  L  L  S  T  V  A  Q  A  V  K  G  Q  G  T  I      80
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C  W  V  D  C  G  D  A  E  S  R  K  L  C  K  K  M  K  V  D     100
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L  S  P  K  D  K  K  V  E  L  F  H  Y  Q  D  G  A  F  H  T     120
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E  Y  N  R  A  V  T  F  K  S  I  V  A  F  L  K  D  P  K  G     140
ccccactgtgggaggaagatcctggagccaaagatgttgtccaccttgacagtgaaaag   534
P  P  L  W  E  E  D  P  G  A  K  D  V  V  H  L  D  S  E  K     160
gacttcagacggctcctgaagaaggaagagaagcgctcctgatcatgttttatgccccc   594
D  F  R  R  L  L  K  K  E  E  K  P  L  L  I  M  F  Y  A  P     180
tggtgcagcatgtgcaagaggatgatgccgcatttccagaaggctgcgactcagctgcga   654
W  C  S  M  C  K  R  M  M  P  H  F  Q  K  A  A  T  Q  L  R     200
ggccacgccgtgctggccgggatgaatgtctactcctctgaatttgaaaacatcaaggag   714
G  H  A  V  L  A  G  M  N  V  Y  S  S  E  F  E  N  I  K  E     220
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E  Y  S  V  R  G  F  P  T  I  C  Y  F  E  K  G  R  F  L  F     240
cagtatgacaactatgggtccacagctgaggacattgtggagtggctgaagaatccgcag   834
Q  Y  D  N  Y  G  S  T  A  E  D  I  V  E  W  L  K  N  P  Q     260
ccgccacagccccaggtccctgagactccctgggcagatgagggcggctccgtttatcac   894
P  P  Q  P  Q  V  P  E  T  P  W  A  D  E  G  G  S  V  Y  H     280
ctgaccgatgaagactttgaccagtttgtgaaggaacactcctctgtcctcgtcatgttc   954
L  T  D  E  D  F  D  Q  F  V  K  E  H  S  S  V  L  V  M  F     300
cacgccccatggtgtggccactgtaagaaaatgaagccggagtttgagaaggcagcagaa  1014
H  A  P  W  C  G  H  C  K  K  M  K  P  E  F  E  K  A  A  E     320
gccctccatggagaagcggatagctctggtgtccttgcagctgtcgatgccactgtcaac  1074
A  L  H  G  E  A  D  S  S  G  V  L  A  A  V  D  A  T  V  N     340
aaggccctggcagaaagattccacatctcagagtttcctacgttgaagtattttaagaat  1134
K  A  L  A  E  R  F  H  I  S  E  F  P  T  L  K  Y  F  K  N     360
ggagagaaatacgcagtgcctgtgctcaggacaaagaagaagtttctcgagtggatgcaa  1194
G  E  K  Y  A  V  P  V  L  R  T  K  K  K  F  L  E  W  M  Q     380
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H  L  V  G  D  N  F  R  E  T  L  K  K  K  K  H  T  L  V  M     420
ttctacgccccttggtgcccacactgtaagaaggtcattccgcactttactgctactgct  1374
F  Y  A  P  W  C  P  H  C  K  K  V  I  P  H  F  T  A  T  A     440
gatgccttcaaagatgaccgaaagattgcctgtgccgctgttgactgtgtcaaagacaag  1434
D  A  F  K  D  D  R  K  I  A  C  A  A  V  D  C  V  K  D  K     460
aaccaagacctgtgccagcaggaggcggtcaagggctaccccacttccactactaccac  1494
N  Q  D  L  C  Q  Q  E  A  V  K  G  Y  P  T  F  H  Y  Y  H     480
tatgggaagttcgcagaaaagtatgacagcgaccgcacagaattgggatttaccaattat  1554
Y  G  K  F  A  E  K  Y  D  S  D  R  T  E  L  G  F  T  N  Y     500
attcgagcctccggaggaggagaccatgaaagactagggaaaaagaaggaagagttataa  1614
I  R  A  L  R  E  G  D  H  E  R  L  G  K  K  K  E  E  L  *     519
ttcctgcctcagaaaaagcttttccattacactgtgaatgatacctgttttgttgtttct  1674
gaatttccccgaattc                                              1691
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Fig. 1. The nucleotide and deduced amino acid sequences of human PDIR cDNA. The three CXXC motifs are underlined. The ER retention signal is indicated by a double line. Transcriptional initiation sites determined by the primer extension described in Fig. 2 are indicated by arrowheads. The termination codon is indicated by an asterisk. Nucleotides are numbered beginning with the first residue of the cDNA insert. Amino acids are numbered starting from the initiation methionine residue.

to the primer extension reaction in 50 mM Tris-HCl buffer, pH 7.6, containing 60 mM KCl, 10 mM $MgCl_2$, 1 mM of each dNTP, 1 mM dithiothreitol, 1 unit/μl placental ribonuclease inhibitor (Takara Shuzo), 50 μg/ml actinomycin D (Sigma), and 2.5 units/μl reverse transcriptase (Takara Shuzo) at 37°C for 2 h. The product of the extension reaction was analyzed on 6% polyacrylamide gels.

2.3. Northern blots

Human poly(A$^+$)RNAs (0.5 μg) isolated from liver, kidney, brain, and lung (Clontech) were fractionated on agarose gels containing formaldehyde and transferred to the hybridization transfer membrane (Hybond-N, Amersham) [13]. The membrane was probed with the radiolabeled PDIR cDNA insert or the *Hin*fI human β-actin gene fragment (Wako Pure Chemical Industry) [14]. Hybridization proceeded overnight at 68°C in 5 × SSPE (1 × SSPE: 0.15 M NaCl, 0.01M sodium phosphate, pH 7.4, and 1 mM EDTA) containing 50% formamide, 0.5% SDSa, 0.2% polyvinylpyrrolidone, 0.2% Ficoll 400, 0.2% BSA, and 20 μg/ml of sheared salmon sperm DNA. The membrane was then washed with 0.1 × SSPE containing 0.1% SDSa at 68°C for 30 min. Autoradiography was performed at −80°C with an intensifying screen. Mouse normal liver cells (BNL CL.2, ATCC No. TIB73) were grown in α-modified Eagle's minimum essential medium supplemented with 10% fetal calf serum for 12 h and treated with tunicamycin (10 μg/ml) or A23187 (7 mM) for 12 h. Total RNAs were purified from the cells as described [15] and 20 μg of each was Northern blotted using the radiolabeled PDIR insert, human PDI cDNA insert [9, 11] and human β-actin genomic fragment [14] as the probes, respectively. The hybridization conditions were as described above. Densitometric measurements were performed using the image analyzer BA100 (Fuji Film).

2.4. Computer analysis

A computer-assisted homology analysis and hydropathy calculation [16] were carried out by BION program provided by IntelliGenetics Inc.

3. Results and discussion

To identify new members of the PDI superfamily [6], we screened a human placental λgt11 cDNA library using human PDI cDNA [9,11] under conditions of low stringency. Through

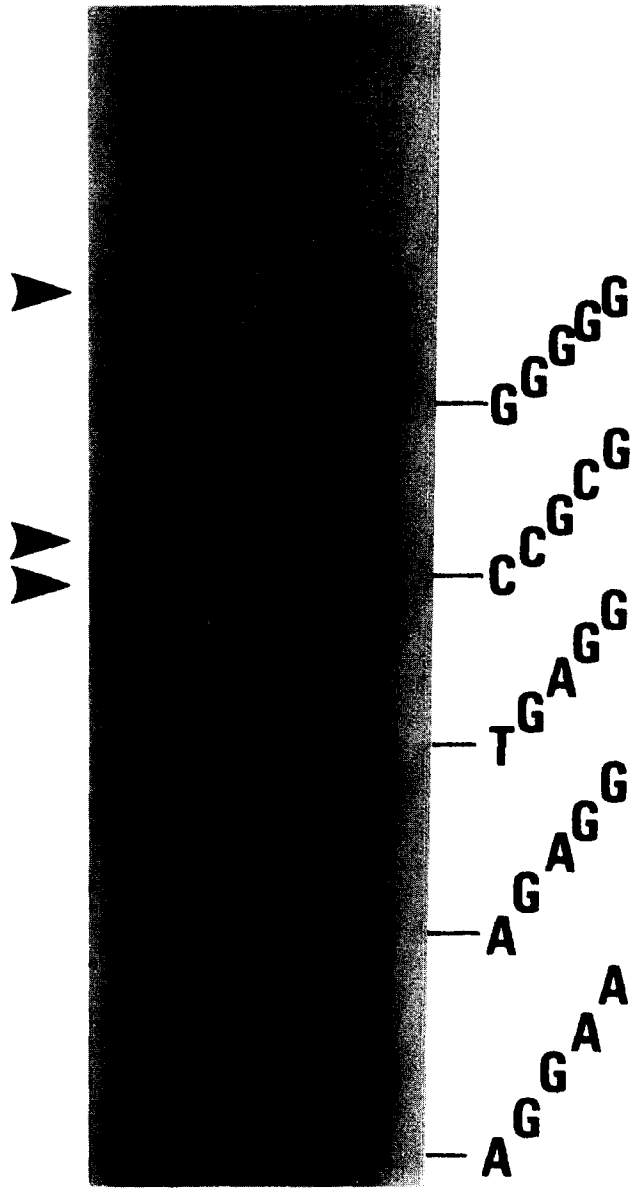

Fig. 2. Identification of the transcriptional initiation site of the human PDIR gene. The products of the primer extension reaction were separated on a 6% polyacrylamide gel with the products of the dideoxy sequencing. The major transcriptional initiation sites are indicated by arrowheads and the nucleotide sequence of the 5'-part of the PDIR cDNA is described.

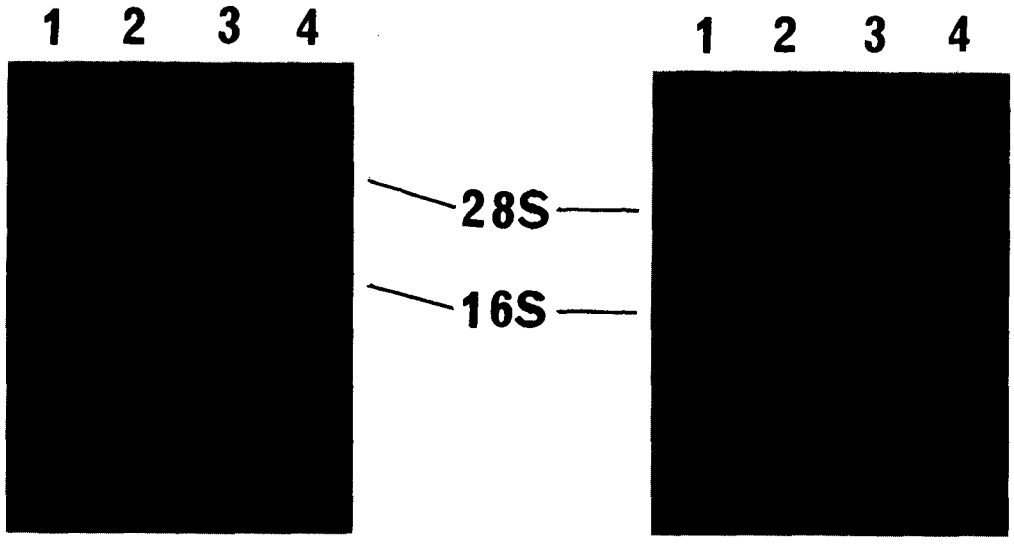

Fig. 3. Northern blots. Human liver (lane 1), kidney (lane 2), brain (lane 3) and lung (lane 4) mRNAs were probed with the human PDIR cDNA insert (left panel) and with the mouse β-actin gene fragment as a control (right panel).

subtracting of positive clones encoding human PDI cDNA under stringent conditions, two positive clones were obtained from about 5×10^4 phages. Sequencing showed that one encoded a polypeptide of 440 amino acids with high similarity to the hamster P5 protein (93% identical in amino acid sequence, [T. Hayano and M. Kikuchi, submitted]), which has two PDI-like sequences. The other clone encoded an unidentified protein with homology to PDI, which was designated PDI-related protein (PDIR). The insert in this clone consisted of 1681 nucleotides and it contained an open reading frame encoding a polypeptide of 519 amino acids with a molecular weight of 59,556 Da (Fig. 1). As no stop codon was found upstream from the putative initiation codon in the open reading frame, the transcriptional initiation site of the corresponding gene was determined by primer extension. The major initiation sites were assigned to the residues G, C and C at 50, 42 and 41 nucleotides respectively, upstream from the putative initiation codon (Figs. 1 and 2). Thus the isolated cDNA covered the 5′ end of human PDIR mRNA and encoded the entire sequence of PDIR. The cDNA insert had no polyadenylation signal and no poly(A) tract. A transcript of about 2,000-bases was detected by Northern blotting using the PDIR cDNA insert (Fig. 3), indicating that the cDNA did not cover about 300 bases of the 3′ untranslated region of PDIR mRNA.

To determine the structural features of human PDIR, we calculated its hydropathy plot using the HPLOT program [6] (data not shown). The first 24-residue sequence had the following characteristics of a signal sequence: (i) a basic amino acid residue close to the amino terminus (Arg at the third position from the initiator Met); (ii) a hydrophobic amino acid stretch in the middle portion; (iii) an amino acid residue having a small side chain (Ala) in the carboxy terminal site. These criteria suggested that this amino terminal stretch serves as the signal peptide to transport PDIR into the lumen of the ER. And PDIR also has the sequence 'Lys-Glu-Glu-Leu' at its carboxy terminus. In mammalian cells, the sequence consisting of Lys-Asp-Glu-Leu and modified versions of the sequence serve as retention signals for the ER resident proteins [17]. The sequence 'Lys-Glu-Glu-Leu' was found in the carboxy terminus of ERp72 and it can function as the retention signal [18]. Thus human PDIR appeared to be located within the luminal space of the ER, where secretory proteins fold.

Human PDIR has three intramolecular homologous domains containing the CXXC motifs Cys-Ser-Met-Cys, Cys-Gly-His-Cys, and Cys-Pro-His-Cys, and it has been classified into the PDI superfamily [6]. The motif, which is characteristic of the active centers of proteins (Fig. 4) catalyzing oxidoreduction reactions, was originally found in *E. coli* thioredoxin, of which active site contains Cys-Gly-Pro-Cys [19]. The flanking amino acid sequences of the motifs are well conserved among mammalian proteins belonging to the PDI superfamily, thioredoxin, and DsbA, which is a bacterial equivalent of PDI [20, 21]. The first (162–247), second (286–367) and third (407–492) thioredoxin-like domains of PDIR reveal 37%, 41% and 40% identity in the amino acid sequences with the first thioredoxin-

PDIR	(1st)	IMFYAPW**CSMC**KRMMP
PDIR	(2nd)	VMFHAPW**CGHC**KKMKP
PDIR	(3rd)	VMFYAPW**CPHC**KKVIP
PDI	(1st)	VEFYAPW**CGHC**KALAP
PDI	(2nd)	VEFYAPW**CGHC**KQLAP
P5	(1st)	VEFYAPW**CGHC**QRLTP
P5	(2nd)	VEFYAPW**CGHC**KNLEP
ERp72	(1st)	LEFYAPW**CGHC**KQFAP
ERp72	(2nd)	VEFYAPW**CGHC**KKLAP
ERp72	(3rd)	IEFYAPW**CGHC**KQLEP
ERp60	(1st)	VEFFAPW**CGHC**KRLAP
ERp60	(2nd)	IEFYAPW**CGHC**KNLEP
thioredoxin		VDFWAEW**CGPC**KMIAP
DsbA		LEFFSFF**CPHC**YQFEE

Fig. 4. Comparison of the CXXC motifs of the members of the PDI superfamily, thioredoxin and DsbA. Three CXXC motifs (highlited) and their flanking amino acids of human PDIR are aligned with those of human PDI [9, 11], human P5 (T. Hayano and M. Kikuchi, submitted), mouse ERp72 [18], rat ERp60 [23], *E. coli* thioredoxin (Trx) [19] and *E. coli* DsbA [20, 21]. PDIR and ERp72 have three CXXC motifs. PDI, P5 and ERp60 have two CXXC motifs.

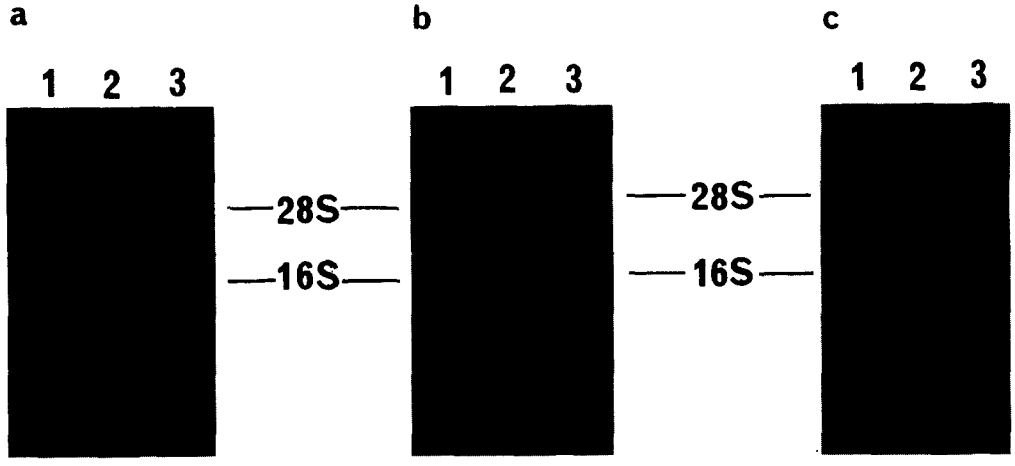

Fig. 5. Response of mouse PDIR gene transcription to stress. Northern blot of total RNAs from control BNL CL.2 cells (lane 1), from BNL CL.2 cells incubated with tunicamycin (lane 2) or A23187 (lane 3). The RNAs were probed with the human PDIR cDNA insert (a), the human PDI cDNA insert (b) and the human β-actin gene fragment (c).

like domain (34–120) of human PDI and 34%, 35% and 27% identity with the second domain (377–461) of human PDI, respectively. The PDI superfamily consists of PDI, P5, ERp60 and ERp72. Whereas the first three contain two CGHC sequences, ERp72 contains three. PDIR also has three CXXC motifs. P5 cDNA was first isolated from a hamster cell line, which is highly resistant to hydroxyurea, which destroys free radicals and which potently inhibits ribonucleotide reductase; P5 may be related to the hydroxyurea-resistant phenotype of the cell [22]. ERp60 is an ER resident protein and it is identical to form-I phosphoinositide-specific phospholipase C (PI-PLC I) [23]. It participates in the degradation of misfolded proteins through its thiol proteinase activity (M. Otsu et al., personal communication). ERp72 is an abundant ER resident protein and its functional role is unknown [18]. From the fact that P5 and ERp72 reveals weak but significant PDI activity [8], the CGHC motif accounts for the disulfide interchange reaction, while the relationship between the CGHC motifs of the members of the PDI superfamily and their functions described above remains obscure. It is notable that the sequences of three CXXC motifs of human PDIR are all different, because only the Cys-Gly-His-Cys sequence has been found in any of the mammalian proteins belonging to the PDI superfamily so far (Fig. 4). The first motif, Cys-Ser-Met-Cys, of PDIR has not been found in any proteins with the CXXC motif, including bacterial oxidoreductase. The third motif, Cys-Pro-His-Cys, has been identified in *E. coli* DsbA. The quantitative difference in redox potential and oxidoreductase activity among PDI, thioredoxin and DsbA is explained by the different dipeptide sequences between the two cysteine residues in the motifs [24], suggesting that PDIR with three separate CXXC motifs and PDI differs in terms of substrate range in the oxidoreductase reaction.

The relative content of PDI mRNA is liver > pancreas ~ kidney > lung > testes ~ spleen > heart > brain [4], indicating that PDI is preferably expressed in cells that actively secrete protein, as a catalyst of protein folding. Northern blotting has shown that the relative content of PDIR mRNA is liver > kidney > lung > brain (Fig. 3). The relative content of mRNA of P5 [8] is lung > kidney > heart > liver > brain; that of ERp60 (PI-PLC I) [22] is lung ~ kidney > liver ~ brain and the relative levels of ERp72 and PDI proteins are significantly different in liver, muscle and spleen [18]. Thus only PDIR, among all the known members of the PDI superfamily, resembles PDI in cellular distribution, suggesting that PDIR is not only structurally, but also functionally related to PDI.

The expression of heat shock proteins in cells is induced by some kinds of stressors such tunicamycin, which inhibits the *N*-glycosylation of nascent polypeptides during protein synthesis, and the calcium ionophore A23187, which affects the permeability of cellular membranes to calcium ions. We investigated the effects of stress on the expression of PDIR. Northern blots and densitometry showed that the level of PDIR mRNA increased 5- and 2-fold after exposing the mouse normal liver cell line BNL CL.2 for 12 h to tunicamycin and A23187, respectively (Fig. 5). PDI gene transcription was stimulated 4- and 6-fold by tunicamycin and A23187, respectively (Fig. 5). Some heat shock proteins assist the folding of nascent polypeptides and refolding of proteins, which are denatured by the stress [25]. The transcriptional inducibility of the PDIR gene by stress was moderate but significant, suggesting that one role of PDIR is the catalysis of protein folding in the cell. It should be noted that the expression level of PDIR is less than that of PDI (Fig. 3 and 5), suggesting that PDIR may catalyze disulfide bond formation in specific proteins while PDI reveals broad range of the substrate specificities.

The functional role of PDIR remains unclear. However, its structural features, cellular compartmentation, tissue distribution and stress inducibility described here revealed the resemblance between PDIR and PDI. Members of the PDI superfamily, including PDIR, could share a variety of substrates during protein folding in the cell. The availability of the PDIR cDNA will lead to the elucidation of its precise role. The novel CXXC motif, Cys-Ser-Met-Cys of PDIR should allow us to know more about the mechanism of the disulfide interchange reaction and the molecular evolution of the PDI superfamily.

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