Functional analysis of the CXXC motif using phage antibodies that cross-react with protein disulphide-isomerase family proteins

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Polyclonal antibodies that had been raised against particular PDI (protein disulphide-isomerase) family proteins did not cross-react with other PDI family proteins. To evade immune tolerance to the important self-motif Cys-Xaa-Xaa-Cys, which is present in PDI family proteins, we used the phage display library [established by Griffiths, Williams, Hartley, Tomlinson, Waterhouse, Crosby, Kontermann, Jones, Low, Allison et al. (1994) EMBO J. 13, 3245–3260] to isolate successfully the phage antibodies that can cross-react with human and bovine PDIs, human P5, human PDI-related protein and yeast PDI. By measuring the binding of scFv (single-chain antibody fragment of variable region) to synthetic peptides and to mutants of PDI family proteins in a surface plas-

mon resonance apparatus, we identified clones that recognized sequences containing the CGHC motif or the CGHCK sequence. By using the isolated phage antibodies, we demonstrated for the first time that a lysine residue following the CXXC motif significantly increases the isomerase activities of PDI family proteins. Moreover, we demonstrated that the affinity of isolated scFvs for mutant PDI family proteins is proportional to the isomerase activities of their active sites.

Key words: CXXC motif, motif mutation, phage antibody library, protein disulphide-isomerase, protein disulphide-isomerase-related protein (PDIR), single-chain antibody fragment.

INTRODUCTION

Protein disulphide-isomerase (PDI; EC 5.3.4.1) catalyses the oxidation, reduction and isomerization of disulphide bonds in proteins as well as the disulphide-coupled folding of proteins in the ER (endoplasmic reticulum) [1]. PDI also has chaperone and anti-chaperone activities [2] and its chaperone activity is inhibited by aminoglycoside antibiotics [3,4]. In addition, it was found that PDI associates with misfolded proteins *in vivo* and may be involved in protein quality control [5,6]. Furthermore, P5 expressed in the embryonic mid-line has been reported to be necessary for the establishment of left/right asymmetries during ontogeny [7]. These findings suggest that the PDI family of proteins may have novel, so far undefined functions. The catalytic mechanism of PDI is believed to involve the acceleration of thiol–disulphide interchange reactions at one or two catalytic sites containing the sequence CGHC [8,9]. This sequence is referred to as a Trx (thioredoxin-like) domain [8] and is often called the CGHC or CXXC motif. PDI and its relatives form a diverse protein family whose members are characterized by the presence of two or three Trx domains in their primary structure [1].

We have isolated cDNA clones encoding the novel hPDI (human PDI)-related protein (hPDIR) [10] and hP5 (human P5) [11]. The former has three CXXC motifs with different amino acid sequences (CSMC, CGHC and CPHC), whereas the latter has two CXXC motifs with the same amino acid sequence (CGHC). Thus, in summary, PDI [12], P5 [11] and ER60 [13] bear two CGHC sequences, whereas ERp72 [14] has three CGHC sequences and PDIR (PDI-related protein) [10] has the three disparate sequences, CSMC, CGHC and CPHC. On the basis of the number and the relative positions of the Trx domains, we have classified PDI and its relatives into four groups [15].

Although the active sites of all PDI family proteins contain the CXXC motif, their isomerase activities differ from protein to protein. The sequence around the active site of PDI and its relatives is highly conserved from yeast to mammals. However, there is little additional homology between the amino acid sequences of the proteins belonging to the PDI family, PDI, P5, PDIR and yPDI (yeast PDI). To understand the relationship between the strength of isomerase activity and the local structure of the active site, we considered the use of antibodies that recognize specifically the active site of each PDI protein. We found that the polyclonal antibodies raised against individual proteins of the PDI family do not cross-react with other PDI family proteins. This indicates that these polyclonal antibodies do not recognize the CXXC motif, although the motif and the sequences in its vicinity are conserved in PDI family proteins. This is because CXXC motif-specific antibodies belong to a type of autoantibody group which are made in response to self molecules and which cannot be obtained in the usual way. However, cross-reactive antibodies are very useful for the rapid identification of new PDI family proteins and the functional analysis of their active sites. With the aim of isolating such antibodies, we have employed a human synthetic phage display antibody library prepared by Griffiths et al. [16].

In the present study, we report the successful isolation of new phage antibodies against both bPDI (bovine PDI) and hPDIR, which cross-react with the hPDI and bPDI proteins, yPDI [17], hP5 and PDIR. Functional analysis revealed that these antibodies recognize sequences containing the CGHC motif or CGHCK. By using isolated phage antibodies, we obtained evidence for the first time that the presence of a lysine residue at the end of the CGHC motif increases the isomerase activities of PDI family proteins. We also found that the affinity of isolated scFvs (single-chain antibody fragment of variable region) for the CGHC motif and its

Abbreviations used: CaBP1, calcium-binding protein 1; CDR, complementarity-determining regions; ER, endoplasmic reticulum; hP5, human P5; PDI, protein disulphide-isomerase; hPDI, bPDI, yPDI, human, bovine and yeast PDI respectively; PDIR, PDI-related protein; hPDIR, hPDI-related protein; pfu, plaque-forming units; RU, resonance unit; scFv, single-chain antibody fragment of variable region; Trx domain, thioredoxin-like domain.

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vicinity correlates with the strength of the isomerase activity of individual members of the PDI family.

MATERIALS AND METHODS

Materials

A human synthetic phage display antibody library (pHEN2), prepared by Griffiths et al. [16], was kindly provided by Dr G. Winter (MRC Centre, Cambridge, U.K.). Recombinant hPDI-related proteins and yeast PDI were prepared by a method described previously [18]. The bPDI protein was purified from bovine liver by a method described previously [19]. The anti-PDI polyclonal antibody was prepared in our laboratory by the standard method [20] and the anti-P5 antibody was a gift from Dr N. Takahashi (Tokyo University of Agriculture and Technology, Japan).

Bacterial strains

Escherichia coli strain TG-1 [K12, $\Delta(lac-pro)$, *supE*, *thi*, *hsd* Δ 5/*F'**traD36*, *proA*⁺*B*⁺, *lacI*^a, *lacZ* Δ M15] was used for the phage rescue. The non-suppressor *E. coli* strain HB2151 [K12, *ara*, $\Delta(lac-pro)$, *thi*/*F'**proA*⁺*B*⁺, *lacI*^a*Z* Δ M15] was used to prepare scFvs. *E. coli* AD494 (DE3) { Δara^- , *leu*7967, $\Delta lacX74$, $\Delta phoA$, *Pvu II*, *phoR*, $\Delta malF3$, *F'*[*lac*⁺, (*lacI*^a), *pro*], *trxB::kan*(DE3)} was used to express the scFvs.

Rescue of phagemid particles from the library

The phage library used for the selection of antibodies has been described previously [21]. This library contains a variety of human VH genes from 50 germ-line VH gene segments with variable third CDRs (complementarity-determining regions) of 4–12 residues [22], which are combined with human light-chain gene segments [23,24]. The rescue of phagemid particles has been described by Hirose et al. [25].

Selection of phages binding to hPDIR and bPDI by panning

The phage particles obtained as described above were subjected to panning for binding using Nunc Maxisorp immuno test tubes (Nalgene, Rochester, NY, U.S.A.) coated overnight with hPDIR or bPDI dissolved in PBS (0.1 M NaCl/33 mM Na₂HPO₄/17 mM NaH₂PO₄) at a concentration of 50 μ g/ml. Panning was performed as described by Hirose et al. [25] and phages were selected as described by Kikuchi et al. [26].

ELISA

ELISAs were used to screen the phages and involved 96-well plates coated with various PDI family proteins (10 μ g/ml). The phage ELISA was performed using a horseradish peroxidase-conjugated mouse anti-M13 monoclonal antibody (Amersham Biosciences, Uppsala, Sweden) and 2,2'-azinobis-6-sulphonic acid diammonium salt (Wako, Osaka, Japan). The binding of the phages to the PDI family proteins was detected at 415 nm using a microplate reader (Bio-Rad, Hercules, CA, U.S.A.; model 680). The phages binding to hPDIR were then examined for their ability to bind to bPDI, and phages that bound to both hPDIR and bPDI were used in subsequent experiments.

Sequencing of clones

The phages were rescued from single colonies of the infected *E. coli* suppressor strain TG-1 using VCS-M13 helper phage [27], and the *E. coli* non-suppressor strain HB2151 was used to deter-

mine the DNA sequences of the clones. The nucleotide sequences were determined by the dideoxy method [28] using an ABI PRISM® BigDye® Terminator (Applied Biosystems, Foster City, CA, U.S.A.) and an ABI PRISM® 3100-Avant Genetic Analyzer.

Construction of the expression plasmids

The pHEN2 DNA was cleaved with *NcoI* and *NotI*, and DNA fragments encoding the scFvs of the selected clones were inserted between the *NcoI* and *NotI* sites of pET22b(+) (Novagen, Madison, WI, U.S.A.). The resulting plasmids were designated as pET22b(+)-5E and pET22b(+)-18F1. Two *NdeI* sites in the hPDI cDNA (cloned in our laboratory) and hP5 cDNA [11] were created by site-directed mutagenesis using PCR as described previously [18]. Two *NdeI* sites in the hPDIR cDNA [10] and *XhoI* and *BamHI* sites in the yPDI (cloned in our laboratory) were created in a similar manner. The above PCR products of the hPDI, hP5 and hPDIR cDNAs and their mutant cDNAs and yPDI DNA and its mutant DNAs were inserted downstream of the His-tagged coding region of pET15b (Novagen) in the *NdeI* site and *XhoI* and *BamHI* sites respectively. Various mutants were produced by site-directed mutagenesis using PCR. For example, hPDI-m1, in which the first active site has been destroyed by replacing both the cysteine residues of the CXXC motif with serine, was produced using 5'-CCCTTGGAGTGGCCACAGCAAGGCT-3' and 5'-AGCCTTGCTGTGGCCACTCCCAAGGG-3' as the forward and reverse primers respectively (the mutated nucleotides are underlined). Other mutants were prepared in the same manner.

Expression and purification of His-tagged scFvs

E. coli AD494(DE3) was transformed with plasmids of the pET22b(+) series, and the transformants were grown at 37 °C in Luria-Bertani medium containing 100 μ g/ml ampicillin with shaking. When the absorbance at 600 nm reached 0.4–0.6, isopropyl β -D-thiogalactoside was added at a final concentration of 1 mM and the incubation was continued at 30 °C for 6 h. Cells were collected by centrifugation, suspended in a 20 mM sodium phosphate buffer (pH 7.4) and disrupted with an ultrasonic cell disrupter. The supernatant was passed through a Minisart (0.2 μ m; Sartorius, Belmont, Surrey, U.K.) and applied on to an Ni²⁺-chelating resin column (Amersham Biosciences) for purification.

Western-blot analysis

SDS/PAGE was performed as described by Laemmli [29] using 12.5 % (w/v) gels. After SDS/PAGE, the gels were electroblotted by the semi-dry method. Hybond-C nitrocellulose (Amersham Biosciences) was blocked with 10 % (w/v) skimmed milk in PBS and, subsequently, phage antibodies (10¹¹ pfu (plaque-forming units)/ml) were added. After 2 h, a mouse anti-M13 monoclonal antibody and a horseradish peroxidase-conjugated goat anti-mouse IgG antibody were added.

Biomolecular interaction

The binding of selected scFvs to immobilized PDI family proteins was measured in a surface plasmon resonance apparatus using a Biacore 3000 instrument (Biacore, Uppsala, Sweden). The hPDI, hPDIR, hP5 and yPDI proteins (approx. 5 $\times$ 10⁻⁹ g) were covalently coupled with the matrix of a CM5 sensor chip via amino groups according to the manufacturer's instructions. The scFvs were then delivered to the protein-coated flow cell at a flow rate of 20 μ l/min to observe the binding. In other experiments, scFvs (approx. 3 $\times$ 10⁻⁹ g) were covalently coupled with CM5 sensor

Figure 1 Western-blot analysis of PDI family proteins using polyclonal antibodies

The ability of an anti-bPDI antibody (A) and an anti-human P5 antibody (B) to detect PDI family proteins was tested by Western-blot analysis. Lane 1, marker proteins; lane 2, hPDI; lane 3, hPDI; lane 4, hPDI; lane 5, hPDI; lane 6, yPDI. Approx. 5 μ g of each protein was used.

chips and several peptides bearing sequences around the active sites of PDI family proteins were used as the analytes. Several tetrapeptides were synthesized by Fmoc (fluoren-9-ylmethoxycarbonyl) solid-phase peptide synthesis using Rink-amide resin [30]. Data analysis was performed using Biacore evaluation version 3.1 software.

Analysis of the isomerase activities of PDI family proteins

The isomerase activities of PDI family proteins were determined by the method of Ibbetson and Freedman [31], in which the enzyme-catalysed reduction of disulphide bonds of insulin by GSH is coupled with the reduction of GSSG to GSH by NADPH and glutathione reductase. The assay was performed in 2 ml cuvettes containing final concentrations of 8 mM GSH, 30 μ M insulin, 120 μ M NADPH, 1 unit of glutathione reductase, 5 mM EDTA and PDI family proteins (usually 0.1 μ M) in 200 mM sodium phosphate buffer (pH 7.5). The reaction was monitored at 25 °C for 10 min in a U-3210 spectrophotometer (Shimadzu, Kyoto, Japan) at 340 nm.

RESULTS

Selection of the phage antibodies specific for PDI family proteins and sequencing

Many organisms, ranging from yeast to mammals, possess PDI enzymes. Whereas the amino acid sequence of hPDI is highly homologous with bPDI, the mammalian and yeast PDIs bear only limited sequence homology. Furthermore, there is little homology between amino acid sequences of the PDI family proteins except the CXXC motif and its neighbouring sequence. We examined the cross-reactivity of the polyclonal antibodies raised against various PDI family proteins with other PDI family proteins. The results are shown in Figure 1, which reveals that a polyclonal antibody raised against bPDI recognizes only hPDI and bPDI, whereas a polyclonal antibody raised against hP5 recognizes only hP5. These observations prompted us to search for cross-reactive anti-(PDI-family protein) antibodies from the human synthetic phage display antibody library established by Griffiths et al. [16]. Candidate clones were screened by panning using hPDIR and bPDI as antigens, and their binding was detected by ELISA. As a result, several phages were found to recognize the PDI family

Figure 2 Detection of PDI family proteins by the 5E phage antibody

The ability of the 5E phage antibody to detect PDI family proteins was tested by Western-blot analysis. Lane 1, marker proteins; lane 2, hPDI; lane 3, hPDI; lane 4, bPDI; lane 5, hP5; lane 6, yPDI. The 5E phage antibody (10¹¹ pfu/ml) and each protein (approx. 5 μ g) were used. The asterisk indicates a breakdown product of hPDIR.

hPDI (1st)	V E F Y A P W C G H C K A L A P
hPDI (2nd)	V E F Y A P W C G H C K Q L A P
hPDIR (1st)	I M F Y A P W C S M C K R M M P
hPDIR (2nd)	V M F H A P W C G H C K K M K P
hPDIR (3rd)	V M F Y A P W C P H C K K V I P
hP5 (1st)	V E F Y A P W C G H C Q R L A P
hP5 (2nd)	V E F Y A P W C G H C K N L E P
yPDI (1st)	E F F Y A P W C G H C K N M A P
yPDI (2nd)	V L Y Y A P W C G H C K R L A P

Figure 3 Comparison of the CXXC motif and its neighbouring sequences in PDI family proteins

The most conserved region (APWCGHCK) is indicated by dark grey boxes.

proteins hPDI, bPDI, yPDI, hP5 and hPDIR by Western blotting (Figure 2). We determined the nucleotide sequences of the coding regions of selected clones, 5E and 18F1, by the dideoxy method and compared the deduced amino acid sequences. The families and germ lines of the VH and VL segments of these clones were determined as described by Tomlinson et al. [22] and Williams and Winter [23]. The VH segment sequences of both clones were identical and were derived from the VH1 family and the DP-3 lineage. The VL segments of the 5E and 18F1 clones varied and were from the V λ 1 and V λ 3 families respectively and from the DP-L3 and DP-L16 germ lines respectively. The deduced VH-CDR3 amino acid sequences of the 5E and 18F1 clones were GVFTYFDY and FPSL respectively.

Determination of dissociation constants (K_D) by the Biacore system

From the low amino acid sequence homology among PDI family proteins, we predicted that the 5E and 18F1 clones selected above would probably recognize the CXXC motif and neighbouring sequences, since these similarities are present in all the PDI family proteins that we tested (Figure 3). However, despite these similarities, hP5 was less strongly recognized by the clones in ELISA compared with the other PDI family proteins (Figure 4). To investigate this, we examined the binding of 5E and 18F1 to the PDI family proteins using the Biacore system. In this experiment, the His-tagged scFvs were expressed as described in the Materials and methods section, extracted and purified to a single band as seen by SDS/PAGE. PDI family proteins that were purified in a similar manner were immobilized in the Biacore 3000 on a

Ligand	Dissociation Constant (M)		ELISA
	Analyte		
	5E	18F1	
hPDI	4.32x10 ⁻⁷	5.02x10 ⁻⁷	
hPDIR	1.01x10 ⁻⁶	3.90x10 ⁻⁶	
hP5	3.11x10 ⁻⁴	2.92x10 ⁻⁴	
yPDI	4.01x10 ⁻⁷	5.63x10 ⁻⁶	

Figure 4 Dissociation constants (K_D) of scFvs for 5E and 18F1 and ELISA of PDI family proteins with 5E

The data analysis was performed using the BIA evaluation version 3.1 software.

Figure 5 Sensorgrams for the binding of the 5E scFv to immobilized hPDIR (A) or hPDI (B)

All analytes [1 ($7.5 \mu\text{M}$), 2 ($3.75 \mu\text{M}$) and 3 ($1.675 \mu\text{M}$)] of the 5E scFv, which are shown in the inset, were injected over immobilized hPDIR (A) or hPDI (B) on the sensor chip. Progress in the binding of the 5E scFv to immobilized hPDI and hPDIR was assessed by following the increase in the signal [expressed in terms of RU (resonance unit)].

CM5 sensor chip, and the binding of the scFvs was monitored. As indicated in Figure 5, the binding curves of 5E scFv to immobilized hPDIR and hPDI demonstrate the typical sensorgram of an antibody to an antigen on the Biacore system, since it shows that the association occurs gradually and the dissociation occurs slowly. The K_D values were determined from the curves and are shown in Figure 4. The K_D values of the 5E scFv for hPDI, hPDIR and yPDI were 4.32×10^{-7} , 1.01×10^{-6} and 4.01×10^{-7} M respectively, whereas that for hP5 was 3.11×10^{-4} M. These observations indicate that only hP5 is recognized weakly by 5E scFv in comparison with other proteins, which is in agreement with the ELISA results (Figure 4). These results indicate that the affinity of the 5E phage for native hP5 is low. The affinity values of our clones are lower than those described by Griffiths et al. [16] (10^{-8} – 10^{-9} M). Since the ligand size is very limited in the present study, it is possible that the library did not contain phages with high affinity for the region common to both bPDI and hPDIR. The K_D value of phage 5E for hPDI was approx. 10^{-9} – 10^{-10} M, indicating that 5E scFv had a lower affinity when compared with the phage antibody (results not shown). The 18F1 scFv showed almost the same K_D value as 5E scFv for PDI family proteins.

Binding specificities of 5E

As shown in Figure 3, the sequence common to PDI and PDIR is APWCGHCK, but K is replaced by Q in hP5. To determine the sequence recognized by the 5E clone, we first performed Western-

Figure 6 The domain structures of hPDI and yPDI, the sequences of their mutants and recognition of the mutants by the 5E phage antibody by Western blotting

(A) Domains a and a' (striped boxes), redox-active Trx domains; domains b and b' (grey boxes), redox-inactive Trx domains; and domain c (white box), a putative calcium-binding domain. The C-terminal KDEL and HDEL sequences are the possible ER-retention signals. (B) Detection of hPDI and yPDI mutants by the 5E phage antibody by Western blotting. Lane 1, marker proteins; lane 2, hPDI-m1; lane 3, hPDI-m2; lane 4, hPDI-m12; lane 5, yPDI-m1; lane 6, yPDI-m2; lane 7, yPDI-m12. hPDI and yPDI are shown in Figure 2. The 5E phage antibody (10^{11} pfu/ml) was used with each protein (approx. $5 \mu\text{g}$).

blot analysis using various hPDI and yPDI mutants (Figure 6). With regard to the designation of these mutants, the mutant in which the first or the second active site has been destroyed by replacing both cysteine residues of the CXXC motif with serine was designated as m1 or m2 respectively. The mutant in which both active sites were destroyed was designated as m12. The hPDI-m12 and yPDI-m12 mutants that had lost both CXXC motifs were not detected by the 5E phage antibody by Western blotting (Figure 6). These results, together with the observation that the active sites of all of the PDI family proteins show high sequence homology (Figure 3), suggest that the 5E clone recognizes the CXXC-motif-containing sequence.

We also examined the ability of the phage antibody to recognize mutants of hPDIR and hP5. As shown in Figure 7(A), hPDIR has three different motifs, namely CSMC, CGHC and CPHC. The hPDIR mutants hPDIR-m2, hPDIR-m12, hPDIR-m23 and hPDIR-m123, which have in common the destruction of the second active site (CGHC) due to the replacement of both cysteine residues with serine, were not detected by the 5E clone (Figure 7B). These results clearly indicate that the 5E clone specifically recognizes the CGHC-motif-containing sequence. In addition, the hPDIR mutants hPDIR-CGHS and hPDIR-SGHC, in both of which one of the cysteine residues in the CGHC motif has been substituted, were not detected by the 5E clone by Western blotting (Figure 7B). This observation reveals that detection of the PDI family proteins by Western-blot analysis using the 5E clone depends on both the cysteine residues of the CGHC motif.

With regard to the hP5 protein, the active sites consist of CGHCQ (Figure 8A, domain a') and CGHCK (domain a). The results obtained using the hP5 mutants demonstrate that hP5-m2, which lacks the second CGHCK, is not detected by the 5E

Figure 7 The domain structure of hPDIR, the sequences of its mutants and recognition of the mutants by the 5E phage antibody by Western blotting

(A) Domains a⁰ and a¹ (striped boxes), redox-active Trx domains; domain b (grey box), redox-inactive Trx domain; and domain c (white box), a putative calcium-binding domain. The C-terminal KEEL sequence is a possible ER-retention signal. (B) Detection of the mutants by the 5E phage antibody by Western blotting. Lane 1, marker proteins; lane 2, hPDIR-m1; lane 3, hPDIR-m3; lane 4, hPDIR-m2; lane 5, hPDIR-m12; lane 6, hPDIR-m13; lane 7, hPDIR-m23; lane 8, hPDIR-m123; lane 9, hPDIR-CGHS; lane 10, hPDIR-SGHC; lane 11, hPDIR-m12-GH; lane 12, hPDIR-m23-GH. hPDIR is shown in Figure 2. The 5E phage antibody (10^{11} pfu/ml) was used with each protein (approx. 5 μ g).

Figure 8 The domain structure of hP5, the sequences of its mutants and recognition of the mutants by the 5E phage antibody by Western blotting

(A) Domains a⁰ and a¹ (striped boxes), redox-active Trx domains; domain b (grey box), redox-inactive Trx domain; and domain c (white box), a putative calcium-binding domain. The C-terminal KDEL sequence is a possible ER-retention signal. (B) Detection of the mutants by the 5E phage antibody by Western blotting. Lane 1, marker proteins; lane 2, hP5-m1; lane 3, hP5-m2; lane 4, hP5-m12; lane 5, hP5-Q40K; lane 6, hP5-m2Q40K. hP5 is shown in Figure 2. The 5E phage antibody (10^{11} pfu/ml) was used with each protein (approx. 5 μ g).

clone, despite the fact that this mutant still has the first CGHCQ sequence. This observation suggests that the 5E clone recognizes the sequence containing CGHCK. Furthermore, we used the

Table 1 Recognition of various synthetic peptides by selected clones

Analyte	K_D (M)	
	Ligand 5E	Ligand 18F1
FYAPWCGHCK	5.81×10^{-6}	8.95×10^{-4}
FHAPWCGHCK	8.96×10^{-4}	6.96×10^{-4}
APWCGHC	1.18×10^{-4}	4.02×10^{-4}
FYAPWC	1.66×10^{-3}	3.03×10^{-3}
CGHCK	3.32×10^{-5}	3.26×10^{-4}
CGHCQ	4.30×10^{-4}	3.76×10^{-4}
CGHC	1.33×10^{-4}	2.16×10^{-4}
PWCG	N.D.*	N.D.
YAPW	3.70×10^{-4}	4.92×10^{-4}

* N.D., not detected.

Biacore system to determine whether these clones react with only the CGHC motif or with the CGHC motif plus residues in its vicinity. Thus purified scFvs of 5E and 18F1 were immobilized as ligands, and various synthetic peptides encoding CGHC alone or with additional flanking residues or mutated CGHC motifs were used as analytes (Table 1). The presence of a lysine residue at the end of the CGHC motif clearly increased the binding strength of 5E (see peptides CGHCK and FYAPWCGHCK in Table 1), but had no effect on the binding affinity of the 18F1 clone (Table 1). The 5E and 18F1 clones only weakly reacted with the other peptides shown in Table 1, as well as with the FYAP, HAPW and FHAP peptides (results not shown). In addition, the 5E and 18F1 clones showed no reaction with PWCG (Table 1). These results suggest that the 5E clone best recognizes the sequence containing YAPWCGHCK, whereas the 18F1 clone may recognize the sequence containing APWCGHC. Thus the 5E clone recognizes mainly CGHCK, and the flanking sequence possibly strengthens the 5E binding.

Correlation between isomerase activity and the affinity of 5E scFv for the active sites

It has also been shown that the N-terminal CGHC motifs of hPDI and hP5 demonstrate higher isomerase activities when compared with the C-terminal motifs [18], whereas in yPDI, the C-terminal motif is stronger than the N-terminal motif [32]. Although the isomerase activities of these sites may be shaped by the primary structures surrounding them, it is also possible that the extent of the exposure of the active sites due to conformational constraints affects their isomerase activity. However, the tertiary structures of most of the PDI family proteins are still not known. Thus we decided to estimate the location of the active site using the 5E clone, which recognizes the CGHCK sequence. We examined how the strength of isomerase activity is affected by the primary structure and location of the active site. We determined the K_D value of the 5E scFv for various PDI family proteins and their mutants bearing one CGHCK sequence by using the Biacore system, since these K_D values provide the accessibility and binding strength of the 5E scFv for the CXXC motif and its vicinity in various PDI family proteins. We also measured the isomerase activities of various PDI family proteins and their mutants. The isomerase activities and K_D values of the m1 and m2 mutants of hPDI and yPDI, which lack the N- and C-terminal active sites respectively, were compared first (Table 2). The N-terminal motif of hPDI (hPDI-m2) showed a stronger affinity for the 5E scFv and showed higher isomerase activity than the C-terminal motif (hPDI-m1), whereas the opposite results were obtained using

Table 2 Dissociation constants (K_D) and isomerase activities of PDI family proteins and their mutants bearing one CGHCK sequence

Ligand	K_D (M)*	Order of affinity	Isomerase activity (%)	Order of activity
hPDI	4.06×10^{-7}		100.00	
hPDI-m2	2.50×10^{-7}	1	92.83	1
hPDI-m1	2.36×10^{-4}	9	53.46	5
hPDI-m12	N.D.†		0.00	
hP5	3.66×10^{-4}		45.00	
hP5-m2Q40K	2.41×10^{-5}	7	60.92	4
hP5-m1	3.83×10^{-5}	8	22.50	6
hP5-m12	N.D.		0.00	
hPDIR	2.36×10^{-6}		2.50	
hPDIR-m13	4.09×10^{-6}	5	1.38	8
hPDIR-m12-GH	1.80×10^{-6}	3	1.84	7
hPDIR-m23-GH	8.19×10^{-6}	6	1.00	9
hPDIR-m123	N.D.		0.00	
yPDI	3.30×10^{-7}		200.00	
yPDI-m2	4.05×10^{-6}	4	62.40	3
yPDI-m1	7.80×10^{-7}	2	86.88	2
yPDI-m12	N.D.		0.00	

* Determined by using the 5E scFv. Results are the means for two separate experiments.

† N.D., not detected.

yPDI, as expected. These observations suggest that the 5E scFv is more accessible to the N-terminal motif and its vicinity of hPDI and the C-terminal motif and its vicinity of yPDI, and the strength of the isomerase activities of hPDI and yPDI correlates with the strength of the binding of the 5E scFv to the CGHC motif and its vicinity. The isomerase activities and K_D values of the hP5 mutants were then examined. The wild-type protein bears an N-terminal CGHCQ sequence and a C-terminal CGHCK sequence (the sequence recognized by 5E; Figure 8A). The hP5-m2Q40K mutant, which bears the CGHCK sequence at the N-terminus and no other CGHC sequence, showed a slightly lower K_D value when compared with hP5-m1, which bears only CGHCK at its C-terminus. These results suggested that the 5E scFv is more accessible to the N-terminal motif of hP5 than to the C-terminal one. In addition, the isomerase activity of the former was higher than that of the latter (Table 2).

The hPDIR protein is unique in that it possesses three different motifs, namely CSMC, CGHC and CPHC. To verify whether the location of the CGHC motif affects the isomerase activity, we produced hPDIR mutants and assessed their isomerase activities and K_D values. On the basis of the isomerase activities and K_D values of hPDIR-m23-GH, hPDIR-m13 and hPDIR-m12-GH (Table 2), the order of accessibility of the 5E scFv to the three motifs in hPDIR was CPHC > CGHC > CSMC (Figure 7A). The order of isomerase activities of these mutants was the same as the order of accessibility of the 5E scFv to their active sites. Results obtained using hP5 and hPDIR mutants were completely in agreement with the results obtained using hPDI and yPDI mutants. These observations strongly suggest that the isomerase activity correlates with the accessibility of the 5E scFv to the active site on the protein. However, the relationship between the strength of isomerase activity and the accessibility of the 5E scFv to the CGHC motif and its vicinity is limited to individual PDI family proteins, and cannot be expanded to all PDI family proteins. As evident from results shown in Table 2, the affinity of hPDIR and its mutants for 5E scFv, for example, was stronger than that of hP5 and its mutants for 5E scFv, whereas the isomerase activities of the former were considerably lower than those of the latter.

Table 3 Effect of lysine at the end of the CGHC motif on the isomerase activity

Each value is shown as the mean of two separate experiments.

hP5, hPDI and their mutants	Isomerase activity (%)
hP5	100.00
hP5-Q40K	145.24
hP5-m2	75.00
hP5-m2Q40K	135.37
hP5-m2Q40R	67.72
hP5-m1K175Q	41.77
hP5-m1	50.00
hP5-m12	0.00
hPDI	100.00
hPDI-m2	92.83
hPDI-m2K40Q	68.50
hPDI-m2K40R	80.23
hPDI-m12	0.00

Importance of lysine residue at the end of the CGHC motif in isomerase activity

As shown in Table 2, the isomerase activity of hP5-m2Q40K, which has one CGHCK sequence, was higher compared with that of wild-type hP5. This result indicates that the lysine residue at the end of the CGHC motif increases isomerase activity. This is supported by the fact that the CGHCQ sequence is unique to hP5 among all PDI family proteins (Figure 3). To confirm this notion, we produced several mutants of hP5 and hPDI and compared their isomerase activities (Table 3). When hP5-Q40K, which has two CGHCK sequences, was compared with wild-type hP5, which has one CGHCK and one CGHCQ sequence, the isomerase activity of the former increased by up to approx. 145 %, whereas that of hP5-m2, which has one CGHCQ sequence, decreased to approx. 75 %. Similarly, the isomerase activities of hP5-m1K175Q, which has one CGHCQ sequence at its C-terminus, and hP5-m1, which has one CGHCK sequence at its C-terminus, were approx. 42 and 50 % respectively when compared with the wild-type. A comparison of the isomerase activities of hPDI-m2K40Q, bearing one CGHCQ, and hPDI-m2, bearing one CGHCK, at the same position indicates that replacement of the lysine residue with glutamine decreases isomerase activity. To examine the effect of the arginine residue on the isomerase activity, we produced two mutants, namely hPDI-m2K40R, bearing one CGHCR, and hP5-m2Q40R, bearing one CGHCR. A comparison of the isomerase activities of hPDI-m2K40R and hPDI-m2 and hP5-m2Q40K and hP5-m1 indicates that replacement of the lysine residue with arginine decreases the isomerase activity (Table 3). These results indicate that the lysine residue at the end of the CGHC motif generally increases the isomerase activity.

DISCUSSION

PDI and the PDI family proteins, in which the CXXC motif acts as the active isomerase site, occur in organisms ranging from yeast to mammals. We have shown for the first time that polyclonal antibodies that have been raised against particular PDI family proteins do not cross-react with other PDI family members. This indicates that the polyclonal antibodies do not recognize the CXXC motif, although the motif and the sequences in its vicinity are conserved in the PDI family proteins. It is probable that the difficulty in raising CXXC-specific antibodies is due to immune

tolerance to this important self-motif that eliminates possible destructive autoimmune reactions. We speculated that the use of different technologies may generate an antibody that recognizes the CXXC motif and that would react not only with the PDI protein used to select it, but also with the PDI protein homologue of other organisms and other PDI family proteins. Indeed, when we used a phage display library and several rounds of panning using hPDIR and bPDI as antigens, we successfully obtained cross-reacting CXXC-specific phage antibodies. These phage antibodies recognized hPDI, bPDI, hP5, hPDIR and yPDI. Additional experiments allowed us to conclude that our clones react with sequences containing the CGHC motif or CGHCK. It has been reported recently that secretory IgA antibodies in human tears and milk recognized regions containing the CGHC motif and the VKVVV sequence, which is another conserved region in the PDI [33]. We believe our clones are more specific than these antibodies and easier to prepare.

CGHC is not the only sequence conserved between the PDI proteins, as shown in Figure 3. Also conserved are the (i) FYA residues, which are part of the β -strand, (ii) PWCG, which is on a loop region, and (iii) HCK, which is located at the beginning of α -helix [34]. However, binding studies using the clones and synthetic peptides revealed that neither 5E nor 18F1 reacted with the peptide PWCG. Biacore analysis also revealed that the lysine residue on the C-terminal side of the CGHC motif is important in the 5E clone epitope, but is not part of the epitope of the 18F1 clone. This difference between the 5E and 18F1 clones may arise from their different VH-CDR3 sequences (GVFTYFDY and FPSL respectively). The Biacore data with the synthetic peptides indicate that the 5E clone best recognizes the peptide YAPW-CGHCK, whereas the clone 18F1 best recognizes the peptide APWCGHC. Based on these observations, we believe that these clones may be useful in the analysis of the CGHC motif and PDI family proteins.

To this end, we used the phage library clones to analyse why the PDI family proteins, which all bear two or three active sites, differ in the strength of their isomerase activities. It is known that the difference in activities of the PDI family proteins depends to some extent on the sequence of the CXXC motif and the primary structure. However, hPDI and hP5, which differ in their isomerase activities, both bear two CGHC motifs. Moreover, their N-terminal CGHC motifs demonstrate higher isomerase activities when compared with the C-terminal motifs [18]. In contrast, the C-terminal CGHC motif of yPDI shows stronger isomerase activity when compared with the N-terminal motif [32]. It has also been reported that when hPDI was expressed in *E. coli* and purified, the active site in the domain 'a' was located on the surface of the molecule [34]. However, the location of the other active site is unknown, since PDI is highly flexible and is difficult to crystallize. These observations suggest that the different isomerase activities of PDI family proteins may also be shaped to some extent by the degree to which their active sites are exposed to the surface. We tested this notion by using the 5E clone that we obtained from the phage library. Whereas the 5E clone recognized the N-terminal CGHC motif of hPDI and hP5 better than the C-terminal one, yPDI gave entirely opposite results. Moreover, when the strength with which the 5E clone recognized hPDI, hP5, hPDIR and yPDI and their mutants was compared with the isomerase activities of the mutants, a positive correlation between the K_D of the 5E scFv binding and isomerase activity was observed. Thus it appears that the affinity of our clone for mutant PDI family proteins correlates positively with the isomerase activity of their active sites. These observations support the notion that the strength of the isomerase activities of the PDI family proteins having the same CXXC motifs depends on the degree of accessibility of

the 5E scFv to their active site at the protein surface. However, this relationship is limited to individual PDI family proteins, since the relationship between PDI family proteins is variable (Table 2).

Our studies with the 5E antibody also revealed that a lysine residue at the end of the CXXC motif heightens the isomerase activity of the protein (Table 2). Although this lysine residue is highly conserved in PDI family proteins (Figure 3), its functional significance has not been reported. An NMR study of the domain 'a' of hPDI indicates that lysine and glycine residues of the CGHC motif form a hydrogen bond, which can be seen using RasMol version 2.7.1 [32]. The alternative sequence, CGHCQ, is found in hP5 and CaBP1 (calcium-binding protein 1), both of which bear an N-terminal CGHCQ and a C-terminal CGHCK sequences [35]. The insulin reduction assay has revealed that the reductive activity of hP5 is lower than that of hPDI, whereas the RNase-refolding assay has shown that the oxidative activity of CaBP1 is also lower when compared with hPDI [35]. Thus it is probable that hydrogen bond formation between lysine and glycine residues is important for isomerase activity and replacement of the lysine residue with glutamine and arginine may reduce isomerase activity, since they cannot engage in hydrogen-bonding. Moreover, hP5 and CaBP1 possess CGHCQ in their N-terminal active sites. Most of the PDI family proteins share the CGHC motif, but isomerase activity differs from protein to protein. This fact suggests the possibility that these proteins bear a different substrate specificity or perform quite different functions from PDI *in vivo*. This notion is supported by a recent finding that P5 expressed in the embryonic midline is required for the establishment of left/right asymmetries [7].

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