The pancreas-specific protein disulphide-isomerase PDIp interacts with a hydroxyaryl group in ligands

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Using a cross-linking approach, we have recently demonstrated that radiolabelled model peptides or misfolded proteins specifically interact *in vitro* with two members of the protein disulphide-isomerase family, namely PDI and PDIp, in a crude extract from sheep pancreas microsomes. In addition, we have shown that tyrosine and tryptophan residues within a peptide are the recognition motifs for the binding to PDIp. Here we examine non-peptide ligands and present evidence that a hydroxyaryl group is a structural motif for the binding to PDIp; simple constructs containing this group and certain xenobiotics and phytoestrogens, which contain an unmodified hydroxyaryl group, can all efficiently inhibit peptide binding to PDIp. To our knowledge this is the first time that the recognition motif of a molecular chaperone or folding catalyst has been specified as a simple chemical structure.

Key words: cross-linking, oestrogen, peptide binding, protein folding, xenoestrogen.

INTRODUCTION

In vitro experiments have shown that the ability of a protein to fold into its functional three-dimensional structure depends on its amino acid sequence and its interaction with folding catalysts and molecular chaperones. The first catalyst of protein folding to be identified was protein disulphide-isomerase (PDI), an enzyme found in the endoplasmic reticulum of higher and lower eukaryotes. The primary function of PDI is thought to be the catalysis of the formation and isomerization of disulphide bonds during the folding pathway of secretory proteins (for a review see [1]).

Internal sequence homologies within PDI have long been recognized, and a multi-domain architecture for the protein has been proposed, but only recently has a clear picture of the domain architecture and domain boundaries emerged, through a combination of limited proteolysis studies of native purified PDI [2] and the structural characterization of putative domains expressed as recombinant polypeptides [2–5]. The model that emerges is of PDI being constructed of four consecutive structural domains, **a**, **b**, **b′** and **a′**, plus a C-terminal acidic extension. The homologous **a** and **a′** domains of PDI, which contain the active-site motif -WCGHC-, share significant sequence identity with thioredoxin, a small protein involved in many cytoplasmic redox functions [6]. On the basis of this homology, PDI was recognized as a member of a superfamily of proteins containing thioredoxin-like sequences.

Several proteins with similarity to PDI have been described in higher eukaryotes on the basis that (i) they contain thioredoxin-like sequences including the active-site motifs -WCXXC-, and that (ii) they are located in the endoplasmic reticulum. These proteins, specifically ERp57 [7], ERp72 [8], ERp5 [9], PDIR [10] and PDIp [11], appear to have similar enzymic properties to PDI *in vitro*, although this has not been tested systematically.

To address the question of the nature of interactions between PDIs and their substrates we used chemical cross-linkers, which have been shown to be a powerful tool to study interactions between proteins and which can be applied to proteins available in small amounts, even in crude cell extracts. Briefly, radiolabelled peptides were added to the crude lysates, the mixture was cross-linked by using the homobifunctional cross-linking reagent disuccinimidyl glutarate (DSG), and samples were subsequently analysed by SDS/PAGE. This method allows peptides and non-native proteins to be specifically cross-linked to purified bovine liver PDI [12], to recombinant fragments of human PDI, expressed in *Escherichia coli* [13], as well as to folding catalysts in microsomal extracts or crude extracts from mammalian tissues [14].

We observed that in the presence of an extract derived from sheep pancreas microsomes, two cross-linking products with apparent molecular masses of 55 and 66 kDa could be detected (results not shown). We demonstrated that the 55 kDa cross-linking product comprised PDI, while the 66 kDa cross-linking product contained PDIp [14]. PDIp shows 45 % identity and 66 % similarity to PDI and it was demonstrated previously that both proteins could interact with presecretory proteins after their translocation into dog pancreas microsomes [15,16]. In contrast with other members of the PDI family found in higher eukaryotes, PDIp is glycosylated in a variety of species [14] and it exhibits a very specific tissue distribution. Northern-blot analysis has revealed that the mRNA coding for PDIp can only be detected in the pancreas [11], while other members of the PDI family do not show such a narrow organ specificity [17].

In order to examine the interaction between PDIp and substrates in more detail, ligands were tested by their ability to block the cross-linking of PDIp to a radiolabelled peptide, specifically Δ-somatostatin. Competition experiments with a variety of different peptides showed that only tyrosine- and tryptophan-containing peptides competed for the binding of radiolabelled Δ-somatostatin to PDIp in a microsomal extract [18]. The interaction between radiolabelled Δ-somatostatin and PDI, however, was little affected by tyrosine-containing peptides, indicating that the binding motif for PDI is different from that of PDIp. Peptides with an acidic amino acid adjacent to the tyrosine or tryptophan residue did not compete for the binding of radiolabelled Δ-somatostatin to PDIp, indicating that negatively charged residues are strongly disfavoured. We also demonstrated

Abbreviations used: DSG, disuccinimidyl glutarate; PDI, protein disulphide-isomerase.

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that a single C-terminally modified tyrosine and tryptophan can compete with the binding of radiolabelled Δ-somatostatin to PDIp. This suggests that only one amino acid in a peptide, either tyrosine or tryptophan, is sufficient to trigger the recognition by PDIp.

We observed that peptides containing tyrosine residues interacted with PDIp, whereas peptides with a phenylalanine residue in place of the tyrosine residue did not bind to PDIp [18]. From this we inferred that a hydroxyaryl group might be a structural motif involved in the binding of peptides to PDIp. In this study we focus on the properties of non-peptide ligands that can interact with the peptide-binding site of PDIp, selecting candidate compounds based on structural similarities with tyrosine residues.

EXPERIMENTAL PROCEDURES

Materials

The following reagents were from Fluka: 4,4-dihydroxydiphenyl, Bisphenol A, 2,6′-dihydroxynaphthalene, coumestrol, apigenin, quercetin and genistein. 'Scrambled' RNase A, the homobifunctional cross-linking reagent DSG and all other chemicals were obtained from Sigma. ^{125}I-labelled Bolton and Hunter labelling reagent, ECL reagent and X-ray films were purchased from Amersham. The somatostatin derivative without cysteine residues (Δ-somatostatin, Ala-Gly-Ser-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Ser) was synthesized as described previously for other peptides [19].

Construction of the expression vectors for PDIp domains

Three expression vectors for domain fragments of PDIp were constructed corresponding to Glu-9–Leu-511 (PDIp), Val-238–Leu-511 (PDIp **b′-a′-c**) and Val-238–Pro-375 (PDIp **b′**) of the full-length sequence of PDIp [11]. These constructs correspond, by sequence alignment, to those of PDI previously used to study peptide binding [13]. The DNA inserts were prepared by PCR from Pa-1 [11], using primers that allowed insertion of a *Nco*I site at the N-terminus and a *Sal*I site at the C-terminus. The primers complementary to the 5′-end of the fragments also included an initiating methionine, while the primers complementary to the 3′-end included a stop codon. The inserts were cloned between the *Nco*I and *Sal*I sites of pET23d.

Gene expression

Protein production was carried out in *E. coli* strain BL21 (DE3) carrying the pLysS plasmid to control leak-through expression and to allow subsequent cell lysis by freeze–thawing. Strains were grown in LB medium at 37 °C and induced for 4 h with 2 mM isopropyl β-D-thiogalactoside.

Preparation of reticuloplasmic proteins

Sheep pancreas microsomes were prepared as described for the preparation of dog pancreas microsomes [20]. Reticuloplasmic proteins from sheep pancreas microsomes were prepared as described recently [14].

Binding of peptides and scrambled RNAse

After precipitation with trichloroacetic acid, the radiolabelled Δ-somatostatin was dissolved in distilled water. Labelled Δ-somatostatin or scrambled RNAse was added to buffer A (100 mM NaCl/25 mM KCl/25 mM sodium phosphate, pH 7.5), containing microsomal extracts and the ligands. The samples (10 μl) were incubated for 10 min on ice before cross-linking. For competition experiments the radiolabelled Δ-somatostatin was mixed with the compound to be tested prior to the addition of the reticuloplasmic extracts.

Cross-linking

Cross-linking was performed using the homobifunctional cross-linking reagent DSG [13]. The samples were supplied with a 1/10 volume of cross-linking solution (10 mM DSG in buffer A). The reaction was carried out for 60 min at 0 °C. Cross-linking was stopped by the addition of SDS/PAGE sample buffer [12].

The samples were subjected to electrophoresis in SDS/polyacrylamide gels (10%) with subsequent autoradiography. Quantification was performed using a Bio-Rad PhosphoImager. For Western blotting the samples were loaded on to SDS/polyacrylamide gels with subsequent electrotransfer on to a PVDF membrane. Immunodecoration was performed with a polyclonal antibody raised against PDIp [16]. The detection was carried out with ECL reagent.

RESULTS AND DISCUSSION

Compounds containing a hydroxyaryl group inhibit the interaction between peptides and PDIp

In a microsomal extract from sheep pancreas, compounds containing a hydroxyaryl group, specifically tyramine, 4n-propylphenol and 4-ethylphenol, competed efficiently with radiolabelled Δ-somatostatin for its interaction with PDIp (Figure 1 and Table

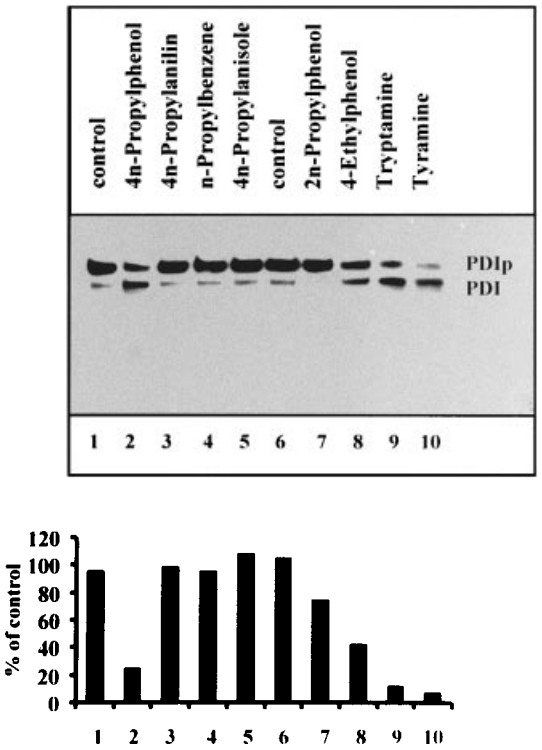

Figure 1 Inhibition of peptide binding to PDIp by compounds with a hydroxyaryl group

Microsomal extracts derived from sheep pancreas were incubated with radiolabelled Δ-somatostatin (3 μM) and with the test compounds (30 μM) with or without a hydroxyaryl group. Samples supplied with buffer served as controls. All samples were then treated with cross-linker as described in the Experimental procedures section. Quantification was performed by PhospholImager analysis.

Table 1 Inhibition of peptide binding to PDIp by various compounds

Microsomal extracts were incubated with radiolabelled Δ-somatostatin (3 μM) and the indicated compounds prior to cross-linking. All compounds were tested at a concentration of 30 μM. Those compounds that showed inhibition were also tested at 20 μM, whereas those that did not show inhibition were also tested at 60 μM. The IC_{50} values of the following compounds were determined in more detail, using a range of concentrations: tyrosine methylester (10 μM), 4n-propylphenol (10 μM), 4-ethylphenol (20 μM), 4-methylphenol (100 μM), phenol (> 450 μM). +, $IC_{50} < 20$ μM; +/−, 20 μM < $IC_{50} < 60$ μM; −, $IC_{50} > 60$ μM.

Compound	Inhibition (30 μM)
Compounds with hydroxyaryl groups	−
Phenol	−
2-Methylphenol	−
3-Methylphenol	−
4-Methylphenol	−
4-Ethylphenol	+
2n-Propylphenol	−
3n-Propylphenol	+/−
4n-Propylphenol	+
2-Phenylphenol	−
3-Phenylphenol	+/−
4-Phenylphenol	+
4,4′-Dihydroxydiphenyl	+
Tyrosine	−
Tyrosine methylester	+
Tyramine	+
Compounds without hydroxyaryl groups	
4-Ethylfluorobenzene	−
4-Ethyliodobenzene	−
4-Ethylcyclohexanol	−
4-Ethylthiophenol	−
4n-Propylaniline	−
1-Propylbenzene	−
4-Acetylbiphenyl	−
4-Biphenylcarboxylic acid	−
4-Biphenylmethanol	−
Phenylphthalide	−
Compounds with modified hydroxyaryl groups	
2,4-Dimethylphenol	−
4-Ethylcatechol	−
4n-Propylanisole	−
4-Acetoxybiphenyl	+
4-Methoxybiphenyl	−
Thyroxine	−
Tri-iodothyronine	−
3-Hydroxytyramine	−

1). Similar results were obtained with tyrosine methylester, 4-phenylphenol and 4,4′-dihydroxydiphenyl (Table 1). An identical inhibition profile was also found for the interaction between ^{125}I-labelled Δ-somatostatin and PDIp in a crude cell extract derived from rat pancreas (results not shown). Compounds with modifications of the aromatic ring, specifically 3-hydroxytyramine and 4-ethylcatechol, inhibited the binding of radiolabelled Δ-somatostatin to a lesser extent. This result is in good agreement with our previous observations that peptides with modified tyrosine residues, e.g. iodination of the aromatic ring, competed less efficiently for the binding of radiolabelled Δ-somatostatin to PDIp [18]. We speculate that the aromatic ring of the ligand interacts with an aromatic amino acid in the peptide-binding site of PDIp via π–π interactions. Such interactions are strongest when either the two aromatic rings interact with the edge of one ring perpendicular to the other, or with the rings parallel but offset. This interaction would clearly be destabilized by modifications of either one of the aromatic rings, in either the protein or substrate.

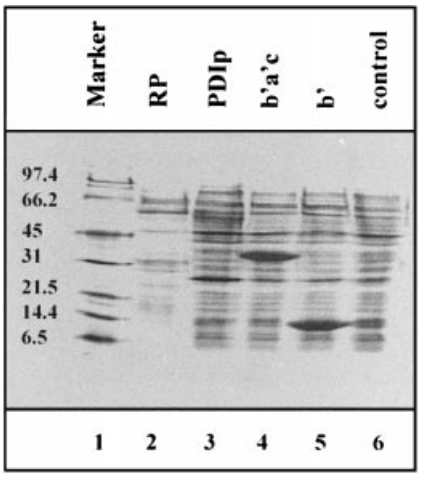

Figure 2 PDIp fragments used in cross-linking studies

Equal amounts of total *E. coli* cellular protein, as estimated by Coomassie Brilliant Blue staining of the SDS gel, were loaded. RP, reticuloplasmic proteins.

Compounds without an aromatic ring (4-ethylcyclohexanol) or without a hydroxy group (4n-propylaniline, 1-propylbenzene, 4-ethylfluorobenzene and 4n-propylthiophenol) did not compete for the binding of radiolabelled Δ-somatostatin to PDIp (Table 1).

This indicates that a hydroxy group within the recognition motif is required for the binding to PDIp and cannot be substituted by other functional groups. It also confirms our results that, unlike a tyrosine residue, a phenylalanine residue within a peptide does not support binding to PDIp [18]. 2n-Propylphenol inhibited the binding of radiolabelled Δ-somatostatin significantly less compared with 4n-propylphenol, indicating that the position of the hydroxy group is an important feature of the recognition motif.

We found that the O-methyl ether of different compounds, including 4n-propylanisole, did not inhibit the binding of radiolabelled Δ-somatostatin to PDIp. However, we found good inhibition when O-acetyl esters were used. Currently, we do not understand fully the nature of the interaction between the hydroxy group and the peptide-binding site in PDIp. It is unlikely that it acts as a simple hydrogen-bond acceptor, since in this case 4n-propylanisole, oestradiol 3-methyl ether and 4n-propylaniline would be expected to bind effectively. It also is unlikely that it is a simple hydrogen-bond donor, because if so 4n-propylaniline, but not 4-acetoxybiphenyl or 3-acetyl-17β-oestradiol, should then inhibit the interaction between radiolabelled Δ-somatostatin and PDIp.

We also noted that tryptamine could inhibit the binding of radiolabelled Δ-somatostatin to PDIp (Figure 1, lane 9). This observation is in line with our previous findings that tryptophan residues within a peptide, under certain circumstances, interfered with the peptide-binding activity of PDIp [18]. Although we currently do not understand the structural requirements for the interaction of this compound with PDIp, it is tempting to speculate that the indole system and the hydroxyaryl moiety interact with the identical residues in PDIp. Clearly, more experiments are needed to reveal the nature of this interaction and to identify residues involved within the binding site of PDIp.

The substituent in the *para* position to the hydroxy group had a major influence on the inhibition of binding of the labelled peptide to PDIp. The IC_{50} values decreased with increasing

length of the alkyl chain of the substituent up to a butyl group. Thus inhibition by 4-methylphenol was less efficient than that by 4-ethylphenol and 4n-propylphenol, whereas phenol did not show any inhibitory effects at concentrations up to 450 μM (Table 1). Since the IC_{50} decreased with increasing hydrophobicity of these substituents, we suggest that hydrophobic interactions may play an important role in the binding process. This is in line with our previous observations that the interaction of peptides with PDIp is sensitive to Triton X-100 [12].

The ligand-binding specificity of PDIp is clearly different from that of PDI. 2n-Propylphenol, 4n-propylaniline and some other tested ligands, which did not significantly inhibit peptide binding to PDIp, did interfere with the binding of radiolabelled Δ-somatostatin to PDI (Figure 1). However, the significance of this observation is not clear in the absence of a more systematic study.

The minimal requirements for efficient inhibition of the peptide binding of PDIp therefore are (i) an aromatic ring with a phenolic hydroxy group and (ii) a substituent in the *para* position with at least two carbon atoms. To our knowledge this is the first time that the recognition motif of a molecular chaperone or folding catalyst has been narrowed down to a simple chemical structure. Clearly, our findings distinguish PDIp from other chaperones, e.g. members of the Hsp70 family, for which it has been shown that the efficient binding of peptides to BiP (heavy-chain binding protein) requires at least seven amino acids, with certain amino acids in alternating positions [21]. In addition, the peptide-binding motif of heat-shock protein DnaK consists of a hydrophobic core of four to five hydrophobic residues, particularly enriched in leucine, but also in isoleucine, valine, phenylalanine and tyrosine [22,23]

The peptide-binding site within the b′-a′-c fragment of PDIp interacts specifically with compounds containing a hydroxyaryl group

Since we could not exclude the possibility that the effect of hydroxyaryl-containing compounds on the binding of radiolabelled peptides to PDIp was unspecific, we wanted to explore whether those compounds specifically interfere with peptide binding to the principal peptide-binding site of PDIp. We concluded from the high overall similarity between PDI and PDIp that the **b′** domain of PDIp contains the principal peptide-binding site; hence we generated recombinant fragments containing the **b′** domain of PDIp.

Figure 2 shows the crude bacterial cell extracts used in this study, stained for total protein. The expression of recombinant human PDIp (Glu-9–Leu-511) in *E. coli* leads to the production of the unglycosylated form, with an apparent molecular mass of 54 kDa. Overexpression of the **b′-a′-c** (Val-238–Leu-511) and the **b′** (Val-238–Pro-375) domain fragments resulted in clearly visible bands in the total extracts (Figure 2, lanes 4 and 5), and the identity of the **b′-a′-c** fragment was confirmed by immunodecoration with antibodies specific for PDIp (results not shown). The identity of the **b′** fragment was confirmed by automated N-terminal sequencing (results not shown), since the fragment was not recognized by the polyclonal antibody.

By subfractionation of the *E. coli* lysates, we found that the **b′** domain of human PDIp formed insoluble inclusion bodies when expressed in *E. coli* under all conditions tested (results not shown). In order to avoid possible artefacts generated by denaturation and subsequent refolding of the **b′** domain, we decided to use the **b′-a′-c** fragment as a model system to study the interaction with peptides and compounds containing a hydroxyaryl group.

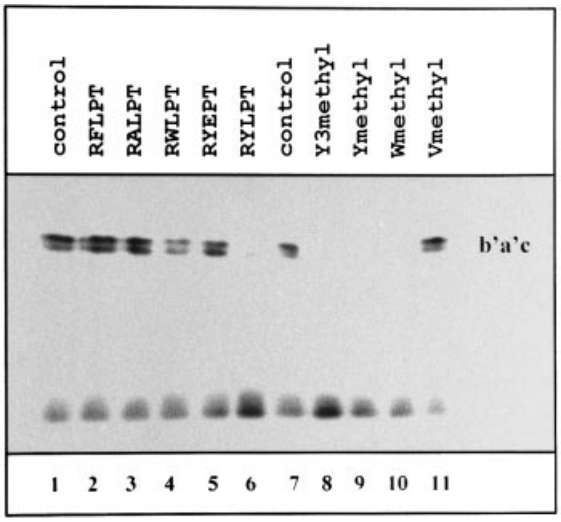

Figure 3 The b′-a′-c fragment contains the principal peptide-binding site of PDIp

An *E. coli* cell lysate, expressing the **b′-a′-c** fragment of PDIp, was incubated with radiolabelled Δ-somatostatin (3 μM) in the presence of 150 μM amino acid methyl esters or 250 μM pentapeptides (shown as single-letter code) prior to cross-linking. Y3methyl, YYY-methyl ester; Ymethyl, Y-methyl ester; Wmethyl, W-methyl ester; Vmethyl, V-methyl ester.

To demonstrate that the peptide-binding properties are the same for the recombinant **b′-a′-c** fragment of PDIp as for the full-length protein from sheep pancreas microsomes [18], we carried out competition experiments with radiolabelled Δ-somatostatin and the **b′-a′-c** fragment of PDIp in the presence of certain pentapeptides and tyrosine derivatives. As shown in Figure 3, only pentapeptides containing tyrosine or tryptophan residues competed efficiently for the interaction between the **b′-a′-c** fragment of PDIp and radiolabelled Δ-somatostatin. Also, C-terminally modified tyrosine or tryptophan competed efficiently with the interaction between the **b′-a′-c** fragment of PDIp and radiolabelled Δ-somatostatin, whereas other modified amino acids did not show any competition (Figure 3, compare lanes 8–10 with lane 11, and results not shown).

PDIp contains three potential glycosylation sites, two of which are located in the **b′-a′-c** fragment [11,24], and it has been demonstrated that PDIp from various sources is a glycoprotein [13]. Although recombinant human PDIp or the **b′-a′-c** fragment, after expression in *E. coli*, were not glycosylated, we detected efficient cross-linking of the radiolabelled Δ-somatostatin to full-length PDIp as well as to the **b′-a′-c** fragment. This result indicates clearly that glycosylation of PDIp or a fragment of it is not essential for the binding of peptides.

The overall peptide-binding properties of the full-length PDIp or the **b′-a′-c** fragment were similar to the properties observed for purified bovine PDI and the **b′** domain of human PDI, i.e. the binding was specific, reversible and sensitive to detergents [12] (results not shown). This result is not surprising, since the **b′** domains of PDI and PDIp, although being the most divergent domains, exhibit 40 % identity and 62 % similarity in their amino acid sequence [11].

As with glycosylated full-length PDIp from a microsomal extract [18], compounds containing a hydroxyaryl group, specifically tyrosine methyl ester, tyramine, 4n-propylphenol and 4-ethylphenol, competed efficiently with radiolabelled Δ-somatostatin for its interaction with the recombinant full-length PDIp

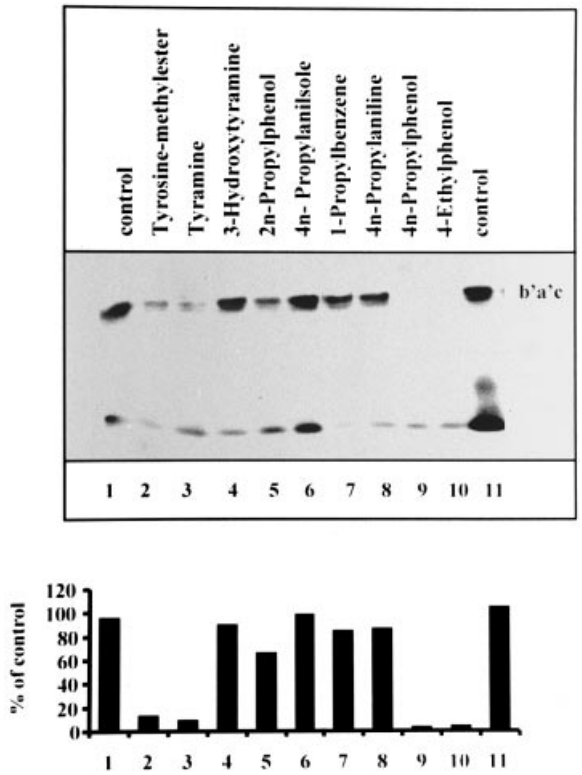

Figure 4 Inhibition of peptide binding to the b′-a′-c fragment by compounds with a hydroxyaryl group

E. coli cell lysates, expressing the **b′-a′-c** fragment of PDIp were incubated with radiolabelled Δ-somatostatin (3 μM) and test compounds (30 μM) with or without hydroxyaryl groups prior to cross-linking. Samples with buffer served as controls. Quantification was performed by PhosphoImager analysis.

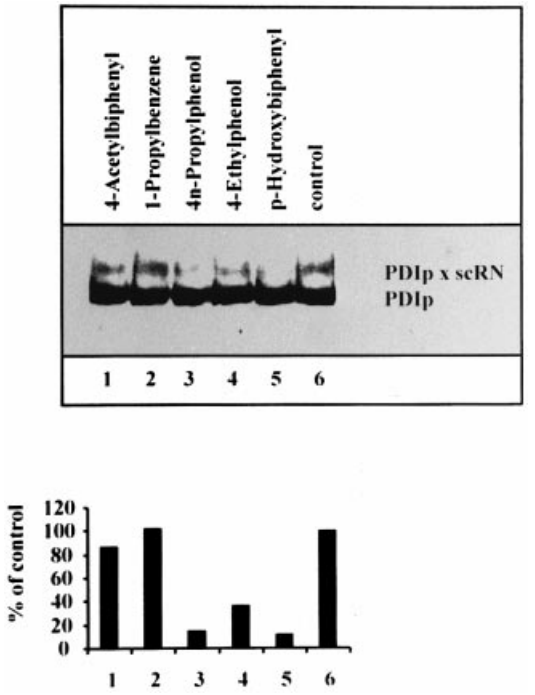

Figure 5 Inhibition of unlabelled scrambled RNase A binding to PDIp by compounds with a hydroxyaryl group

Test compounds (450 μM) with or without a hydroxyaryl group were incubated with a microsomal extract and unlabelled scrambled RNase A (scRN; 3 μM) prior to cross-linking. A sample supplemented with buffer served as a control. Endogenous PDIp (PDIp) and the cross-linking product (PDIp × scRN) were detected after Western blotting with a specific antibody raised against PDIp. Quantification was performed by densitometric analysis of the film.

(results not shown) or the **b′-a′-c** fragment of PDIp (Figure 4). Compounds with modifications of the aromatic ring, specifically 4-ethylcatechol (results not shown) and 3-hydroxytyramine, affected the binding of radiolabelled Δ-somatostatin significantly less. Compounds without an aromatic ring (4-ethylcyclohexanol, results not shown) or without a hydroxy group (4n-propylaniline, 1-propylbenzene) did not compete for the binding of radiolabelled Δ-somatostatin to the **b′-a′-c** fragment of PDIp. 2n-Propylphenol inhibited the binding of radiolabelled Δ-somatostatin significantly less, compared with 4n-propylphenol.

These results are consistent with the findings for full-length PDIp from a microsomal extract, demonstrating that a fragment of PDIp that contains the putative peptide-binding site interacts with the same pentapeptides, tyrosine derivatives or compounds containing hydroxyaryl groups.

Taken together, these results demonstrate that the glycosylation of PDIp is not essential for peptide binding and that, in analogy to PDI, the **b′-a′-c** fragment of PDIp contains the principal peptide-binding site. Furthermore, our results also showed that compounds containing hydroxyaryl groups interfere with the principal peptide-binding site located within the **b′-a′-c** fragment of PDIp.

Hydroxyaryl-containing compounds inhibit the binding of scrambled RNase A to PDIp

[^{125}I]Bolton–Hunter labelling reagent introduced a modified tyrosine residue into Δ-somatostatin, so it was essential to demonstrate that our results were independent of the specific labelled ligand used in competitive binding studies. Scrambled RNase A was cross-linked to a reticuloplasmic extract from sheep pancreas microsomes, and the cross-linking product ($\approx$ 75 kDa), comprising PDIp and scrambled RNase A, was detected by immunodecoration with antibodies directed against PDIp [18]. In this method there is no extrinsic labelling; cross-linking is detected by a shift in molecular mass. As shown in Figure 5, this higher-molecular-mass cross-linking product can be detected clearly in the presence of scrambled RNase A and the chemical cross-linker, whereas there was no cross-linking product observable in the absence of scrambled RNase A or chemical cross-linker (results not shown). In the presence of 4-hydroxybiphenyl or 4n-propylphenol, competition for the interaction between PDIp and unlabelled scrambled RNase A was observed. This confirmed the specificity of competitive ligand binding and demonstrates that the interaction is independent of the cross-linked substrate. However, we noted that, compared with radiolabelled Δ-somatostatin, much higher concentrations of the competitor were needed to inhibit the interaction between PDIp and unlabelled scrambled RNase. The most likely explanation for this result is that sites other than the principal peptide-binding site in the **b′** domain might also contribute to the binding of a misfolded protein, and therefore a higher concentration of an inhibitor is needed to shift the equilibrium between bound and unbound scrambled RNase. This would be in excellent agreement with our earlier observations that the **b′** domain of PDI is essential and sufficient for the binding of small peptide ligands,

but that the contributions of other domains are required for the binding of larger peptides or misfolded proteins.

The peptide-binding site of PDIp interacts specifically with hydroxyaryl-group-containing xenoestrogens and phytoestrogens

As demonstrated recently, the interaction between radiolabelled Δ-somatostatin and PDIp, but not PDI, was significantly diminished in the presence of 17β-oestradiol. Since PDIp does not exhibit any significant homology to oestrogen-binding proteins, we suggested that certain steroid hormones might interfere with the interactions that play an important role in the peptide-binding process [12]. Interestingly, Tsibris and co-workers [25] observed that the interaction between PDI and insulin also can be inhibited in the presence of 17β-oestradiol, and hence the existence of an oestrogen-receptor-like domain (e-domain) in PDI was proposed. Although we were able to detect some inhibitory effects of 17β-oestradiol on the peptide binding to PDI, the required concentrations were 10 times higher than that observed for the inhibition of peptide binding to PDIp. We therefore proposed that PDIp has a much higher affinity for 17β-oestradiol than PDI, which led us to the conclusion that the interaction between 17β-oestradiol and PDIp is specific. This result now can be explained by the fact that 17β-oestradiol contains a hydroxyaryl group and therefore might interfere with the binding of peptides to PDIp. From the observation that 17β-oestradiol competed efficiently for the binding of radiolabelled Δ-somatostatin to PDIp, while 17α-oestradiol showed no effect, it is clear that, in addition to the requirement for a hydroxyaryl group and a hydrophobic stretch in the *para* position, there are also other structural/steric requirements for efficient binding to PDIp. It is therefore tempting to speculate that the position of the 17α-hydroxy group interferes sterically with residues in the proximity of the peptide-binding site, thus preventing 17α-oestradiol from binding, while 17β-oestradiol interacts efficiently with PDIp.

Since many compounds that are classified as oestrogenic (xenoestrogens, phytoestrogens) contain hydroxyaryl groups, we addressed the question of whether other oestrogenic compounds can be seen to interact specifically with PDIp. As shown in Table 2, a wide range of oestrogenic compounds containing a hydroxyaryl group interfered with the binding of radiolabelled Δ-somatostatin to PDIp in a microsomal extract. A similar result was obtained when the interaction between radiolabelled Δ-somatostatin and the **b′-a′-c** fragment of PDIp was probed with these compounds (results not shown) or when the interaction with full-length human recombinant PDIp in a crude *E. coli* cell lysate was tested. However, we did not detect inhibitory effects with other steroid hormones.

Some of the compounds used in this study are classified as oestrogenic, either as phytoestrogens [26,27] (apigenin, tamoxifen, coumestrol, quercetin and genistein) or as xenobiotics with oestrogenic activities [28,29] (Bisphenol A, hexestrol and diethylstilbestrol). Our results demonstrate that most of these compounds can interact directly with the peptide-binding site of PDIp *in vitro*. Therefore it can be speculated that such compounds, containing a hydroxyaryl group, might interfere with the binding of folding substrates to PDIp *in vivo*. This could affect the formation and isomerization of disulphide bonds and hence the correct folding of newly translocated polypeptides in the acinar cells of the pancreas.

We recently demonstrated that peptides derived from digestive enzymes could bind to PDIp and that this interaction was inhibited in the presence of 17β-oestradiol [14]. We therefore speculate that high levels of oestrogens or compounds containing a hydroxyaryl group might interfere with the correct folding and subsequent secretion of zymogens or zymogen-inhibitors, like pancreatic trypsin-inhibitor, by interfering with the peptide-binding site of PDIp.

Table 2 Inhibition of peptide binding to PDIp by various compounds with or without oestrogenic activity

Microsomal extracts were incubated with radiolabelled Δ-somatostatin (3 μM) and the indicated compounds (at 30 μM) prior to cross-linking. All compounds were tested at a concentration of 30 μM. Those compounds that showed inhibition were also tested at 20 μM, whereas those that did not show inhibition were also tested at 60 μM. The following compounds without oestrogenic activity showed no inhibition at 60 μM: androsterone, epiandrosterone, dehydroepiandrosterone, cortisone, dexamethasone, aldosterone, corticosterone, pregnenolone, progesterone, testosterone, nortestosterone, prednisolone, triamcinolone, 19-nortestosterone, 20α-hydroxycholesterol, 25α-hydroxycholesterol, chenodeoxycholic acid, cholic acid, cholic acid methylester, deoxycholic acid, lithocholic acid, cholesterol and 5-cholesten-3-one. +, IC_{50} < 20 μM; −, IC_{50} > 60 μM.

Compound with oestrogenic activity	Inhibition (30 μM)
17α-Oestradiol	−
17β-Oestradiol	+
Oestriol	+
2α-Hydroxyoestradiol	+
4α-Hydroxyoestradiol	+
6α-Hydroxyoestradiol	+
Oestrone	+
17α-Ethynyloestradiol	+
Oestrone 3-methyl ether	−
17β-Oestradiol 3-methyl ether	−
3,17β-Diacetyloestradiol	+
3-Acetyl-17β-oestradiol	+
4,4-Dihydroxydiphenyl	+
Bisphenol A	+
p-Hydroxybiphenyl	+
2,6-Dihydroxynaphthalene	+
Diethylstilbestrol	+
4-Hydroxytamoxifen	+
Hexestrol	+
[*p*-Hydroxyphenyl]$_4$-ethane	+
Quercetin	+
Coumestrol	+
Apigenin	+
Genistein	+
Tamoxifen	−

Although xenobiotic compounds containing a hydroxyaryl group are not likely to be *bona fide* regulators of PDIp activity under physiological conditions, it might be possible that PDIp is inhibited *in vivo* by high levels of oestrogens. It has been suggested that high oestrogen levels, e.g. during pregnancy or due to the use of certain contraceptives or oestrogen-replacement therapy, can cause specific forms of maldigestion combined with nausea and hyperemesis as well as hypertriglyceridaemia and, in severe cases, pancreatitis [30–32]. In addition, it has been observed that oestrogens can influence cholecystokinin-stimulated pancreatic amylase release and acinar cell-membrane cholecystokinin receptors in rats [33,34].

Our results indicate that hydroxyaryl-containing xenobiotic compounds, although probably not being physiological ligands of PDIp, might have effects on protein folding. Clearly, further experiments are needed to reveal to what extent PDIp can interact with these compounds *in vivo*. This eventually should further our understanding of the biological impact of oestrogenic compounds.

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