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Specificity in substrate binding by protein folding catalysts: Tyrosine and tryptophan residues are the recognition motifs for the binding of peptides to the pancreas-specific protein disulfide isomerase PDIp

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Abstract

Using a cross-linking approach, we recently demonstrated that radiolabeled peptides or misfolded proteins specifically interact in vitro with two luminal proteins in crude extracts from pancreas microsomes. The proteins were the folding catalysts protein disulfide isomerase (PDI) and PDIp, a glycosylated, PDI-related protein, expressed exclusively in the pancreas. In this study, we explore the specificity of these proteins in binding peptides and related ligands and show that tyrosine and tryptophan residues in peptides are the recognition motifs for their binding by PDIp. This peptide-binding specificity may reflect the selectivity of PDIp in binding regions of unfolded polypeptide during catalysis of protein folding.

Keywords: cross-linking; peptide binding; protein disulfide isomerase; tryptophan; tyrosine

Native disulfide bond formation in the endoplasmic reticulum (ER) of eukaryotes is an important, but as yet, a poorly understood process. For over 30 years, the process of the formation, reduction, and isomerization of disulfide bonds during the folding pathway of secretory proteins in the ER has been thought to be catalyzed by the enzyme protein disulfide isomerase (PDI; for review see Freedman et al., 1994). By using chemical cross-linkers, it has been shown that PDI interacts with nascent and newly-translocated secretory proteins (Roth & Pierce, 1987; Klappa et al., 1995). The role of PDI in the formation of native disulfide bonds has also been demonstrated by reconstitution studies in mammalian systems and by genetic studies in yeast.

Within the last few years, there have been several major developments that have advanced our understanding of how PDI operates as a catalyst of protein folding associated with native disulfide bond formation. First, the domain architecture of PDI has been clearly established together with the structural resolution of several of the individual domains (Kemmink et al., 1995, 1997; Freedman et al., 1998). Second, the functional roles of the individual domains have become clear, together with a picture of how their roles interact to produce catalysis of folding and thiol:disulfide interchange (Darby et al., 1998; Klappa et al., 1998a). From a combination of these, it appears that in PDI, as in trigger factor (a bacterial peptidyl prolyl *cis-trans* isomerase), domains responsible for catalysis of a chemical isomerization are linked in sequence to domains responsible for binding of peptides and unfolded proteins (Scholz et al., 1997; Zarnt et al., 1997; Klappa et al., 1998a) to produce coupling in the catalysis of protein folding.

Recently, a number of papers have reported on proteins implicated in completing the catalytic cycle for native disulfide bond formation in the ER of the lower eukaryote *Saccharomyces cerevisiae*, including the recently described protein Ero1p (Frand & Kaiser, 1998; Pollard et al., 1998) and flavin-containing monooxygenase FMO (Suh et al., 1999). The in vivo situation in lower eukaryotes is further complicated by the presence in the ER of the gene products of *MPD1* (Tachikawa et al., 1995), *MPD2* (Tachikawa et al., 1997) and the open reading frame YIL005w, which all have similar active sites to PDI and probably contain similar thioredoxin-like domains. The exact role played by these gene products in native disulfide bond formation is still unclear, but it is acknowledged that *S. cerevisiae* has only a single PDI, Pdi1p (Eug1p, which shares sequence and structural homology with Pdi1p, does not contain the CXXC active site motif).

While the processes for native disulfide bond formation in higher eukaryotes are even less well understood, it is apparent that there is at least one additional complication, the presence of multiple proteins with similarity to PDI. During the past decade, several

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Abbreviations: DSG, disuccinimidyl glutarate; ER, endoplasmic reticulum; PDI, protein disulfide isomerase; scRN, "scrambled" ribonuclease A; SDS, sodium dodecyl sulfate.

proteins with similarity to PDI have been described in the ER of higher eukaryotes, specifically ERp57 (Oliver et al., 1997), ERp72 (Mazzarella et al., 1990), ERp5 (Lundstrom-Ljung et al., 1995), PDIR (Hayano & Kikuchi, 1995), and PDIp (DeSilva et al., 1996). All the members of this PDI family have similar active sites with the amino acid sequence WCXXC, and probably contain similar thioredoxin-like domains. Their activities have not been compared systematically, but they appear to have similar enzymatic properties in vitro.

The question arises why there are different members of this protein family present in the same intracellular compartment, i.e., the ER. One inevitable speculation is that they may differ in substrate specificity, but this has not to date been systematically examined. To address the question of the nature of the interaction between protein disulfide isomerases and their substrates, we used chemical cross-linkers that have been shown to be a powerful tool to study interactions between proteins and that can be applied to proteins available in small amounts even in crude cell extracts.

Recently, we demonstrated that peptides and nonnative proteins can be cross-linked specifically to purified bovine liver PDI (Klappa et al., 1997), to recombinant fragments of human PDI, expressed in *Escherichia coli* (Klappa et al., 1998a), as well as to PDI and PDIp in microsomal extracts or crude extracts from mammalian tissues (Klappa et al., 1998b). We added radiolabeled peptides to the crude lysates, the mixture was cross-linked by using the homobifunctional cross-linking reagent disuccinimidyl glutarate (DSG) and subsequently analyzed by SDS-PAGE. Interaction of other ligands could be studied by their ability to compete against cross-linking of the labeled peptides.

In the presence of an extract derived from sheep pancreas microsomes, two cross-linking products with an apparent molecular mass of 55 and 66 kDa, respectively, could be detected (Fig. 1). We demonstrated that the 55 kDa cross-linking product comprised PDI, while the 66 kDa cross-linking product contained glycosylated PDIp (Klappa et al., 1998b). We now explore in detail the peptide binding specificity of these two homologous proteins, demonstrate that they show differences in specificity, and analyze the requirement for substrate binding to PDIp.

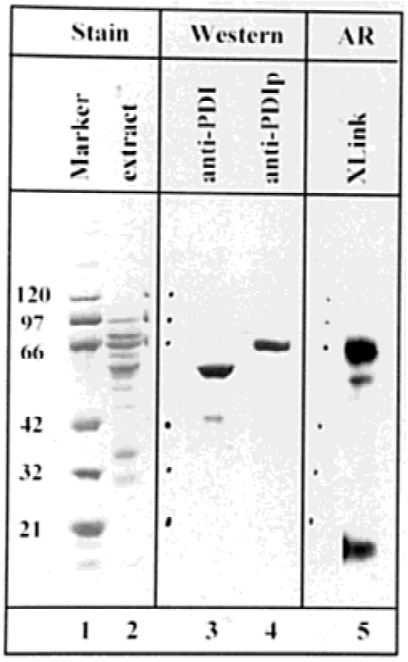

Fig. 1. A radiolabeled peptide interacts with PDI and PDIp. [^{125}I]-Bolton–Hunter labeled Δ-somatostatin was incubated with reticuloplasmic proteins and chemical cross-linker. Autoradiography (AR) of cross-linking products (Xlink); Coomassie-stained extract (Stain) containing reticuloplasmic proteins (extract); immunodecoration (Western) of reticuloplasmic extract using antibodies raised against PDI and PDIp, respectively. M, molecular weight marker.

Results

Tyrosine and tryptophan residues within a peptide are the recognition motifs for the binding of PDIp

To address the question whether PDI and PDIp interact with specific motifs within a peptide, we synthesised several (>40) pentapeptides and tested them for their ability to compete with the binding of a radiolabeled peptide, specifically radiolabeled Δ-somatostatin (AGSKNFFWKTFTSS). As shown in Figure 2A, only pentapeptides containing tyrosine or tryptophan residues competed with the binding of Δ-somatostatin, and hence bound efficiently to PDIp as judged by this assay. In contrast, pentapeptides that contained tyrosine residues did not inhibit the interaction between PDI and Δ-somatostatin (Fig. 2A). This clearly indicates that PDI and PDIp have different substrate binding specificities. The precise nature and context dependence of this recognition motif for PDIp is explored in detail here; the more complex recognition motif of PDI is still under investigation.

The position of the tyrosine residue within a pentapeptide did not have a major influence on its inhibition of labeled peptide binding to PDIp (Fig. 2B), since the apparent IC_{50} values for all these peptides were of the same order of magnitude (100–250 μM). The small differences in apparent IC_{50} values observed here should not be seen as significant. Quantification in the whole cell lysate system used is, by its nature, prone to a higher degree of variation than that obtainable in a system using a purified protein. To ensure reproducibility, all peptides and amino acids (see below) were tested at least three times and all of the results obtained were internally consistent (within ±10%).

Interestingly, we found that pentapeptides containing acidic amino acids (Glu or Asp) immediately adjacent to the tyrosine or tryptophan residues did not inhibit the interaction between PDIp and radiolabeled Δ-somatostatin, indicating that these peptides did not compete for the interaction between PDIp and Δ-somatostatin (Fig. 2C). In contrast, peptides with a tyrosine residue plus a nonadjacent acidic amino acid showed competition for the interaction between PDIp and Δ-somatostatin. This clearly demonstrates that acidic amino acid residues only abolish the interaction between PDIp and a tyrosine-containing peptide when they are adjacent to the tyrosine residue. In contrast, the position of the C-terminal carboxylate of the pentapeptide, with respect to the tyrosine, did not appear to significantly alter the apparent binding affinity, though it is probable from the results with single amino acids (see below) that it does have an overall inhibitory effect.

[^{125}I] Bolton–Hunter labeling reagent introduced a modified tyrosine residue into Δ-somatostatin, hence it was essential to demonstrate that these results were independent of the labeled substrate and the method of labeling. "Scrambled" ribonuclease A was cross-linked to a reticuloplasmic extract and the cross-linking product, comprising PDIp and "scrambled" ribonuclease A, was detected by

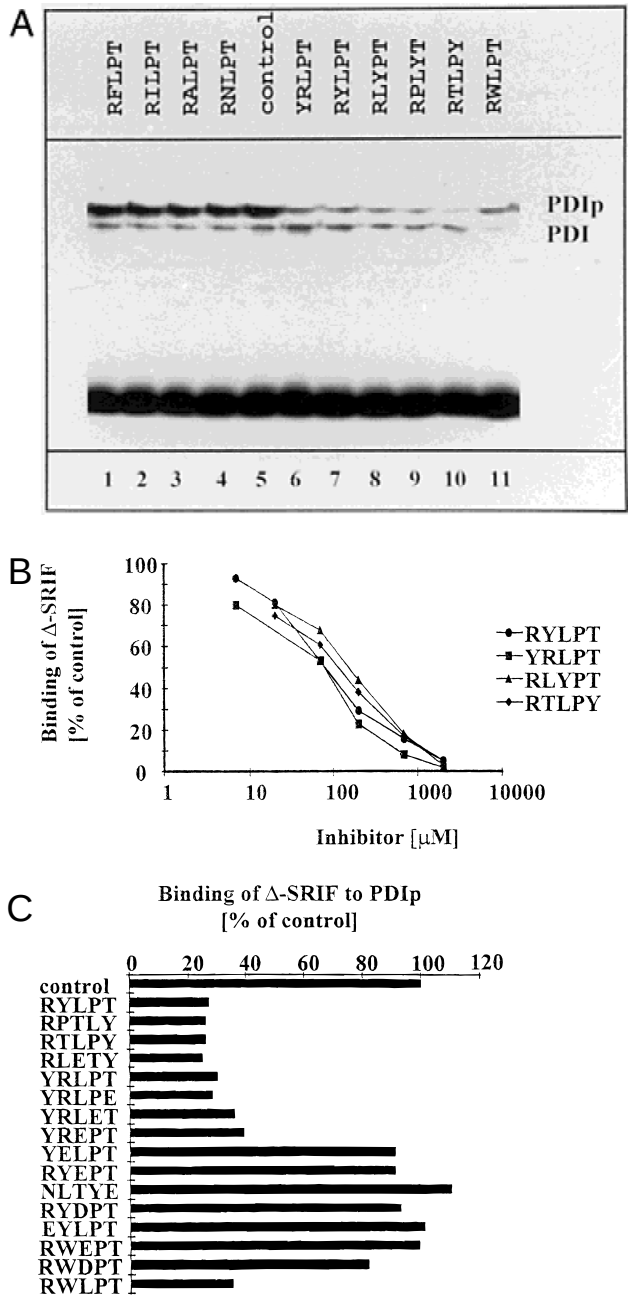

Fig. 2. Inhibition of peptide binding to PDIp by pentapeptides. **A:** Pentapeptides (250 μM) were incubated with a microsomal extract and radiolabeled Δ-somatostatin (3 μM) prior to cross-linking. A sample without peptide served as a control. **B:** A microsomal extract and radiolabeled Δ-somatostatin (3 μM) was incubated with the indicated concentrations of various pentapeptides. **C:** Pentapeptides (250 μM), containing negatively charged residues, were incubated with a microsomal extract and radiolabeled Δ-somatostatin (3 μM) prior to cross-linking. Samples without peptide served as a control. Quantification was performed with a BioRad PhosphoImager.

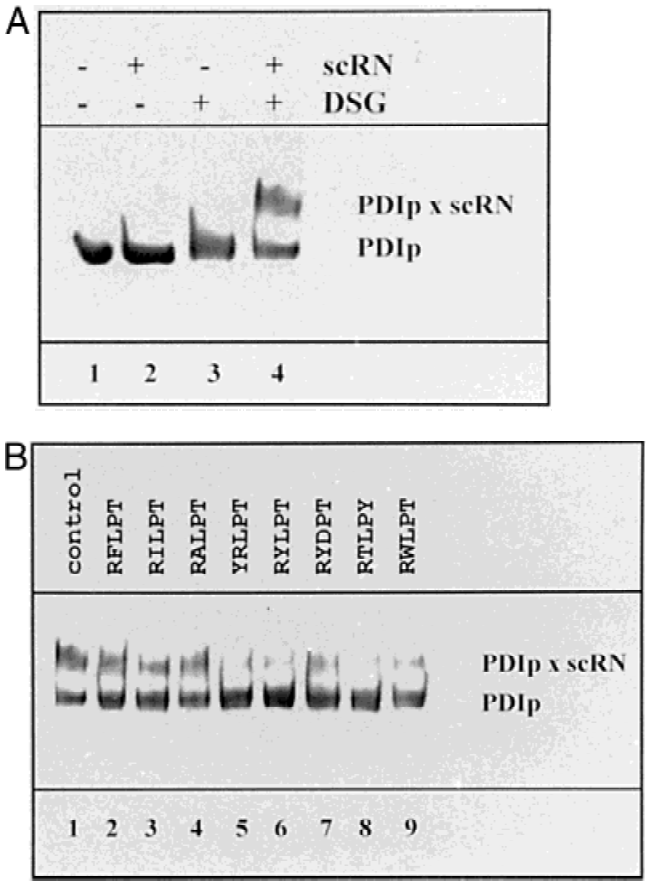

Fig. 3. Inhibition of unlabeled substrate binding to PDIp by pentapeptides. **A:** A microsomal extract was incubated in the presence or absence of unlabeled "scrambled" ribonuclease A (scRN) (3 μM) with or without the chemical cross-linker DSG. **B:** Pentapeptides (250 μM) were incubated with a microsomal extract and unlabeled "scrambled" ribonuclease A (scRN) (3 μM) prior to cross-linking. A sample without peptide 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.

immunodecoration with antibodies directed against PDIp. As shown in Figure 3A, this cross-linking product can be only detected in the presence of "scrambled" ribonuclease A and the chemical cross-linker. When various pentapeptides containing tyrosine or tryptophan residues were also present, they competed for the interaction between PDIp and "scrambled" ribonuclease A (Fig. 3B). In contrast, when pentapeptides which did not contain tyrosine or tryptophan residues were also present no competition with the interaction between PDIp and "scrambled" ribonuclease A was observed. This confirmed the specificity of competitive ligand binding and demonstrates that the interaction is independent of the identity of the indicator cross-linked substrate or its method of labeling.

C-terminally modified tyrosine and tryptophan can compete for the interaction between PDIp and peptides

To extend the results we obtained with the pentapeptides, we carried out competition experiments with single amino acids. To overcome the potentially inhibiting effects of the negatively charged C-terminus, we used C-terminally modified amino acids. Only modified tyrosine or tryptophan competed efficiently with the interaction between PDIp and radiolabeled Δ-somatostatin in microsomal extracts, while other modified amino acids did not show any competition (Fig. 4A and data not shown). No single

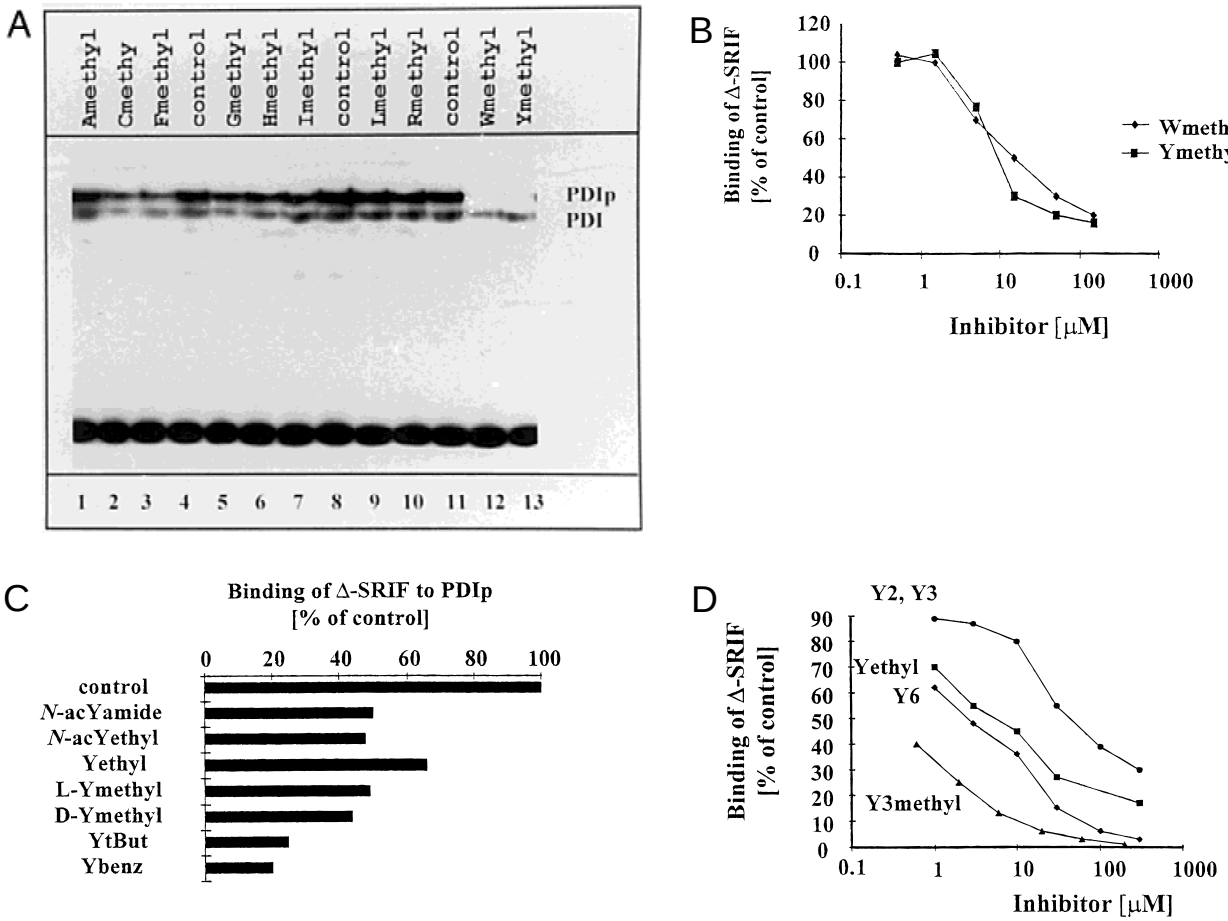

Fig. 4. Inhibition of peptide binding to PDIp by amino acid derivatives. **A:** Amino acid derivatives (150 μM) were incubated with a microsomal extract and radiolabeled Δ-somatostatin (3 μM) prior to cross-linking. **B:** Microsomal extracts and radiolabeled Δ-somatostatin (3 μM) were incubated with the indicated concentrations of tyrosine-methylester and tryptophan-methylester, respectively. Quantification was performed with a BioRad PhosphoImager. **C:** Tyrosine derivatives (15 μM) were incubated with microsomal extracts and radiolabeled Δ-somatostatin (3 μM) prior to cross-linking. **D:** Microsomal extracts and radiolabeled Δ-somatostatin (3 μM) were incubated with the indicated concentrations of various tyrosine derivatives. Quantification was performed with a BioRad PhosphoImager.

amino acid, modified or unmodified, competed efficiently with the interaction between PDI and Δ-somatostatin (Fig. 4A and data not shown).

We did not observe any significant differences between tyrosine methylester and tryptophan methylester with respect to inhibition (Fig. 4B). The apparent IC_{50} values were lower than for the pentapeptides, and in both cases within the same order of magnitude (15–30 μM). The chemical nature of the C-terminal modification played only a minor role (Fig. 4C). However, the introduction of a benzyl group or *t*-butyl group lowered the apparent IC_{50} value significantly. Unmodified tyrosine or tryptophan, i.e., still containing the negatively charged carboxylate, did not compete for the interaction between PDIp and radiolabeled Δ-somatostatin at concentrations up to 500 μM (data not shown). Interestingly, oligomers of tyrosine showed a reduction in the apparent IC_{50} value with increasing length (Fig. 4D). While di-tyrosine and tri-tyrosine had an apparent IC_{50} value of 100 μM, comparable to tyrosine-containing pentapeptides, hexa-tyrosine showed an apparent IC_{50} value of 7 μM, comparable to C-terminally modified tyrosine. Tri-tyrosine methylester, however, had an apparent IC_{50} value of ~1 μM, which is of the same order of magnitude as our previously reported apparent IC_{50} for 17β-oestradiol (Klappa et al., 1998b). Given the structural similarities between 17β-oestradiol and tyrosine, these may be interacting at the same site on PDIp.

To examine the influence of tyrosine modifications within a peptide, we employed angiotensin (sequence DRVYIHPF) and tyrosine-modified angiotensin derivatives in the competition experiments (Fig. 5). Tyrosine-modified angiotensin derivatives only competed with the interaction between PDIp and radiolabeled Δ-somatostatin to a limited degree: the (3′,5′-di-I) derivative and the tyrosine-*O*-methylether derivative inhibited the binding of Δ-somatostatin significantly less than did unmodified angiotensin, while no inhibition could be observed with the phosphorylated derivative.

This clearly indicates that the recognition motif for the binding of angiotensin to PDIp is tyrosyl side chain with a free phenolic hydroxy group.

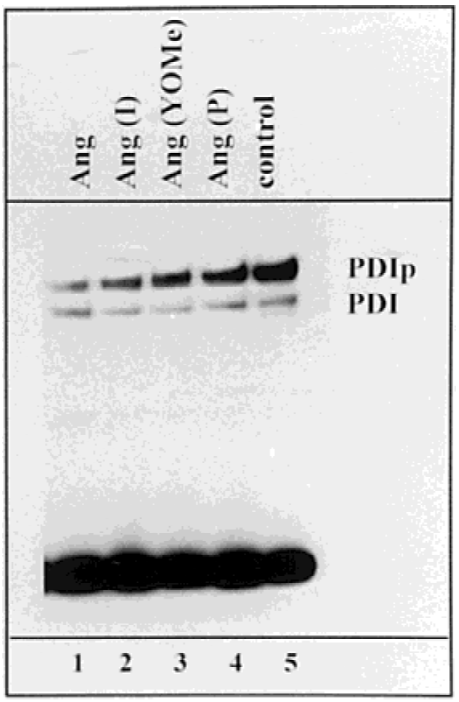

Fig. 5. Inhibition of peptide binding to PDIp by modified tyrosine derivatives. Angiotensin derivatives with modified tyrosine residues (250 μM) were incubated with a microsomal extract and radiolabeled Δ-somatostatin (3 μM) prior to cross-linking. [Ang, Asp-Arg-Val-Tyr-Ile-His-Pro-Phe; Ang(I), Asp-Arg-Val-Tyr(3′,5′-di-I)-Ile-His-Pro-Phe; Ang(YOMe), Sar-Arg-Val-Tyr(Me)-Ile-His-Pro-Phe; Ang(P), Asp-Arg-Val-Tyr(PO_3H_2)-Ile-His-Pro-Phe].

Discussion

There is currently no complete rationalization for the existence of multiple members of the PDI family in mammalian cells, particularly for the coexistence of many members of the family in the ER of some cells, such as the pancreatic acinar cell. It is conceivable that they fulfill equivalent functions but act on specific protein substrates. Erp57 has been found to interact exclusively with glycosylated secretory proteins after their translocation into the ER (Oliver et al., 1997). However, it has been shown that this specificity is not an intrinsic property of ERp57 but is due to the interaction between ERp57 and calnexin, a glycoprotein-specific chaperone of the ER (Zapun et al., 1998). The expression levels of ERp72 were found to be highest in plasma cells, and hence it has been speculated that ERp72 might be involved in the assembly and folding of antibodies (Iida et al., 1996). However, cross-linking experiments demonstrated that ERp72 can also interact with a variety of other denatured proteins in vitro (Kuznetsov et al., 1997). Similarly, Northern blot analysis revealed that the mRNA of PDIp can be detected exclusively in the exocrine pancreas (DeSilva et al., 1997) suggesting that PDIp might play a role in the folding pathway of certain pancreatic enzymes. However, translocation of presecretory proteins into dog pancreas microsomes with subsequent cross-linking showed that PDIp and PDI can interact with the same substrates (Klappa et al., 1995). It therefore seems very likely that most members of the PDI family have an overlapping substrate specificity.

Rather than acting on distinct protein substrates, a more subtle possibility is that these proteins might interact with different parts of the folding polypeptide. Thus, different members of the PDI family might show different affinities for regions of unfolded polypeptide, related to the amino acid sequence or composition of those regions. Similar issues arise in relation to other families of folding factors such as the hsp70 family of molecular chaperones. It is therefore useful to investigate how such proteins interact with model substrates. To date, this issue has not been addressed systematically in the PDI family, because the various family members have not been readily available in purified form and because a range of discriminating specific assays has not been developed. In this work, for the first time, the specificity of peptide binding by a specific member of the family has been explored.

The motifs for PDIp binding are tyrosine and tryptophan residues within a peptide

Competition experiments with a variety of different peptides showed that only tyrosine- and tryptophan-containing peptides competed for the binding of radiolabeled Δ-somatostatin to PDIp in a microsomal extract. The interaction between radiolabeled Δ-somatostatin and PDI, however, was not affected by tyrosine-containing peptides, indicating that the binding motif for PDI is different from that of PDIp. Earlier work of Noiva et al. (1991, 1993) showed that a radiolabeled tripeptide could be specifically cross-linked to residues within the carboxy-terminal 50 amino acid residues of rat liver PDI. However, it was not demonstrated that the binding of this species was saturable, nor that its binding was competitive with binding of the unlabeled peptide or of standard PDI substrates. Indeed, Morjana and Gilbert (1991) showed that PDI exhibits only very weak affinity for tripeptides. It was also demonstrated that the affinity of various peptides toward PDI is largely dependent on the peptide length rather than amino acid composition, hydrophobicity, or charge (Morjana & Gilbert, 1991; Westphal et al., 1998).

This clear difference in the peptide binding specificity of PDI and PDIp was employed as an internal control: The inhibitory effect of peptides on PDIp but not on PDI demonstrated that this was not a trivial nonspecific effect, e.g., by quenching the cross-linking reagent.

Peptides with an acidic amino acid adjacent to the tyrosine or tryptophan residue did not compete for the binding of radiolabeled Δ-somatostatin to PDIp, indicating that negatively charged residues are disfavored. This is very similar to recent findings with different members of the hsp 70 family and their respective co-chaperones: It was demonstrated that bacterial DnaK, bacterial DnaJ, mammalian cytosolic hsp 70, and BiP from mammalian ER interact with peptides containing hydrophobic residues (Flynn et al., 1991; Gragerov & Gottesman, 1994; Gragerov et al., 1994; Rudiger et al., 1997b). Although these molecular chaperones have different peptide binding specificities, it was shown that all of them strongly disfavor negatively charged residues.

The peptide binding affinity of PDIp, as measured indirectly by competition experiments and calculating apparent IC_{50} values after cross-linking, was in the range of 100–250 μM. However, the negatively charged C-terminus probably lowers the affinity significantly, since inhibition by tyrosine-methylester or tryptophan-methylester was observed at lower concentrations (apparent IC_{50} 15–30 μM). The difference in the magnitude of the effect of the C-terminal carboxylate and an adjacent acidic amino acid probably

reflects the different spatial location of a side-chain carboxylate compared with a main-chain carboxylate in the substrate binding site. Thus, we speculate that the binding affinity of PDIp to a folding polypeptide that exposes a tyrosine residue or tryptophan residue in an apolar environment might be within the same order of magnitude. This would be in excellent agreement with the peptide binding affinities of DnaK and BiP, as measured by stimulation of their ATPase activity (Flynn et al., 1991; Gragerov et al., 1994).

A single amino acid can inhibit the interaction between PDIp and a peptide

Our results clearly demonstrated that C-terminally modified tyrosine and tryptophan can compete with the binding of radiolabeled 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. This observation is in stark contrast to that seen for PDI where no single modified or unmodified amino acid can compete with its binding to Δ-somatostatin. Overall, substrate recognition by PDI appears to be much more complex than that for PDIp, indeed the two peptide inhibitors with the lowest apparent IC_{50} for inhibiting Δ-somatostatin binding to PDI found to date have no amino acids in common. Much more work needs to be done to clarify the nature of substrate recognition by PDI.

These findings distinguish PDIp from other chaperones, e.g., members of the hsp 70 family. It has been shown that the efficient binding of peptides to BiP requires at least seven amino acids with certain amino acids in alternating positions (Flynn et al., 1991). In addition, the peptide binding motif of DnaK consists of a hydrophobic core of four to five hydrophobic residues particularly enriched in leucine, but also in isoleucine, valine, phenylalanine, and tyrosine (Rudiger et al., 1997a, 1997b).

C-terminally modified D-tyrosine was as efficient as C-terminally modified L-tyrosine suggesting that the interaction is not stereospecific. This was confirmed by recent observations that a peptide containing exclusively D-amino acids interacted efficiently with PDI and PDIp from dog pancreas microsomes (M. Frien, pers. comm.).

From these results, we conclude that tyrosine residues or tryptophan residues within a folding polypeptide trigger its binding to PDIp, except when adjacent to a negative charge. We speculate that these exposed aromatic residues might act as a molecular tag, indicating an incompletely folded protein. PDIp can interact with this tag, thus preventing aggregation or misfolding of the polypeptide.

Although PDI and PDIp can bind to the same substrates in vitro, they clearly show different binding specificities. This resembles the situation of the members of the hsp 70 family: Although BiP and hsp 70 potentially can bind to the same substrates, their binding specificities are clearly different (Blond-Elguindi et al., 1993; Fourie et al., 1994), indicating that these molecules might have different biological functions. This was confirmed by the finding that BiP and hsp 70 cannot substitute for each other for polypeptide translocation into the lumen of the ER in a reconstituted system (Brodsky et al., 1993).

Clearly, further experiments are needed to determine whether or to what extent PDI and PDIp can substitute for each other in vivo. This eventually should further our understanding of the biological functions of the various members of the PDI family and clarify as to why there are multiple members of the PDI family.

Materials and methods

Materials

"Scrambled" ribonuclease A, di-Tyr, tri-Tyr, hexa-Tyr, tri-Tyr-OMe, Ala-OMe, Arg-OMe, Cys-OMe, Gly-OMe, His-OMe, Ile-OMe, Leu-OMe, Phe-OMe, Ser-OMe, Trp-OMe, Tyr-OMe, 17 α- and 17β-oestradiol, and the homobifunctional cross-linking reagent disuccinimidyl glutarate (DSG) were obtained from Sigma (St. Louis, Missouri). The following reagents were from Novabiochem (Läufelfingen, Switzerland): angiotensin and derivatives, Asp-OtBu, Asn-OtBu, Glu-NH_2, Gln-NH_2, Tyr-OtBu, Met-OMe, Lys-OMe, Pro-OMe, Ser-OMe, Thr-OMe, D-Tyr-OMe, Val-OMe. [^{125}I] Bolton–Hunter labeling reagent and X-ray films were purchased from Amersham (Little Challant, UK). The somatostatin derivative without cysteine residues (Δ-somatostatin, Ala-Gly-Ser-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Ser) and the pentapeptides were synthesized as described previously for other peptides (Klappa et al., 1991). The amino acid sequence of each pentapeptide used in this study is given in the one letter code. The polyclonal antibody raised against dog PDIp was a kind gift from R. Zimmermann (Homburg). The polyclonal antibody raised against bovine PDI was from Stressgen (Victoria, Canada).

Methods

[^{125}I] Bolton–Hunter labeling of Δ-somatostatin was performed as described by the manufacturer.

Preparation of reticuloplasmic proteins and cell extracts

Sheep pancreas microsomes were prepared as described for the preparation of dog pancreas microsomes (Schlenstedt et al., 1990). Reticuloplasmic proteins were prepared as follows: Sheep pancreas microsomes were diluted with the same volume of distilled water and sonicated for 5 min. Reticuloplasmic proteins (supernatant) were separated from the membrane proteins (pellet) by subsequent centrifugation (5 min, 50,000 $\times$ g). The concentration of reticuloplasmic proteins was adjusted to 2 μg PDI/μL as estimated by Coomassie-staining after gel-electrophoresis.

Binding of peptides and "scrambled" RNAse

After precipitation with trichloroacetic acid, the radiolabeled Δ-somatostatin was dissolved in distilled water. Labeled Δ-somatostatin (3 μM) or "scrambled" RNAse (3 μM) were added to buffer A (100 mM NaCl, 25 mM KCl, 25 mM phosphate buffer pH 7.5), containing reticuloplasmic extracts ($\sim$3 μM PDI and PDIp final concentration). For competition experiments, the labeled probe was mixed with unlabeled probe prior to the addition of the reticuloplasmic extracts. The samples (10 μL) were incubated for 10 min on ice before cross-linking.

Cross-linking

Cross-linking was performed using the homobifunctional cross-linking reagent disuccinimidyl glutarate (DSG) (Klappa et al., 1994). The samples were supplied with 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 (Klappa et al., 1995, 1997).

The samples were subjected to electrophoresis in 12.5% SDS polyacrylamide gels with subsequent autoradiography. Quantification was performed using a BioRad PhosphoImager. For "Western-blotting" the samples were loaded on 12.5% SDS polyacrylamide gels with subsequent electrotransfer onto a polyvinylidene fluoride membrane. Immunodecoration was performed with a polyclonal antibody raised against PDIp.

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