Thiol-independent interaction of protein disulphide isomerase with type X collagen during intra-cellular folding and assembly

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Protein disulphide isomerase (PDI) has been shown to be a multifunctional protein capable of catalysing disulphide-bond formation and isomerization, and of participating as a non-catalytic subunit of prolyl 4-hydroxylase (P4-H) and microsomal triacylglycerol transfer protein. It has also been proposed to function as a molecular chaperone during the refolding of denatured proteins *in vitro*. To investigate its potential role as a molecular chaperone within a cellular context, we studied the folding, modification and assembly of type X collagen in semi-permeabilized cells. Using this approach, we demonstrate that depletion of ATP has no effect on the rate or extent of helix formation, indicating that the individual triple helical regions do not interact with the molecular chaperone immunoglobulin

heavy-chain binding protein (BiP). However, PDI was shown to interact transiently with type X during helix formation in a role related to its function as the β subunit of P4-H. Once the collagen triple helix was formed, PDI re-associated, indicating a role in preventing the premature assembly of this molecule into higher-order structures. This interaction was not thiol dependent, as a type X polypeptide that did not contain any cysteine residues was able to fold correctly and interact with PDI. Both PDI and the collagen-binding protein hsp47 showed a similar pH-dependent interaction with folded collagen, dissociating when the pH was lowered to pH 6.0. These results suggest a role for PDI in chaperoning type X collagen during its transport through the cell.

INTRODUCTION

The role of protein disulphide isomerase (PDI) in catalysing disulphide-bond formation and re-arrangement in the cell is now well established [1]. Not only is the gene in *Saccharomyces cerevisiae* required for disulphide-bond formation [2,3], but over-expression of recombinant PDI can increase the yield of heterologously expressed disulphide-bonded proteins [4]. There is growing evidence that the role of PDI in protein folding *in vivo* may not be strictly limited to its catalytic activity but that it may also assist in the efficiency of the folding process by suppressing aggregation. Evidence to support this view has come from studies of the effect of PDI on the refolding of denatured proteins *in vitro*. PDI has been shown to suppress the aggregation of non-disulphide-bonded proteins such as rhodanase [5] and glyceraldehyde-3-phosphate dehydrogenase [6] during refolding. This chaperone function has also been shown to enhance isomerase activity *in vitro*. PDI chemically treated to inactivate its catalytic site was shown to enhance the isomerase activity of unmodified PDI when added at various ratios during the refolding of acidic phospholipase A₂ [7]. Evidence that PDI interacts with nascent polypeptide chains within the lumen of the endoplasmic reticulum (ER) comes from the use of chemical cross-linking agents. A range of polypeptides, including mis-folded lysozyme [8], Igs [9], nascent glycoproteins [10] and non-cysteine-containing peptides [11], has been shown to form complexes with PDI, indicating a transient association during biosynthesis. Whether these transient associations aid directly in the folding of the protein or in protein transport is still to be determined.

In the present study, we have examined the potential chaperone role of PDI in the pathway of collagen assembly in the ER, using bovine type X collagen as a model collagen. Type X collagen is a small non-fibrillar collagen (*M*_r 59000), consisting of three identical chains, and is synthesized exclusively in the epiphyseal

growth plate during chondrocyte hypertrophy. The bovine gene has been cloned, and the deduced amino acid sequence reveals that it shares a number of properties with other collagen molecules [12]. Thus, it consists of three distinct domains: a short N-terminal domain of 38 amino acids, a collagenous triple-helical domain of 463 amino acids and a non-collagenous C-terminal domain of 155 amino acids. There are no cysteine residues in the N-terminal domain and only one in the C-terminal domain, which indicates that intra-molecular disulphide bonds do not form in these domains. However, there are two cysteine residues, separated by only two amino acids, within the triple-helical domain that have been shown to form inter-molecular disulphide bonds between individual polypeptides within the collagen trimer [13,14].

The initial stages in the folding and assembly of type X collagen within the cell are thought to occur in a similar fashion to the fibrillar procollagens. Once the individual chains are translocated into the lumen of the ER, they associate via their C-terminal globular domains into homotrimers, allowing the triple-helical domain to fold in a zipper-like fashion from the C- to the N-terminal direction [15]. One of the functions of the fibrillar collagen propeptides is to maintain the molecule in a soluble form before its secretion from the cell. As the protein is secreted the propeptides are removed, resulting in a self-assembly process to form collagen fibrils [16]. Unlike the fibrillar procollagens, type X collagen C- and N-propeptides are not cleaved; however, the molecules are still able to self assemble within the extra-cellular matrix to form hexagonal lattice-like structures [17]. This suggests that a mechanism may exist to prevent this self-assembly process from taking place within the cell before secretion. Consequently, the efficiency of type X collagen secretion may be limited by the potential for aggregation both of the non-triple helical chains before their association and the triple-helical trimers before their secretion.

Abbreviations used: ER, endoplasmic reticulum; SP-cells, semi-permeabilized HT1080 fibroblasts; PDI, protein disulphide isomerase; P4-H, prolyl 4-hydroxylase; DSP, dithiobis[succinimidylpropionate]; NEM, *N*-ethylmaleimide; BiP, immunoglobulin heavy-chain binding protein; DTT, dithiothreitol.

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We have used a semi-permeabilized cell system that reconstitutes the initial stages in the assembly and modification of procollagen as they would occur in an intact cell [18], to investigate the role of PDI in the folding, assembly and transport of type X collagen. Our results indicate that PDI binds to type X during folding by an interaction that is not dependent on the presence of cysteines in the polypeptide chain, but which can be regulated by changes in pH. These results provide compelling evidence for a chaperone function of PDI during the intracellular maturation of type X collagen.

EXPERIMENTAL

Materials

Restriction enzymes and nucleotides were obtained from Boehringer Mannheim (Lewes, East Sussex, U.K.). T7 RNA polymerase, nuclease-treated rabbit reticulocyte lysate (Flexi-Lysate), amino acids minus methionine, KCl and RNasin ribonuclease inhibitor were purchased from Promega (Southampton, U.K.). EASY-TAG [³⁵S]methionine was purchased from ICN Biomedicals (Thane, Oxfordshire, U.K.). RNeasy RNA purification kit was obtained from Qiagen (Dorking, Surrey, U.K.). Quickchange mutagenesis kit was obtained from Stratagene (Cambridge, U.K.). HT-1080 human fibrosarcoma cells (ATCC-CCL121) were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). Tissue-culture reagents were purchased from Gibco Life Technologies (Glasgow, Scotland, U.K.). Digitonin was obtained from Calbiochem (Nottingham, U.K.). The antibodies to bovine PDI and prolyl 4-hydroxylase (P4-H) α subunit were prepared as previously described [19]. The monoclonal anti-(type X collagen) antibody was kindly donated by Dr. A. P. L. Kwan (University of Cardiff, U.K.). The antibody to CaBP1 was a gift from Professor H.-D. Söling (Universität Göttingen, Germany). The anti-Erp57 was a gift from Dr. T. Wileman (Institute for Animal Health, Pirbright, U.K.). The anti-hsp47 antibody was obtained from Bioquote (York, U.K.). All other reagents were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.).

Human fibrosarcoma cell culture

HT-1080 cells were maintained in Dulbecco's modified Eagle medium supplemented with 10 % (v/v) fetal calf serum, glutamine (2 mM), penicillin (1000 units/ml) and streptomycin (1000 μ g/ml). Ascorbate (50 μ g/ml) was added to the medium 24 h before cells were used.

Construction of recombinant plasmids and mutagenesis

The coding sequence for bovine type X maintained in pGEM-3Z [14] was sub-cloned into the vector pBluescript as an *Eco*RI fragment. The recombinant plasmid was designated pSKBX. Site-directed mutagenesis was carried out using the Quickchange kit (Stratagene, Cambridge, U.K.) using the accompanying protocol. Using two complementary primers to the following sequence 5'-GGAATCTTCACCTCTAAGATTCCAGGGAT-A-3' that introduced one base substitution (underlined), pSKBX was used as a template to generate bovine type X with a single Cys-567 $\rightarrow$ Ser mutation in the C-terminal NC1 domain, designated pSHM12 (BXNC1cys⁻). This plasmid was used as a template to generate the triple mutant (Cys-176 $\rightarrow$ Ser, Cys-179 $\rightarrow$ Ser, Cys-567 $\rightarrow$ Ser) using two complementary primers to the sequence 5'-ATGGGGCATTCTACTCCTTCCCGCCCA-GGT-3' that introduced two base changes (underlined). The resulting plasmid was designated pSHM13 (BXcys⁻).

In vitro transcription

Transcription reactions were carried out as described previously [20]. Full-length transcripts were obtained from 10 μ g of either pSKBX or pSHM13 (BXcys⁻) linearized with *Not*I. Transcription was initiated with T7 RNA polymerase and carried out for 2 h at 37 °C. RNA was purified on Qiagen RNeasy spin columns using the supplied buffers eluting with 50 μ l of DEPC-treated sterile distilled water containing 40 units of RNasin and 5 mM dithiothreitol (DTT).

In vitro translation in semi-permeabilized human fibroblasts (HT1080)

Cultured HT1080 fibroblasts were permeabilized with the detergent digitonin (40 μ g/ml) using a modification of a method published previously [21] as described previously [18]. RNA was translated using a rabbit reticulocyte lysate (Flexi-Lysate, Promega) at 30 °C in the absence of exogenous DTT. The translation reaction (25 μ l) contained 17.5 μ l of reticulocyte lysate, 0.5 μ l of 1 mM amino acids (minus methionine), 0.5 μ l of 2.5 M KCl, 1.5 μ l of L-[³⁵S]methionine (10 mCi/ml; ICN Biomedicals, Thane, Oxfordshire, U.K.), 1 μ l of transcribed RNA and 4 μ l of semi-permeabilized HT1080. Ascorbate was added to a final concentration of 0.25 mM.

Pulse-chase analysis of type X folding

Translations were incubated for 1 h at 30 °C in the presence of 0.5 mM $\alpha\alpha'$ dipyriddy, an iron chelator that inhibits prolyl and lysyl hydroxylases. Further protein synthesis was stopped by addition of cycloheximide to 1 mM final concentration. Post-translational folding was initiated by addition of Fe²⁺ to 5 mM and ATP to 1 mM final concentrations.

In experiments where ATP was depleted, samples were incubated with or without 5 units of Apyrase (Sigma) for 2 min at 37 °C before the addition of Fe²⁺ to 5 mM. After the pulse and chase, *N*-ethylmaleimide (NEM) was added to 20 mM and aliquots were incubated on ice for 10 min. Semi-permeabilized cells were pelleted by centrifugation. Cell pellets were resuspended in 25 μ l of KHM buffer (110 mM potassium acetate, 20 mM Hepes, pH 7.2, and 2 mM magnesium acetate) and treated with proteinase K as described previously [22].

Chemical cross-linking

After proteinase K treatment, cell pellets were resuspended in 100 μ l of KHM buffer and dithiobis[succinimidylpropionate] (DSP) was added to 1 mM final concentration from a 100 mM stock in DMSO, incubating on ice for 30 min. The cross-linker was quenched by addition of glycine to 40 mM, and incubating for a further 10 min on ice.

Immunoprecipitations

Cells were resuspended in 100 μ l of NET-gel buffer [50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.25 % (w/v) gelatin and 0.05 % (v/v) Nonidet P-40] and incubated for 30 min on ice. Cell debris was removed by centrifugation for 10 min. For 'denatured' immunoprecipitations, SDS was added to 0.5 % (w/v) and the samples were heated to 95 °C for 1 min. 'Native' and 'denatured' samples were diluted to 1 ml with NET-gel buffer and pre-cleared by incubation with 40 μ l of Protein A-Sepharose [10 % (v/v) pre-incubated in NET-gel buffer containing 5 % BSA] for 40 min at 4 °C. After removal of Protein A-Sepharose-bound material by centrifugation, the appropriate anti-serum was added at a dilution of 1:500 and samples were

incubated for 16 h at 4 °C. After incubation with 80 μ l of 10 % (v/v) Protein A-Sepharose for 1 h at 4 °C, pelleted complexes were washed three times with 1 ml of 50 mM Tris/HCl (pH 7.4)/150 mM NaCl/2 mM EDTA/1 % (v/v) Triton X-100.

Effect of pH on association of PDI and hsp47 with type X

Pelleted cells were lysed in 100 μ l of KHM buffer, pH 7.2 or pH 6.0, containing 0.05 % (v/v) Nonidet P-40 and incubated on ice for 30 min. The samples were diluted to 1 ml of NET buffer (50 mM Tris/HCl, pH 7.4, 150 mM NaCl and 5 mM EDTA), and cell debris was removed by centrifugation before precipitation with the appropriate anti-sera as described above.

Chymotrypsin/trypsin digestion

Cell pellets or immunoprecipitates were resuspended in 50 mM Tris/HCl, pH 7.4, containing 0.15 M NaCl, 10 mM EDTA and 0.5 % (v/v) Triton X-100. Chymotrypsin and trypsin were added to 250 μ g/ml and 100 μ g/ml final concentrations, and samples were incubated at room temperature for 5 min. Further digestion was stopped by the addition of soya-bean trypsin inhibitor to a final concentration of 5 mg/ml and 2.5 vol. of boiling SDS/PAGE loading buffer containing 5 % (v/v) 2-mercaptoethanol, and the samples were boiled for 5 min.

SDS/PAGE

Samples were prepared for electrophoresis by mixing with an equal volume of 2 $\times$ SDS-loading buffer [0.125 M Tris/HCl, pH 6.8, 2 % (w/v) SDS, 20 % (v/v) glycerol and 0.004 % (w/v) Bromophenol Blue]. If necessary samples were reduced by addition of 5 % (v/v) 2-mercaptoethanol. The samples were boiled for 5 min before electrophoresis. After electrophoresis, dried gels were exposed to Kodak X-Omat AR film or analysed on a Fuji BAS 2000TR Phosphorimager.

RESULTS

Triple-helix formation of bovine type X in SP-cells

To study the folding pathway of type X collagen and the role of ER-resident proteins in this process, pulse-chase analysis was carried out in an *in vitro* translation system supplemented with semi-permeabilized HT1080 cells (SP-cells). We have previously demonstrated that this system reconstitutes the initial folding and assembly of fibrillar collagens [23]. We took advantage of the fact that synthesis of collagen in the presence of $\alpha\alpha'$ -dipyridyl, which chelates iron (an essential co-substrate of P4-H), inhibits proline hydroxylation and consequently helix formation [23,24]. This inhibition can be reversed by addition of excess iron during the chase.

Translation of bovine type X mRNA in the SP-cell translation system in the presence of $\alpha\alpha'$ dipyridyl and [35 S]methionine resulted in the appearance of a single major radiolabelled product (Figure 1A, lane 9). As these chains are resistant to proteinase K digestion, they represent fully translocated type X collagen. Triple-helix formation was assayed by resistance to protease digestion with a mixture of chymotrypsin/trypsin [25]. Only chains within a correctly aligned triple-helical domain are resistant to protease treatment, resulting in the loss of both the N- and C-terminal globular domains and consequently an increase in mobility of the denatured helix (compare Figure 1A, lanes 8 and 9). Synthesis in the presence of $\alpha\alpha'$ dipyridyl inhibits hydroxylation and hence triple-helix formation, and all the translocated chains were protease sensitive (Figure 1A, lane 1).

Figure 1 Time course of triple-helix formation

Bovine type X mRNA was translated in reticulocyte lysate supplemented with SP-cells and in the presence of $\alpha\alpha'$ dipyridyl for 60 min at 30 °C. Cycloheximide was added to a final concentration of 1 mM to inhibit further translation. Two equal aliquots were taken at the end of translation (lanes 1 and 9), and one aliquot was treated with proteinase K to remove untranslocated material (lane 9). FeSO_4 and ATP were added to 5 mM and 1 mM respectively, and after further incubation at 30 °C for the times indicated equal aliquots were taken and NEM was added to a final concentration of 25 mM, incubating on ice for 10 min. Isolated cells were lysed in digest buffer and digested with chymotrypsin and trypsin (lanes 1–8), and together with an untreated control (ut, lane 9) they were analysed under reducing conditions by SDS/PAGE (7.5% polyacrylamide) and autoradiography (A). The intensities of the protease-digested bands were quantified by Phosphorimaging and were plotted relative to the untreated control (100%), correcting for the loss of [35 S]methionines in the digested C-globular domains (B).

The inhibition is overcome in the chase by the addition of excess iron, which initiates hydroxylation and hence triple-helix formation. With increased times of incubation during the chase, more resistant chains accumulated (Figure 1A, lanes 2–8).

The percentage of resistant chains at each time point was plotted relative to the total translated chains (Figure 1A, lane 9), taking into account the loss of radiolabelled methionines within the cleaved C-terminal globular domains (Figure 1B). The half-time for conversion of the chains to protease resistance was approx. $8\frac{1}{2}$ min, with a yield of approx. 80 % of translocated chains after 30 min. There was little significant increase in the total of resistant chains when incubations were carried out for up to 2 h (results not shown). It is interesting to note that there was

Figure 2 Effect of ATP depletion on helix formation

Translations were carried out in the presence of $\alpha\alpha'$ dipyridyl as described in the legend to Figure 1 except that, before addition of FeSO_4 to 5 mM, samples were incubated at 37 °C for 2 min and ATP was added to 1 mM (+ATP, lanes 1–5) or ATP was depleted with 5 units of Apyrase for 2 min at 37 °C (–ATP, lanes 6–10). After further incubation at 30 °C for the times indicated, aliquots were treated on ice for 10 min with 25 mM NEM. Isolated cells were lysed and treated with chymotrypsin and trypsin, and the digests were analysed under reducing conditions by SDS/PAGE (7.5% polyacrylamide) and autoradiography. The intensities of the protease-digested bands were quantified by PhosphorImaging.

no lag phase during the chase, which would be expected if the three constituent chains of the helix had to associate before helix formation could commence [26,27]. Digestion of unhydroxylated type X at the end of the pulse with bacterial collagenase cleaves the chains within the triple-helical region, leaving the globular N- and C-terminal globular domains. Analysis of these fragments by SDS/PAGE under reducing conditions after heating the samples at 60 °C for 10 min in SDS-loading buffer revealed that the C-terminal globular domains were associated in an SDS-resistant trimer (results not shown), which has previously been demonstrated for both chicken and human type X [28,29]. Therefore, folding and association of the C-terminal globular domains is independent of helix formation when bovine type X is synthesized in the presence of $\alpha\alpha'$ dipyridyl. Hence, this folding

intermediate accumulates in the ER and can be efficiently folded to a correctly aligned triple helix after reversal of inhibition of proline hydroxylation.

Effect of ATP on helix formation

The binding of many molecular chaperones involved in preventing aggregation of proteins is coupled to the hydrolysis of ATP [30]. To investigate the possibility of such a role in type X folding, the time course of helix formation, as judged by protease resistance, was compared in the presence (Figure 2, lanes 1–5) and absence of ATP (Figure 2, lanes 6–10). There was no apparent difference in the kinetics of helix formation in the presence or absence of ATP, suggesting that ATP-dependent molecular chaperones such as immunoglobulin heavy-chain binding protein (BiP) are not involved in protecting the chains from aggregation. The resistant bands in the absence of ATP appear to be less diffuse and of a slightly greater mobility than those in the presence of ATP, suggesting that a post-translational modification that is not critical to helix formation of procollagen is ATP dependent.

P4-H binds to unhydroxylated type X and dissociates during helix formation

To investigate the role of P4-H and PDI in triple-helix formation, samples were removed at various times during post-translational folding and immunoprecipitated with antisera specific to both the α and β (PDI) subunits of P4-H. As the antiserum to the α subunit only interacts with the denatured form of the protein, it was necessary to stabilize any complexes formed between P4-H and type X collagen by cross-linking with the membrane-permeant cross-linker DSP before denaturation with 0.5% (w/v) SDS at 95 °C for 1 min. Reduction before analysis by SDS/PAGE cleaves the cross-linker, allowing resolution of the radio-labelled type X (Figure 3, lanes 2–9). The α -subunit of P4-H interacted most strongly with the non-hydroxylated type X during the chase (Figure 3, lane 2) and dissociated within 3 min of the reversal of inhibition during the chase (Figure 3, lanes 3–9). At these early time points most of the α -subunit of P4-H

Figure 3 Interaction of P4-H and PDI with type X during helix formation

In a parallel translation of type X in SP-cells to that described in the legend to Figure 1, incubations were carried out at 30 °C for the times indicated after the addition of iron. Then, after NEM treatment as previously described, cell pellets were resuspended and treated with proteinase K. For precipitations with the anti-P4-H α antiserum (lanes 2–9), samples were cross-linked with DSP and denatured as described in the Experimental section. Precipitations with anti-P4-H β (PDI) (lanes 10–17), anti-type X (lane 1), and pre-immune (Pre-I, lane 18) antisera were carried out under native conditions without cross-linking. Immunoprecipitates were analysed under reducing conditions by SDS/PAGE (7.5% polyacrylamide) and autoradiography.

Figure 4 Interaction between PDI and type X during inter-chain disulphide-bond formation in type X

Translations of type X were carried out in the presence of $\alpha\alpha'$ dipyriddy as described in the legend to Figure 1 and incubated at 30 °C for the times indicated after the addition of Fe^{2+} . Samples were treated with 25 mM NEM for 10 min on ice to prevent further disulphide-bond rearrangement. Then, after proteinase K treatment, samples were split and either analysed under non-reducing conditions by SDS/PAGE (7.5% polyacrylamide) and by autoradiography (**A**) or precipitated (**B**) with anti-PDI (lanes 3–7), anti-type X (lane 2) and pre-immune (Pre-I, lane 1) antisera. Immunoprecipitates were analysed under reducing conditions by SDS/PAGE (7.5% polyacrylamide) and autoradiography. In (**A**), type X isoforms are indicated on the right.

has dissociated before the chains have acquired a protease-resistant triple helix (Figure 1B).

When the β subunit (PDI) was immunoprecipitated, a much slower dissociation from type X collagen was revealed (Figure 3, lanes 10–17), with a minimum being reached at 30 min (Figure 3, lane 17). This is in contrast with the rate of dissociation of the α -subunit; however, it should be noted that the PDI immunoprecipitations were carried out without cross-linking, so differences in rates of dissociation could reflect different affinities of the two antibodies used. The interaction of PDI was specific, as no labelled type X was precipitated with pre-immune anti-serum (Figure 3, lane 18). These results demonstrate that P4-H interacts strongly with unhydroxylated collagen chains, preventing their premature association before hydroxylation of proline residues. Once proline hydroxylation occurs, P4-H dissociates and formation of the triple helix is initiated. The lag between the dissociation of P4-H and the completion of triple-helix formation,

as judged by protease resistance, could be due to slow conformation changes within the helix, such as *cis-trans* isomerization of proline residues that limit the rate of helix formation [26].

Time course of disulphide bond formation within the triple helix

To determine the time course of disulphide-bond formation, translations of type X synthesized in the presence of $\alpha\alpha'$ dipyriddy were chased for various times in Fe^{2+} and then treated with NEM (25 mM) to prevent any further disulphide-bond rearrangement. Samples were either analysed by SDS/PAGE under non-reducing (Figure 4A) or immunoprecipitated with PDI antisera and analysed by SDS/PAGE under reducing conditions (Figure 4B).

At the end of the pulse, only monomeric unhydroxylated type X was seen (Figure 4A, lane 1). The addition of Fe^{2+} during the chase resulted in a decrease in mobility and broadening of the denatured monomeric chains, indicative of hydroxylation and glycosylation [31,32] and the appearance of interchain disulphide-linked dimers and trimers (Figure 4A, lane 2). Digestion with chymotrypsin/trypsin confirmed that these disulphides are formed within the triple-helical region; the dimeric and trimeric species were resistant to digestion with an increase in mobility, consistent with the loss of the C- and N-globular domains (results not shown). As the chase proceeded, the trimeric species became the dominant form, reaching a maximum between 90 and 120 min of the chase (Figure 4A, lanes 4 and 5). Hence the formation of interchain disulphide bonds within the triple helix proceeds after the helix has been completed.

Immunoprecipitations with antisera to PDI during the time-course of disulphide-bond formation were analysed by SDS/PAGE under reducing conditions (Figure 4B, lanes 3–7). As previously seen in Figure 3, there was an initial drop in the interaction at 30 min after triple-helix formation is complete (Figure 4B, lane 4). This was then followed by re-association of PDI during disulphide-bond formation (Figure 4B, lanes 5–7), reaching a maximum at 120 min of chase (Figure 4B, lane 7), at which point the majority of type X chains are covalently linked in dimers and trimers. In a parallel experiment with antisera to the α -subunit of P4-H the only interaction was seen at the start of the chase (results not shown), correlating with the results in Figure 3. Again, no labelled type X was immunoprecipitated with pre-immune antisera (Figure 4B, lane 1).

Effect of replacement of cysteine residues in bovine type X on the interaction with PDI

One possibility to account for the prolonged interaction of PDI with type X during disulphide-bond formation is that it interacts with free thiols. These could be either free cysteines within the triple-helical domain or the single cysteine residues within the C-terminal globular domain. To ascertain if these cysteines are crucial to the PDI interaction, two rounds of site-directed mutagenesis were performed to replace the three cysteines within bovine type X collagen with serine. Both wild-type and cysteine-minus type X mRNAs were translated in the presence of $\alpha\alpha'$ -dipyriddy and chased for 120 min after the addition of iron and then either analysed for disulphide-bond formation or immunoprecipitated with anti-PDI antisera.

Analysis of the translation products by SDS/PAGE under non-reducing conditions clearly demonstrates that the cysteine-minus type X does not form disulphide-linked dimers and trimers (Figure 5A, lane 4) in contrast with the wild type (Figure 5A, lanes 2). It is also clear that replacement of the cysteines in type X does not disrupt the interaction of PDI after 120 min

Figure 5 Interaction between PDI and type X is not dependent on the presence of cysteines in type X

Translations of type X [wild type (Wt): (A) lanes 1 and 2; (B) lanes 1–3] and the cysteine-minus type X mutant [Cys⁻: (A) lanes 3 and 4; (B) lanes 4–6] were carried out in the presence of $\alpha\alpha'$ dipyridyl as described in the legend to Figure 1. (A) Aliquots were taken before the addition of Fe^{2+} (lanes 1 and 3) or incubated for 120 min at 30 °C in the presence of 5 mM FeSO_4 (time 120, lanes 2 and 4). After proteinase K treatment, samples were analysed under non-reducing conditions by SDS/PAGE (7.5% polyacrylamide) and Phosphorimaging. Type X isoforms are indicated on the left. (B) After incubation in the presence of Fe^{2+} for 120 min, cell lysates were split and precipitated with anti-PDI antisera (lanes 1, 2, 4 and 5) or with pre-immune serum (lanes 3 and 6). One anti-PDI immunoprecipitate of wild-type and Cys⁻ type X was digested with chymotrypsin/trypsin (lanes 3 and 5). Samples were analysed under reducing conditions by SDS/PAGE (7.5% polyacrylamide) and Phosphorimaging.

chase, as judged by the result of immunoprecipitation with PDI antisera of the wild-type (Figure 5B, lane 1) and the cysteine-minus type X (Figure 5B, lane 4) when analysed by reducing SDS/PAGE. Denaturation of the samples before immunoprecipitation disrupted the interaction with type X, even though the antibody recognizes denatured PDI, confirming that the association is non-covalent (results not shown). The majority of type X collagen that is associated with PDI is triple helical, as it is resistant to protease digestion after immunoprecipitation with PDI antisera (Figure 5B, lanes 2 and 5). These interactions are specific, as no labelled type X is precipitated by pre-immune sera after 120 min of chase (Figure 5B, lanes 3 and 6). When the immunoprecipitates were separated without prior reduction, higher-molecular-mass bands were observed only in the wild-type sample, corresponding to disulphide-linked dimers and trimers, whereas the mutant remained monomeric (results not shown). These results demonstrate that the PDI interacts at latter stages with type X collagen that has folded into a triple-helical molecule and that this interaction is not dependent on the presence of free thiols within the polypeptide chain.

Interaction of PDI-like proteins and hsp47 with type X

To compare the specificity of the association of PDI with type X, we examined the interactions of the PDI-like proteins CaBP1 [33] and Erp57 [34] and the collagen-binding protein hsp47 [35]. Type X was synthesized in the presence of SP-cells and $\alpha\alpha'$ -dipyridyl for 60 min before a 120 min chase in the presence of Fe^{2+} . In order to preserve any transient interactions, cells were treated

with the thiol-cleavable chemical cross-linker DSP as previously described before immunoprecipitation under denaturing conditions. The immunoprecipitates were revealed by SDS/PAGE under reducing conditions to allow resolution of the labelled type X.

The PDI-like proteins CaBP1 and Erp57 interacted with the unhydroxylated type X collagen chains at the end of synthesis (Figure 6, lanes 3 and 5) but there was little apparent association with the fully folded collagen after 120 min of the chase (Figure 6, lanes 4 and 6). In contrast, hsp47 appeared to interact at both the start and end of folding (Figure 6, lanes 7 and 8) but to a lesser extent than PDI (Figure 6, lanes 1 and 2). These interactions are specific, as the addition of cross-linker did not cause non-specific precipitation of type X by pre-immune antisera. Hence this would suggest that the binding specificity of PDI for hydroxylated folded type X is not shared with the PDI-like proteins.

Dependence of the interaction of PDI and hsp47 with type X on pH

The previous experiment indicates that both hsp47 and PDI interact with folded type X. The interaction of hsp47 with collagen has been shown to be pH sensitive in both *in vivo* and *in vitro* binding studies [35]. To examine if the interaction of PDI with folded disulphide-bonded type X is similarly pH dependent, SP-cells were lysed with Nonidet P-40 after a 120 min chase, and the lysates were incubated on ice for 30 min in buffer at either pH 7.2 or pH 6.0. The complexes were not stabilized by the

Figure 6 Comparison of the interaction of PDI, PDI-like proteins and hsp47 with type X

Translations of type X were carried out in the presence of $\alpha\alpha'$ dipyrpyridyl as described in the legend to Figure 1. Equal aliquots were taken before the initiation of the chase (lanes 1, 3, 5 and 7) and after 120 min incubation chase in the presence of 5 mM FeSO_4 (lanes 2, 4, 6, 8 and 9). After proteinase K treatment, the samples were cross-linked with DSP and denatured as described in the Experimental section. The cells were lysed and split into equal aliquots. The cell lysates were precipitated with anti-PDI (lanes 1 and 2), anti-CaBP1 (lanes 3 and 4), anti-Erp57 (lanes 5 and 6), anti-hsp47 (lanes 7 and 8) or pre-immune (lane 9) antisera. Immunoprecipitates were analysed under reducing conditions by SDS/PAGE (7.5% polyacrylamide) and PhosphorImaging.

addition of cross-linker during the incubation, to avoid the complication of the pH dependence of the cross-linking reaction. The pH was equilibrated by dilution in buffer at pH 7.4 before immunoprecipitation. Immunoprecipitates were analysed by SDS/PAGE under reducing conditions, and the monomeric labelled type X was quantified by PhosphorImage analysis.

PDI and hsp47 both showed a pH-dependent interaction with type X, dissociating to 5% and 50% of the level at pH 7.0 respectively, when the pH is lowered to pH 6.0. These results should be considered in the context of the effect a decrease in pH has on the total amount of type X that can be immunoprecipitated with an anti-type X antibody. Quantification of the relative levels of type X by PhosphorImage analysis revealed that the pH of lysis has no significant effect on the total amount of type X that can be precipitated with the anti-type X antibody. Therefore, the reduction in the detectable interaction with PDI and hsp47 is not due to protein aggregation and precipitation of the protein before immunoprecipitation.

DISCUSSION

The function of PDI as a chaperone in assisting protein folding has mainly been investigated by studying the refolding of proteins *in vitro*. Evidence for such a role in the cell has been limited. Indications that PDI may be involved in assisting protein folding beyond its catalytic function came from the ability of a mutant PDI that is inactive in catalysing disulphide-bond formation to increase the secretion of recombinant lysozyme in *S. cerevisiae* [36]. PDI has also been shown to interact with a range of misfolded proteins and polypeptides [8,10,11], but whether this interaction is part of the normal folding pathway of these proteins is unclear. In this present study, we have investigated the association of PDI with type X collagen in the ER lumen of semi-permeabilized fibroblasts. During the normal folding pathway of this protein, the efficiency of triple-helix formation is dependent on the extent of proline hydroxylation, a modification that is catalysed by P4-H. The activity of this enzyme is dependent on a number of co-factors, including ascorbic acid and iron; any deficiency in these co-factors leads to inefficient proline hy-

droxylation and a build up of unhydroxylated collagen chains within the cell. We took advantage of the dependence of triple-helix formation on hydroxylation by P4-H to enable the naturally occurring folding intermediate of type X to be generated in the ER lumen. Thus by depleting iron we were able to accumulate unhydroxylated chains. Addition of iron to these cells initiated hydroxylation and hence triple-helix formation. The interaction of PDI and other ER residents could then be examined in relation to the formation of the triple helix and subsequent disulphide-bond formation within the triple helix.

As PDI is a component of P4-H, it was important to determine at each stage whether PDI itself or as part of P4-H interacted with type X collagen. The binding specificity of P4-H has been well characterized [37], and it has previously been shown that stable enzyme-substrate complexes can be formed *in vitro* when the activity of P4-H is inhibited, which subsequently dissociate after partial hydroxylation of the substrate [38]. We also showed that this is the case in the ER lumen, where during inhibition of hydroxylation P4-H forms a stable complex with type X but dissociates within 3 min after the initiation of hydroxylation, when only approx. 10% of the chains have become triple helical. It has previously been reported that association of PDI and/or P4-H with type I collagen occurs where the folding of the triple-helical domain is disturbed by naturally occurring mutations [39]. Hence, in addition to binding of collagen during helix formation, P4-H may also retain collagens with malformed triple helices in the ER as part of a quality control mechanism [40].

The interaction of PDI during disulphide-bond formation in type X might be expected, due to its catalytic function. It has been proposed that PDI catalyses disulphide-bond formation via the transient formation of a mixed disulphide between the substrate and the PDI active-site cysteine [41]. Formation of a mixed disulphide between an active-site mutant PDI and RNaseA has been detected [42]. However, replacement of cysteines in type X does not affect the interaction of PDI, and therefore the formation of a mixed disulphide between PDI and type X is not critical for the association in the ER.

In addition to PDI, a number of ER-resident proteins have been identified that contain thioredoxin-like domains with PDI-like active sites: Cys-Xaa-Xaa-Cys [1]. The highest similarity among PDI and Erp57 (Grp58, Erp60) and CaBP1 is within these thioredoxin-like domains, with little similarity outside these regions. Though they have been shown to possess oxido-reductase activity [43,44], it is unclear what their functional role is in protein folding *in vivo*. During the inhibition of hydroxylation in semi-permeabilized cells, Erp57 and CaBP1 both bind to the non-triple-helical chains of type X in addition to PDI and P4-H but, in contrast with PDI, have little affinity for triple-helical and disulphide-bonded type X. This implies a difference in binding specificity of the PDI-like proteins compared with PDI and potentially different functions in the ER lumen. Erp57 is unable to substitute for the essential function of PDI in *S. cerevisiae* when PDI gene is disrupted [45] or as a subunit in P4-H [46]. Isolated thioredoxin domains of PDI, though active in some of the oxido-reductase activity of the full-length molecule, are relatively inactive in catalysing isomerization of disulphide bonds [47], suggesting a role for the non-thioredoxin-like domains in the catalytic activity. The low level of similarity of these regions among Erp57, CaBP1 and PDI proteins may explain the difference in binding specificity.

The binding and release of substrate from ATP-dependent molecular chaperones such as BiP is coupled with the binding and hydrolysis of ATP [30], so that depletion of ATP will trap BiP-substrate complexes. A role for ATP-dependent molecular chaperones in helix formation of type X would seem to be

precluded by the lack of an effect of ATP depletion on the kinetics and yield of triple-helical chains. BiP has previously been shown to bind to procollagens with mutations in the globular C-terminal domains that cause aberrant association of the chains but not with mutations within the triple helix [39,48], and so may have an important role in association of the chains before helix formation. As the type X chains were found to have associated during the inhibition of hydroxylation, the role of BiP in this system could not be further investigated. The ATP-independent collagen-binding protein hsp47 was found in association with unhydroxylated type X chains and with triple-helical and disulphide-bonded type X, in agreement with the proposed function as a collagen-specific chaperone in the folding and transport of types I–V procollagen [35].

The association of PDI with triple-helical and disulphide-bonded type X raises the question as to the function of PDI in type X secretion. Unlike the fibrillar procollagens, the globular N- and C-terminal domains of type X collagen are not cleaved, and the secreted mature molecule forms supramolecular assemblies in the extracellular matrix [17]. Therefore, it is possible that the association of PDI with type X prevents this from occurring in the ER lumen by binding to the sites of intermolecular interaction.

Immunofluorescence data suggest that hsp47 dissociates from collagen in the Golgi [49], and this may correlate with its pH-sensitive binding to collagen both *in vivo* and *in vitro* [35]. Like hsp47, PDI also shows a pH-dependent dissociation from triple-helical type X, and so may allow recycling of PDI back to the ER via the receptor for the ER retention motif (KDEL receptor). It is interesting to note that the KDEL receptor also shows a pH-sensitive binding *in vitro*, associating with substrate at pH below 7 [50]. Therefore, it is possible to envisage that in a post-ER acidic secretory compartment PDI and hsp47 will dissociate from type X and bind to the KDEL receptor.

The results demonstrate that PDI interacts with type X collagen during its folding and assembly in the ER lumen of cultured fibroblasts. The interaction is not mediated by the formation of a mixed disulphide between type X and PDI, implying that the chaperone activity of PDI is distinct from its catalytic role. It extends the role of PDI in the assembly of collagen beyond its function in catalysing disulphide-bond formation in the globular domains of type I and type II collagen [27] and as a subunit of P4-H, and suggests that PDI can function as a chaperone in the ER lumen.

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