Quality control in the endoplasmic reticulum: PDI mediates the ER retention of unassembled procollagen C-propeptides

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Quality control within the endoplasmic reticulum (ER) is thought to be mediated by the interaction of a folding protein with one or several resident ER proteins [1]. Protein disulphide isomerase (PDI) is one such ER resident protein that has been previously shown to interact with proteins during their folding and assembly pathways [2, 3]. It has been assumed that, as a consequence of this interaction, unassembled proteins are retained within the ER. Here, we experimentally show that this is indeed the case. We have taken advantage of our previous finding that PDI interacts with procollagen chains early on in their assembly pathway [2] to address the role of this protein in directly retaining unassembled chains within the ER. Our experimental approach involved expressing individual C-propeptide domains from different procollagen chains in mammalian cells and determining the ability of these domains to interact with PDI and to be secreted. The C-propeptide from the proα2(I) chain was retained within the cell, where it formed a complex with PDI. Conversely, the C-propeptide from the proα1(III) chain did not form a complex with PDI and was secreted. Both domains were secreted, however, from a stable cell line expressing a secreted form of PDI lacking its ER retrieval signal. Hence, we have demonstrated directly that the intracellular retention of one substrate for ER quality control is due to an interaction with PDI.

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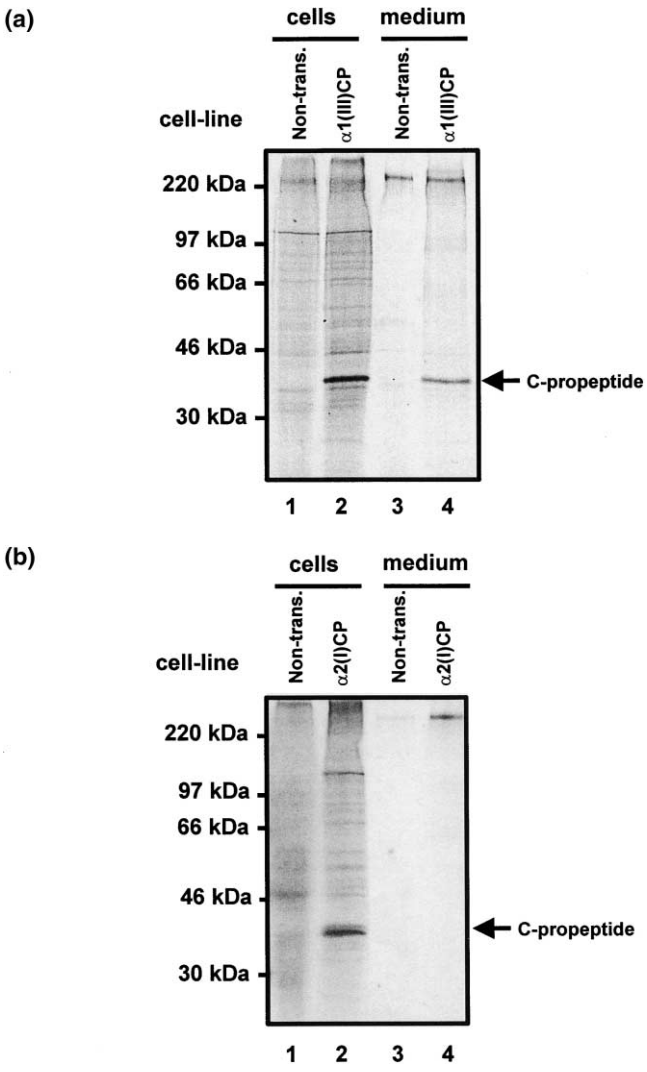
Results and discussion

The fibrillar procollagen molecule is a trimer consisting of three distinct regions, the N- and C-propeptides separated by a long triple-helical domain [4]. Along with others, we have previously shown that the procollagen chain interacts with a number of ER resident proteins during its folding and assembly and prior to its transport from the ER. Each domain within the procollagen chain seems to interact with a distinct set of ER resident proteins; thus, the triple-helical domain interacts with prolyl4-hydroxylase [5, 6] and Hsp47 [7, 8], whereas the C-propeptide may interact with BiP [9] or PDI [2]. These interactions could facilitate the retention of unassembled chains within the ER, directly preventing the secretion of procollagen until the correct native structure is reached. Alternatively, secretion could be a selective process, with only the native structure being incorporated into secretory vesicles with the consequence that unassembled chains remain within the ER.

To address this question, we studied the folding, assembly, and secretion of an independently folding domain of procollagen, the C-propeptide. When expressed individually, the C-propeptide from the homotrimer forming procollagen type III has been shown to fold correctly and assemble into trimers [10, 11]. For our experiments, we studied the expression of the C-propeptides in mammalian cells from the episomally replicating plasmid pCEP4 after the selection of cell lines with hygromycin. Our results show that when the proα1(III) C-propeptide was expressed in HT1080 cells, the expressed protein could be immunoprecipitated from the medium as well as the cell lysate, demonstrating that the protein was secreted (Figure 1a). In contrast, when the C-propeptide from a procollagen chain that does not self-associate to form trimers (proα2[I]) was expressed, the protein could only be immunoprecipitated from the cell lysate and not the medium (Figure 1b). Hence, the proα2(I) C-propeptide is retained within the cell in the absence of the proα1(I) C-propeptide, with which it would normally associate to form a heterotrimer.

To determine whether PDI associated with the C-propeptide domains, we first translated the individual C-propeptides into semipermeabilized HT1080 cells, added the thiol-specific crosslinking reagent BMH, and immunoprecipitated the crosslinked products with an antibody raised to PDI. We have previously shown that PDI can be crosslinked to procollagen chains that remain monomeric, but

Figure 1

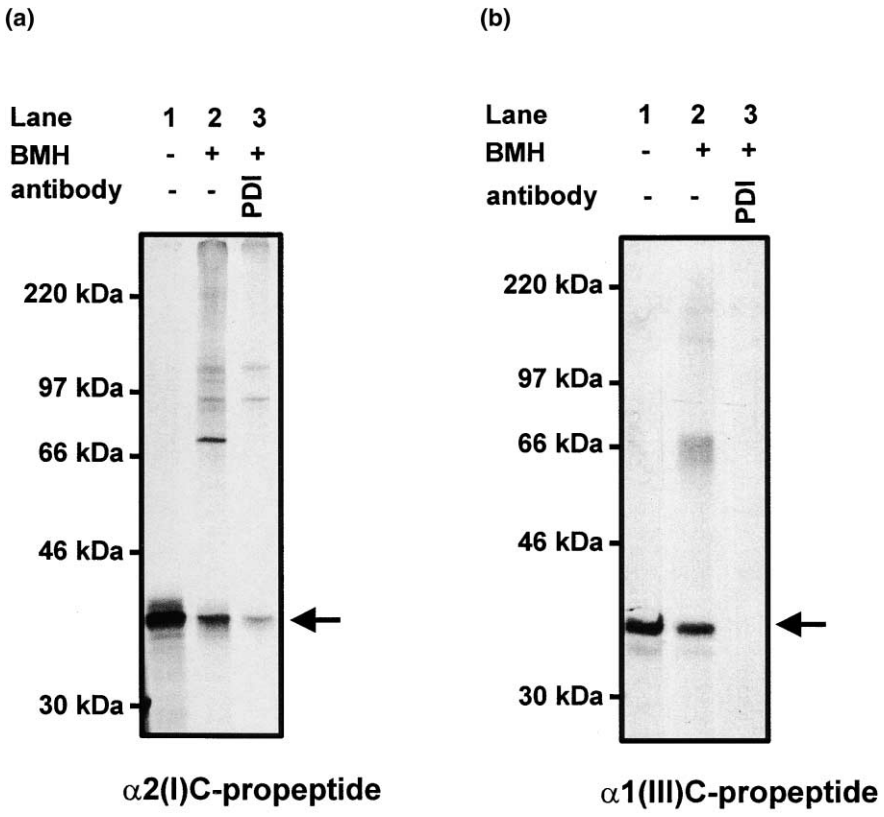


C-propeptides that remain monomeric are retained in mammalian cells. The two C-propeptide constructs were generated from the proα1(III) and proα2(I) procollagen chains by PCR and subcloned into the expression vector pCEP4. Both constructs contained a HA-epitope tag between the signal sequence and the start of the C-propeptide sequence to allow for immunoprecipitation. The HT1080 cell line was transfected and left for 24 hr before 250 μg/ml hygromycin was added and stable cell lines were selected. For labeling, cells were washed twice with PBS and then incubated for 40 min in starve medium (cysteine- and methionine-free minimum essential eagle medium supplemented with 2 mM glutamine). Starve medium was removed, and cells were then pulsed with 125 μCi/ml S^{35}-labeled methionine/cysteine in starve medium for 1 hr. After the pulse, the medium was removed, and the chase was carried out in complete medium supplemented with 125 μg/ml l-methionine and 0.5 mM cycloheximide for 90 min. Cell lysates were prepared by washing cells twice in cold PBS, then incubating with low salt IP buffer (50 mM Tris-HCl, 0.15 M NaCl, 2 mM EDTA, 1% Triton X-100) supplemented with 1 mM PMSF for 20 min on ice. Cell debris was removed by centrifugation at 13,000 rpm for 30 min at 4°C. Pulse-chase medium and cell lysates were preincubated with 50 μl protein A-Sepharose (10% [w/v] in PBS) for 30 min at 4°C to remove protein A binding components. Immunoprecipitation was carried out using anti-HA antibody and 50 μl protein A-Sepharose overnight at 4°C. Immunoprecipitated complexes were retrieved by centrifugation at 13,000 × g for 30 s, and they were washed three times in IP buffer prior to separation by SDS-PAGE and visualized by autoradiography. Cell lysates (lanes 1 and 2) or medium (lanes 3 and 4) were prepared from nontransfected HT1080 cells (lanes 1 and 3) or cells transfected with the C-propeptide from **(a)** α1(III) (lanes 2 and 4) or from **(b)** α2(I) (lanes 2 and 4).

not to procollagen chains that form trimers [2]. When the C-propeptide from the proα2(I) chain was expressed in semipermeabilized cells, a single major translation product was formed (Figure 2a, lane 1). After crosslinking with BMH, crosslinked products were observed (lane 2). Two of these crosslinked products were immunoprecipitated with the PDI antibody, demonstrating that PDI associates with the C-propeptide (lane 3). The appearance of two crosslinked products immunoprecipitated with the PDI antibody could represent crosslinks between different regions of the proteins, crosslinks to dimers, or crosslinks to a third as yet unidentified protein. Some noncrosslinked material was also immunoprecipitated, suggesting that some of the C-propeptide could be coimmunoprecipitated with antibody to PDI. The other crosslinked product with a relative molecular weight of approximately 70 kDa is likely to arise from the self-association of the C-propeptide to form a dimer, which may occur at low efficiency. When the C-propeptide from the proα1(III) chain was expressed in semipermeabilized cells, a single major translation product was formed (Figure 2b, lane 1), representing the translocated, glycosylated protein. Several crosslinked products were seen; however, none of these were immunoprecipitated with PDI antibody (lanes 2 and 3). We have previously shown that this protein forms interchain disulphide-bonded trimers when expressed in SP cells [9]; therefore, the crosslinked products observed in this case are most likely formed between individual C-propeptide chains. Taken together with our previous results [2], we have demonstrated that PDI interacts specifically with monomeric C-propeptide chains within the ER when these chains are expressed individually. A direct consequence of this interaction could be that the C-propeptides that remain monomeric are retained within the ER; alternatively, this retention may be due to a lack of recognition of the monomeric C-propeptides during cargo selection for vesicular transport from the ER to the Golgi apparatus.

To specifically address this point, we made a stable HT1080 cell line expressing a PDI molecule that lacks its ER retrieval carboxy-terminal sequence, KDEL. Here, cells were transfected with linearized DNA, and stable cell lines were selected by their resistance to neomycin. To distinguish the recombinant PDI protein from endogenous PDI, we replaced the KDEL sequence with a myc-epitope tag. When nontransfected HT1080 cells were

Figure 2



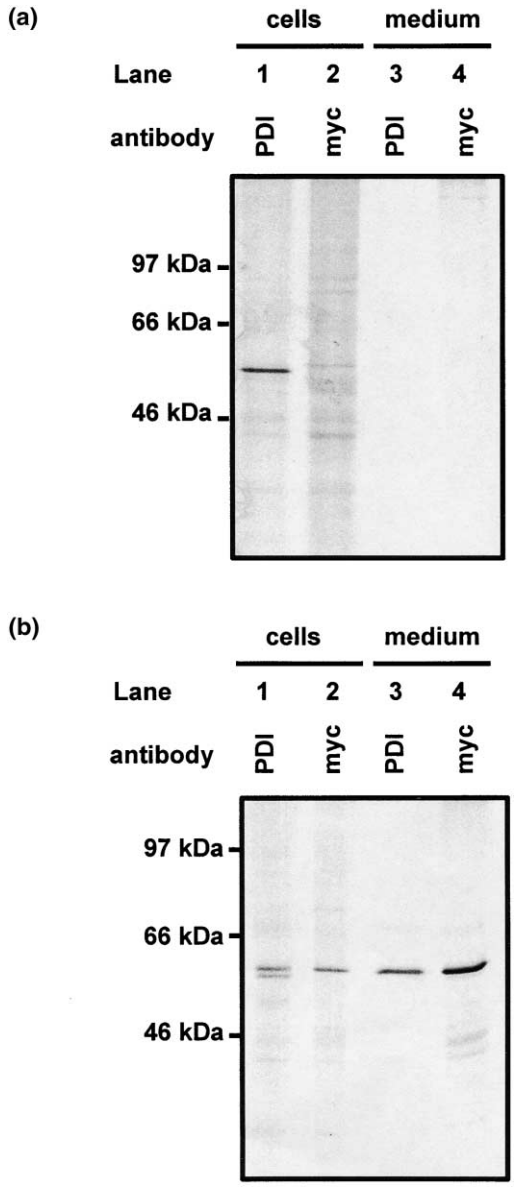
The monomeric C-propeptide can be crosslinked to PDI. Transcription and translation of the C-propeptides of procollagen in the presence of semipermeabilized cells [18] was carried out as described previously [10]. Following in vitro translation, semipermeabilized cells were isolated and resuspended in 100 mM KOAc, 2 mM MgOAc, 20 mM HEPES (pH 7.2) buffer containing DMSO (solvent control) or 50 μM bismaleimidohexane (BMH) and incubated at room temperature for 10 min. Crosslinking was then quenched by the addition of 10 mM DTT, and the samples were left on ice for 10 min. Samples were then immunoprecipitated with an antibody raised to PDI [19], as described in the legend of Figure 1. Noncrosslinked samples (lane 1), crosslinked samples (lane 2), and products immunoprecipitated with PDI antibody (lane 3) were separated by SDS-PAGE and visualized by autoradiography. The presence of the full-length C-propeptide is indicated with an arrow. **(a)** Translation products from the α2(I) C-propeptide. **(b)** Translation products from the α1(III) C-propeptide.

pulse-labeled and proteins were immunoprecipitated with antibodies to PDI, endogenous PDI was immunoprecipitated exclusively from the cell lysate (Figure 3a, lane 1). No PDI was immunoprecipitated with the myc antibody, and no PDI was present in the medium. In the transfected cell line, both endogenous and recombinant PDI was immunoprecipitated from the cell lysate with antibody to PDI (Figure 3b, lane 1). The recombinant PDI has a slightly slower mobility due to the presence of the myc-tag. The myc antibody immunoprecipitated the recombinant PDI, but not endogenous PDI from the cell lysate, while both the PDI and the myc antibody immunoprecipitated the recombinant PDI from the medium. These results confirm those published previously [12] and demonstrate that the removal of the KDEL sequence from PDI results in secretion of PDI. These results also show that the endogenous PDI was not cosecreted with the recombinant PDI, indicating that the overexpression of PDI-KDEL does not result in a general breakdown of the ER retention and retrieval mechanism. This was further verified by a lack of secretion of two other ER resident proteins, BiP and ERp57 (data not shown).

The cell line stably expressing PDI-KDEL (designated K2) was then transfected with the individual C-propeptides, and the effect of coexpressing PDI-KDEL on the secretion of both the proα1(III) and proα2(I) C-propeptides was assessed. The C-propeptides were expressed from the episomally replicating vector pCEP4, and the hygromycin-resistant cell lines were selected. The transfected PDI continued to be secreted from both cell lines, as evidenced by the appearance of PDI in the medium (Figure 4a,b, lane 3). The proα1(III) C-propeptide was secreted from the K2 cell line (Figure 4a, lane 4), as was seen previously with the transfected wild-type HT1080 cell line. The proα2(I) C-propeptide, on the other hand, in contrast to transfected wild-type HT1080 cells, was secreted from the K2 cell line (Figure 4b, lane 4), indicating that the coexpression of PDI-KDEL overcomes the cellular retention of this protein.

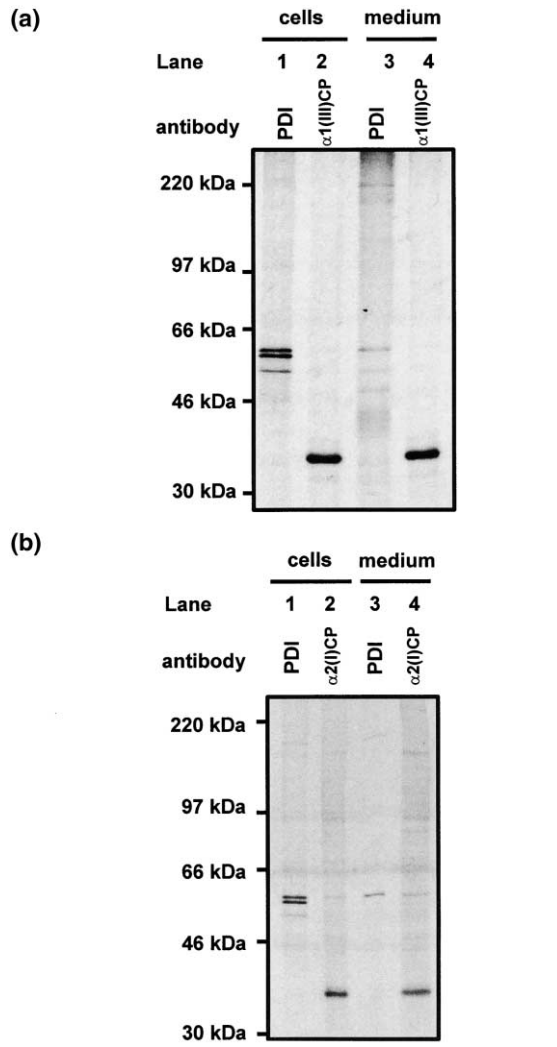
PDI is a multifunctional protein that is involved in the isomerization of disulphide bonds during protein folding and functions as a subunit for both prolyl4-hydroxylase and microsomal triglyceride transfer protein [14, 15]. One property that PDI seems to have is the ability to bind to peptides and polypeptides, and this has given rise to the suggestion that it functions as a molecular chaperone during the folding of certain proteins within the ER lumen [15, 16]. One consequence of this polypeptide binding function is that it will prevent the nonspecific association of procollagen propeptides prior to the synthesis of their

Figure 3



The expression and secretion of PDI-KDEL. The PDI-KDEL construct was prepared by PCR from a human cDNA clone and involved replacing the nucleotide sequence coding for the last four amino acids (KDEL) with the myc-epitope sequence. The resulting PDI-KDEL sequence was subcloned into pCIneo. HT1080 cells were transfected with a linearized vector, and stable cell lines were selected by their resistance to G148 and cloned by limited dilution. Pulse-labeling of **(a)** nontransfected HT1080 cells or **(b)** the transfected stable cell line was carried out as described in Figure 1. After a 90 min chase, cell lysates (lanes 1 and 2) or medium (lanes 3 and 4) were immunoprecipitated with antibody against PDI (lanes 1 and 3) or myc-peptide (lanes 2 and 4).

Figure 4



The secretion of procollagen C-propeptides form cells expressing PDI-KDEL. The cell line expressing PDI-KDEL was transfected with either the **(a)** α1(III) C-propeptide or the **(b)** α2(I) C-propeptide. Cells were pulse-labeled for 1 hr, and after a 90 min chase, cell lysates (lanes 1 and 2) or medium (lanes 3 and 4) were immunoprecipitated with either PDI antibody (lanes 1 and 3) or anti-HA antibody (lanes 2 and 4).

corresponding partners. Since the monomeric C-propeptides contain free thiol residues (the proα2[1] C-propeptide has seven cysteines, and the proα1[III] C-propeptide has eight), the mechanism of PDI association may result in part from the formation of mixed disulphides [17]. However, we have not been able to detect mixed disulphides between PDI and procollagen C-propeptides (data not shown). Rather, we have found that the interaction can be disrupted with nonionic detergents such as Triton X-100, suggesting that the interaction is stabilized by hydrophobic interactions (R.A.L. and N.J.B., unpublished data). Irrespective of the nature of the interaction of PDI with the monomeric C-propeptide, we show here that this association directly leads to the retention of the unassembled chains within the ER lumen, thereby facilitating quality control.

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