Procollagen folding and assembly: The role of endoplasmic reticulum enzymes and molecular chaperones

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Procollagen assembly occurs within the endoplasmic reticulum, where the C-propeptide domains of three polypeptide α-chains fold individually, and then interact and trimerise to initiate folding of the triple helical region. This highly complex folding and assembly pathway requires the co-ordinated action of a large number of endoplasmic reticulum-resident enzymes and molecular chaperones. Disease-causing mutations in the procollagens disturb folding and assembly and lead to prolonged interactions with molecular chaperones, retention in the endoplasmic reticulum, and intracellular degradation. This review focuses predominantly on prolyl 4-hydroxylase, an essential collagen modifying enzyme, and HSP47, a collagen-specific binding protein, and their proposed roles as molecular chaperones involved in fibrillar procollagen folding and assembly, quality control, and secretion.

Key words: chaperone / collagen / HSP47 / prolyl 4-hydroxylase / PDI

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The collagens are a large family of structural proteins that form a diverse range of highly organised supramolecular assemblies in the extracellular matrix. Their defining feature is the initial association within the endoplasmic reticulum (ER) of three polypeptide proα-chains containing the repeating triplet sequence Gly-X-Y, which then wind together and form a triple helix. Although at least 19 different collagen types, made up of 33 distinct gene products have been identified (for detailed reviews see refs. 1,2), not all of them have been well characterised, and the biosynthetic pathway of the fibril forming class of collagens (collagens I, II, III, V, and XI) has been the most thoroughly studied. Fibrillar collagens are synthesised as large precursor proα-chains, containing a central triple helix-forming domain in which the Gly-X-Y repeat motif is continuous for approximately 1000 amino acids, flanked by N- and C-propeptide globular domains (Figure 1). Biosynthesis, folding, and assembly of procollagens is complex and involves post-translational modification by at least nine ER-resident enzymes.2 Some of these modifications, particularly hydroxylation of proline residues, are essential for efficient folding and secretion. Several studies have now demonstrated that newly synthesised procollagens are associated in the ER with the molecular chaperones HSP47, BiP, GRP94 and protein disulphide isomerase (PDI),3–6 suggesting that they assist in the procollagen folding pathway. While some steps in procollagen biosynthesis, including binding of BiP and GRP94, N-linked glycosylation, and PDI-catalysed disulphide bonding, are common in other secreted proteins, hydroxylation of proline residues, and interaction with the molecular chaperone HSP47 are unique collagen biosynthetic events. Detailed reviews of the structure and action of BiP, GRP94, and PDI can be found in this issue, and will only be discussed here in relation to their possible roles in procollagen biosynthesis and assembly. This review will focus predominantly on prolyl 4-hydroxylase/PDI and HSP47, and their proposed roles as molecular chaperones involved in fibrillar procollagen folding and assembly, quality control, and secretion. Much of what is known of the molecular chaperone functions of these proteins has come from studies on the brittle bone disease osteogenesis imperfecta (OI), where mutations in the procollagen I subunits disturb folding and assembly, and from experiments where normal folding is prevented by inhibiting the catalytic activity of prolyl 4-hydroxylase.
Figure 1. Molecular chaperones involved in procollagen folding and assembly. Three stages of type I procollagen folding and assembly are shown. (a) Synthesis of a proα1(I) chain and folding of the C-propeptide domain. (b) Trimerisation of two proα1(I) and one proα2(I) chain and folding of the triple helix. (c) A fully folded procollagen molecule. Enzymes and molecular chaperones that interact with particular domains during each stage are indicated below the protein chains in each panel. For simplicity, a high-mannose oligosaccharide is shown on only one of the proα1(I) chains, although the C-propeptides of all three chains are substituted with an N-linked carbohydrate.

Protein disulphide isomerase: a collagen molecular chaperone and anti-chaperone?

Protein disulphide isomerase (PDI) assists protein folding in the ER by catalysing the formation, reduction and isomerisation of disulphide bonds (for reviews see ref. 7 and Noiva, this issue). In procollagen, PDI-catalysed intrachain disulphide bonds form in the C- and N-propeptide and procollagen timers are stabilised by the formation of interchain disulphide bonds between the C-propeptide domains. PDI also plays a second role in collagen biosynthesis. As a subunit of the enzyme prolyl 4-hydroxylase, PDI is also essential for proline hydroxylation, the key post-translational modification underpinning helix formation. However, the role of PDI in the prolyl 4-hydroxylase complex is not related to its disulphide isomerase activity since mutant PDI, in which both catalytic sites have been inactivated by site-directed mutagenesis, forms a fully active prolyl 4-hydroxylase tetramer. The main function of PDI in prolyl 4-hydroxylase seems to be to keep the highly insoluble α subunits in a catalytically active, non-aggregated state.

The dual established roles of PDI in collagen biosynthesis as both a foldase catalysing disulphide bond formation, and a subunit of the essential enzyme prolyl 4-hydroxylase, has complicated the analysis of any potential further action it may have as a molecular chaperone during collagen folding.

While a further role of PDI as a molecular chaperone during fibrillar collagen folding has not been demonstrated unequivocally, there is growing evidence from in vitro refolding experiments that PDI can function as a molecular chaperone by suppressing aggregation and increasing the yield of correctly refolded proteins. This activity has been demonstrated during refolding of the disulphide-containing proteins lysozyme and acid phospholipase A2, as well as α-glyceroldehyde-3-phosphate dehydrogenase and rhodanese, which do not contain disulphide bonds. Furthermore, in an in vitro translation system that uses semi-permeabilised cells to mimic the initial stages in collagen assembly and modification, PDI was associated with the C-propeptides of fully hydroxylated but monomeric type I collagen proα1(I) and proα2(I) chains. The C-propeptides of these chains had folded and contained intrachain disulphide bonds, and it was suggested that this indicated a distinct role for PDI as a molecular chaperone. However, in this semi-permeabilised cell system, the majority of the chains had not associated via their C-propeptides and had not formed interchain disulphide linkages and thus it is possible that PDI may have been interacting with free C-propeptide thiols in its disulphide bond-catalysing role.

However, PDI does interact with fully folded, triple helical collagen X. This interaction is not mediated by the formation of a mixed disulphide between collagen X and PDI, implying a distinct binding mechanism and molecular chaperone activity during collagen X biosynthesis. In contrast to the fibrillar collagens, where extracellular assembly requires pro-
teolytic removal of the N- and C-propeptides, the secreted collagen X molecule is not cleaved prior to supramolecular assembly, and association of PDI with fully folded collagen X may prevent this assembly from occurring prematurely within the ER.

Another quite different role for PDI during collagen assembly has also been proposed. The initial interactions in collagen subunit assembly occur between the C-propeptides, and the triple helix then folds in a zipper-like fashion from the C- to the N-terminus (Figure 1). One consequence of this sequence of events is that the individual chains must be maintained in a soluble state, and non-productive interactions between the triple helix-forming domains prevented, until translocation of the C-propeptides, the last domain to be synthesised, is complete. These problems may be solved within the cell by formation of large aggregates that may exceed 1 500 000 Da, containing newly synthesised fibrillar collagen chains and PDI (, see Noiva, this issue). Collagen/PDI aggregates are transient, contain non-helical collagen chains, and predominantly involve formation of aberrant disulphide bonds. Formation of these large aggregates of unfolded collagen chains may be an in vivo example of the anti-chaperone activity of PDI previously identified in in vitro refolding experiments which demonstrated that low concentrations of PDI facilitated the aggregation of both lysozyme and alcohol dehydrogenase. However, whether PDI alone or PDI as a prolyl 4-hydroxylase inhibitor participates in these complexes is not known if other molecular chaperones, such as BiP also participate in these complexes.

C-propeptide folding and quality control

When folded into their native conformation, fibrillar collagen C-propeptides contain two PDI-catalysed intrachain disulphide bonds, and the correct formation of these disulphides is critical for C-propeptide folding and interactions between the C-propeptides leading to subunit assembly. In in vitro refolding experiments, individual C-propeptides fold and form intrachain disulphide bonds prior to recognition between folded C-propeptides, and when disulphide bond formation is completely prevented by incubation of fibroblasts with the reducing agent dithiothreitol, type I collagen does not assemble and is not secreted (Lamande and Bateman, unpublished data). Impaired chain association and assembly is also seen in fibroblasts from osteogenesis imperfecta (OI) patients with mutations in the C-propeptides of the type I collagen proα(I) chain. The mutations in these patients impair the formation of native intrachain disulphide bonds, and result in the C-propeptides migrating as diffuse bands on SDS-PAGE under non-reducing conditions.

It is likely that the molecular chaperone BiP (see Gething, this issue) is also involved in collagen folding, as BiP synthesis is increased in the OI patients with C-propeptide mutations and stably associates with the mutant chains. Mutations that do not affect C-propeptide folding but disturb the formation of the triple helix do not result in BiP binding or increased synthesis, implying that BiP may be restricted to assisting the folding of the C-propeptides. Collagen chains with C-propeptide mutations are selectively degraded within the cell, before transport to the Golgi. This degradation can be prevented by specific proteasomal inhibitors suggesting that it involves recognition of mutant proteins within the ER, reverse translocation, and ultimately, degradation by cytoplasmic proteasomes (Fitzgerald, Lamande and Bate- man, submitted for publication, see Brodsky and McCracken, this issue). While increased BiP synthesis, and its stable association with chains containing malfolded C-propeptides demonstrates that BiP plays a role in the cellular response to the presence of these mutant chains, the possibility that BiP specifically targets them for proteasomal degradation has not been investigated.

The molecular chaperones calnexin and GRP94 may also assist C-propeptide folding. GRP94 synthesis is increased along with that of BiP in the OI cells with C-propeptide mutations, suggesting that it too, may be interacting with malfolded C-propeptides, but specific co-immunoprecipitation of GRP94 and mutant chains was not demonstrated. Most proteins that calnexin binds are N-glycosylated and recognition of the oligosaccharide Glc3Man3GlcNAcβ is important in the initial interaction between glycoproteins and calnexin. The C-propeptides of the fibrillar collagens are each substituted with a single N-linked oligosaccharide, making them potential targets for calnexin interaction. However, if calnexin does bind to the N-linked oligosaccharide of newly synthesised collagen chains, this interaction is not essential for efficient folding of the C-propeptides, since assembly of chains in which the N-glycosylation site has been deleted by site-directed mutagenesis is normal.
Prolyl 4-hydroxylase: role in helix folding and retention of unfolded and abnormal chains

Prolyl 4-hydroxylase plays a critical role in collagen biosynthesis by catalysing the hydroxylation of proline residues in X-Pro-Gly triplets (for a detailed review of its catalytic action see ref. 31). Vertebrate prolyl 4-hydroxylase is an $\alpha_2\beta_2$ tetramer, in which the $\beta$ subunit is PDI. The subsequent formation of the triple helix requires that only trans-peptide bonds are present, and the conversion of cis-peptide bonds (16% of the X-Pro bonds and 8% of the X-Hyp bonds) is achieved by the action of a second enzyme, peptidyl-prolyl cis-trans isomerase (PPIase). A member of a family of protein folding enzymes which include cyclophilins, FK506-binding proteins and parvulins (for a review see ref. 36).

Almost complete hydroxylation of appropriate proline residues is necessary for stability of the fibrillar collagen triple helix at $37^\circ C$ and when hydroxylation is inhibited by incubation of cells in the absence of ascorbate, an essential enzyme co-factor, or by the addition of the iron chelator a, a′-dipyridyl, unfolded chains accumulate within the ER. Upon reactivation of prolyl 4-hydroxylase, collagen folding and secretion is rapidly restored. ER-retention and delayed secretion is also a common consequence of disease causing mutations in the triple helix-forming domains that slow folding of the helix and disturb its structure and stability. Prolyl 4-hydroxylase is one candidate molecule that may monitor collagen conformation and regulate secretion.

In vitro experiments have demonstrated that the substrate binding affinity of prolyl 4-hydroxylase is reduced by three orders of magnitude when collagen is fully hydroxylated, and is reduced even further when a triple helical conformation is achieved. Prolyl 4-hydroxylase might therefore be expected to continuously interact with collagen chains synthesised in the presence of enzyme inhibitors, and this has been shown to be the case. Inhibition of prolyl 4-hydroxylase in semi-permeabilised cells by a, a′-dipyridyl results in stable binding of prolyl 4-hydroxylase to unhydroxylated, non-helical proa(I)III chains, and when prolyl 4-hydroxylase is reactivated by the addition of excess iron, hydroxylation and triple helix folding resumes, and the majority of the prolyl 4-hydroxylase dissociates.

There is also evidence that prolyl 4-hydroxylase may help prevent secretion of type I collagen molecules with abnormal triple helical domains. In fibroblasts from an OI patient with a 180 amino acid deletion within the triple helix, there is almost complete intracellular retention of the mutant-containing molecules. Mutant molecules are hydroxylated and form pepsin-resistant triple helices, but the conformation of the helix N-terminal to the deletion is abnormal and can be digested with a mixture of trypsin and chymotrypsin, more sensitive probes of triple helical structure. Unlike collagen from control cells, and despite being fully hydroxylated, these abnormal collagen molecules are stably associated with prolyl 4-hydroxylase. It is not yet known if prolyl 4-hydroxylase also associates with collagen molecules containing single amino acid substitutions within the triple helix. While the precise mechanisms responsible for recognition and ER-retention of unfolded chains and abnormal molecules is not known, together these data suggest that stable interaction with prolyl 4-hydroxylase may be another of the quality control mechanisms acting to prevent secretion of misfolded collagens.

HSP47

HSP47 is a collagen-binding, heat-inducible protein, resident in the vertebrate endoplasmic reticulum. The human, rat, mouse, and chicken cDNAs have been cloned, and many studies have demonstrated its expression in collagen producing cells and tissues, and its ability to bind a wide range of collagens, both in vitro and in cells. The intracellular location, binding, and expression characteristics have led to the proposal that HSP47 is a collagen-specific molecular chaperone. The literature describing HSP47 (47 kDa heat shock protein) is complicated by the simultaneous and persistent use of multiple names, including colligin, J6, gp46 and CB48, but for clarity, only the most commonly used name, HSP47 will be used here.

Following cleavage of the signal peptide, mature human HSP47 is a 400 amino acid protein, with two N-linked carbohydrate attachment sites, both of which are substituted with high-mannose oligosaccharides, and a C-terminal RDEL sequence that acts as an ER-retention signal. There are a number of potential phosphorylation sites in the HSP47 sequence, and phosphorylated forms of HSP47 can be detected in some, but not all cells, although the function and importance of HSP47 phosphorylation is not known. HSP47 shares homology with the serpin family of serine protease inhibitors, but it is not active as a protease inhibitor in in vitro assays, probably because of sequence differences at the active site.

Shortly after its initial discovery as a collagen-bind-
The expression of HSP47 closely correlates with that of collagen in a range of cell lines, during cell differentiation in vitro, and during development. HSP47 is found in cells that synthesise collagens I and III, such as primary fibroblasts, 34 3T3 cells,50 and in cultured chondrocytes synthesising collagen II.51 However, it is not expressed in cells that do not produce collagens, e.g. E. coli, or 293 embryonic kidney cells.64 HSP47 and collagen IV are both induced during differentiation of F9 embryonic carcinoma cells following retinoic acid treatment,65 and the levels of HSP47 and collagen I decline together during differentiation of L6 myoblasts.66 During mouse embryo development, HSP47 is expressed mainly in mesoderm and mesoderm derived tissues, such as cartilage, bone, notochord and somites, and its expression correlates both temporally and spatially with that of collagens I and II.67 Numerous additional examples of HSP47 and collagen co-expression, and their coincident up- and down-regulation, exist in the literature. This large body of data supports the idea that HSP47 has an important function in collagen biosynthesis.

**In vitro binding properties of HSP47**

HSP47 was first identified because of its ability to bind to gelatin, a mixture of denatured collagens I and III, and was later shown to bind also to triple helical collagen I.48 Individual pro a1(I) and pro a2(I) chains can compete with native collagen for HSP47 binding in solid phase assays, and native collagen I competes as efficiently as gelatin for binding to native collagen,69 suggesting that HSP47 does not distinguish between unfolded chains, and mature folded collagen molecules. The affinity of HSP47 for native collagens I–V has been studied using a BIAcore™ system which allows quantification of protein–protein interactions.70 Collagens I–V all have a similar affinity for HSP47, with dissociation constants of ~10^-7 M, relatively low for molecular interactions. The low dissociation constant results from rapid dissociation (k_diss > 10^-3 s^-1) and a relatively high association rate constant (k_ass ~ 2 x 10^4 M^-1 s^-1). These data predict that high concentrations of HSP47 would be required for stable HSP47/collagen interactions within cells.70

The crystal structure of HSP47 has not been solved, however, a three-dimensional molecular model based on the structure of another serpin, protein C inhibitor, predicts a long cleft that could accommodate collagen chains.51 Binding of HSP47 to gelatin and native collagen is pH-dependent, being abolished below pH 6.3.52 HSP47 undergoes reversible pH-induced conformational changes that can be measured by circular dichroism, and this may explain its pH-dependence.53 HSP47 has also been shown to bind to gelatin, a mixture of denatured collagens I and III,68 and was later shown to bind also to triple helical collagen I.48 Individual pro a1(I) and pro a2(I) chains can compete with native collagen for HSP47 binding in solid phase assays, and native collagen I competes as efficiently as gelatin for binding to native collagen,69 suggesting that HSP47 does not distinguish between unfolded chains, and mature folded collagen molecules. The affinity of HSP47 for native collagens I–V has been studied using a BIAcore™ system which allows quantification of protein–protein interactions.70 Collagens I–V all have a similar affinity for HSP47, with dissociation constants of ~10^-7 M, relatively low for molecular interactions. The low dissociation constant results from rapid dissociation (k_diss > 10^-3 s^-1) and a relatively high association rate constant (k_ass ~ 2 x 10^4 M^-1 s^-1). These data predict that high concentrations of HSP47 would be required for stable HSP47/collagen interactions within cells.70

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Potential functions of HSP47 in collagen biosynthesis

Within the ER, procollagen is continuously bound to HSP47, from synthesis, through to chain association and triple helix folding. HSP47 continues to bind completely folded molecules and only dissociates when procollagen enters the Golgi. This ability to recognise both unfolded and mature folded proteins is unusual, and distinguishes HSP47 from other molecular chaperones.

HSP47 binding to collagen occurs at a very early stage in biosynthesis, probably during co-translational import into the ER. This was demonstrated by the co-immunoprecipitation of HSP47 with nascent pro-α1(I) chains that were still associated with polysomes. It is unusual, and distinguishes HSP47 from other molecular chaperones.

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Molecular chaperones and collagen folding

dicted to dissociate in the Golgi where the pH has been estimated to be approximately 5.7. An essential role for HSP47 in collagen assembly and secretion has not been unequivocally established. Recent studies demonstrated that type III procollagen was able to fold and assemble, and was secreted by transfected 293 cells which do not express HSP47, demonstrating that, in this cell type at least, HSP47 is not required for secretion. When these cells were co-transfected with HSP47 cDNA, secretion of the collagen was apparently reduced, suggesting that HSP47 delays secretion. Based on these results, it was suggested that HSP47/collagen interactions slow the intracellular transit time of procollagens and thus may play a role in regulating the level of post-translational modifications, which are, in part, controlled by the rate of collagen folding and secretion. In contrast, a preliminary report suggests that HSP47 facilitates procollagen secretion (cited in ref. 76). Secretion of procollagen was reported to be slower in cells that had been depleted of HSP47 by transfection with an antisense HSP47 construct. Further studies will be required to resolve these apparently conflicting findings, and determine the role of HSP47 in regulating the rate of procollagen secretion.

Procollagens are not secreted as individual molecules. Once inside the Golgi, procollagen molecules align side-by-side in parallel, and form large electron dense aggregates. These aggregates are seen in distensions at all levels of the Golgi, and procollagen cannot be detected in non-distended regions of the Golgi cisternae. Aggregates are also seen at the cell surface during secretion, and secretion in this form has been proposed to facilitate extracellular fibril formation. Formation of procollagen aggregates in the Golgi coincides with its release from HSP47, raising the possibility that when HSP47 concentrations are lowered in the cis-Golgi, it may exhibit anti-chaperone characteristics and facilitate or promote aggregate formation. While procollagen can be secreted in the absence of HSP47, it is not known if it is secreted in an aggregated or monomeric form, and the ability of these molecules to efficiently assemble into fibrils in the extracellular matrix has not been examined.

Perspectives

The particularly complex collagen biosynthetic and folding pathway involves the concerted, coordinated action of a large number of essential enzymes and molecular chaperones. Prolonged interaction of BiP and prolyl 4-hydroxylase with mutant misfolded procollagen I from OI patients suggests that these molecules are also important in quality control processes, such as ER retention and intracellular degradation. Molecular chaperones, such as BiP and PDI also participate in the folding of many other secreted and membrane-associated proteins and this has assisted in understanding their role in procollagen folding. In contrast, HSP47 is thought to be a collagen-specific molecular chaperone, and despite numerous studies exploring its expression patterns and binding characteristics, the precise function of HSP47 remains elusive. Every possible role has been suggested, from assisting in translocation, to preventing misfolding of individual chains, retaining unfolded molecules in the ER, and regulating secretion, however, essential participation in any of these events has not yet been convincingly demonstrated, and many questions remain. Is it required for translocation into the ER? What is its function during heat shock? Is its synthesis up-regulated in response to the presence of unfolded chains? Does HSP47 regulate the aggregation state of secreted procollagen, and is this important in extracellular fibril formation? The answers to some of these questions may lie in the phenotype of an HSP47 knock-out mouse, which is currently being produced, however, the presence of two HSP47 genes may mean that a double knock-out will be required. The possibility remains that the functions of HSP47 may overlap sufficiently with those of other ER-resident molecular chaperones to allow collagen biosynthesis to continue relatively normally in its absence.

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462
S. R. Lamande and J. F. Bateman


