Intracellular Retention of Procollagen within the Endoplasmic Reticulum Is Mediated by Prolyl 4-Hydroxylase*

(Received for publication, February 9, 1999, and in revised form, March 10, 1999)

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The correct folding and assembly of proteins within the endoplasmic reticulum (ER) are prerequisites for subsequent transport from this organelle to the Golgi apparatus. The mechanisms underlying the ability of the cell to recognize and retain unassembled or malformed proteins generally require binding to molecular chaperones within the ER. One classic example of this process occurs during the biosynthesis of procollagen. Here partially folded intermediates are retained and prevented from secretion, leading to a build up of unfolded chains within the cell. The accumulation of these partially folded intermediates occurs during vitamin C deficiency due to incomplete proline hydroxylation, as vitamin C is an essential co-factor of the enzyme prolyl 4-hydroxylase. In this report we show that this retention is tightly regulated with little or no secretion occurring under conditions preventing proline hydroxylation. We studied the molecular mechanism underlying retention by determining which proteins associate with partially folded procollagen intermediates within the ER. By using a combination of cross-linking and sucrose gradient analysis, we show that the major protein binding to procollagen during its biosynthesis is prolyl 4-hydroxylase, and no binding to other ER resident proteins including Hsp47 was detected. This binding is regulated by the folding status rather than the extent of hydroxylation of the chains demonstrating that this enzyme can recognize and retain unfolded procollagen chains and can release these chains for further transport once they have folded correctly.

The biosynthesis of multi-subunit proteins entering the secretory pathway is regulated at the endoplasmic reticulum (ER) where the individual subunits are synthesized and their assembly is coordinated. This regulation ensures that unassembled subunits are prevented from being transported out of the ER and are either degraded or maintained in an assembly-competent state by interacting with ER resident proteins (1). The mechanism underlying this “quality control” appears to involve the binding of unassembled subunits to a variety of ER proteins until assembly occurs. The assembled complex is then released and can be transported from the ER. Such a mechanism has been likened to affinity chromatography, with the “matrix” being the resident proteins in the ER and the selective interactions occurring via oligosaccharide side chains (2), hydrophobic regions in the protein (3), or free thiol residues (4).

To investigate further the generality of these mechanisms of retention, we have studied the folding, assembly, and secretion of procollagen. The folding and assembly of procollagen occurs in a vectorial manner; trimerization of individual polypeptides called the pro-α-chains occurs via type-specific association of their C-propeptides (5, 6). This association facilitates the formation of a stable nucleus of triple helix at the C-terminal end of the triple helical domain (7) that is subsequently propagated to the N terminus in a zipper-like fashion (8). The prerequisite for the formation of a triple helix that is stable at physiological temperatures is the 4-hydroxylation of a certain proportion of proline residues within the triple helical domain of the pro-α-chains (9), a modification catalyzed by the ER resident enzyme prolyl 4-hydroxylase (P4H) (10).

Evidence that the procollagens of fibril-forming collagens are subject to a retention mechanism has been provided by studies on type I procollagen synthesized by chick fibroblasts (11, 12). It was demonstrated that the rate of secretion of the protein is significantly depressed when the formation of the triple helix is prevented either by the incorporation of helix-destabilizing proline analogues into the pro-α-chains or by the inhibition of hydroxylation of the pro-α-chains. Furthermore, point mutations in the genes for the pro-α-chains of type I procollagen which cause the human disease osteogenesis imperfecta have been shown to disrupt the folding of the triple helix and result in the retention and subsequent degradation of the mutant protein within the cell (13, 14).

A number of ER resident proteins have been proposed to mediate the retention of non-native procollagen within the cell, one of these being Hsp47, a collagen-binding heat-shock protein (15). Hsp47 has been shown to bind to a wide range of collagens and procollagens in vitro (16, 17). It also associates with type I procollagen in cellulo (18, 19), leading to the suggestion that Hsp47 is a chaperone of procollagen biosynthesis. In addition BiP and PDI have been individually shown to associate with certain mutant forms of type I procollagen that were retained within the fibroblasts of patients with osteogenesis imperfecta (20, 21). Furthermore, it has been proposed that P4H is a candidate for the ER resident protein involved in mediating procollagen retention. This was based on the fact that the binding affinity of this enzyme for procollagen substrates in vitro was in compliance with the selectivity of the retention mechanism for procollagens in cellulo (22). More recently, PDI has been shown to interact specifically with individual C-propeptide chains prior to trimerization (23) and with assembled type X collagen (24).

* This work was supported by Wellcome Trust Grant 50600, The Royal Society, and the Biotechnology and Biological Sciences Research Council. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: ER, endoplasmic reticulum; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; DSP, dithiobis(succinimidyl pro- pionate); PDI, protein-disulfide isomerase; SBTI, soybean trypsin inhibitor; P4H, prolyl 4-hydroxylase.
To address the question of which ER resident proteins are involved in the retention of procollagen, we have prepared stable cell lines expressing a type III procollagen mini-gene containing either the authentic C-propeptide or a construct where the C-propeptide has been replaced with a transmembrane domain. Both chains have been shown previously to fold and assemble correctly when expressed in the presence of semi-permeabilized cells (7, 25). These cell lines were used to investigate the regulation of procollagen secretion and to identify proteins interacting with partially folded procollagen chains. The results clearly demonstrate a novel role for a post-translational modifying enzyme in the regulation of secretion of its substrate, and the results show that this regulation is not influenced directly by enzymatic activity.

**MATERIALS AND METHODS**

HT1080 cells (ATCC CCL121) were obtained from the American Type Culture Collection (Rockville, MD). A mouse monoclonal antibody to Hsp47 (SPA-470) and a rat monoclonal antibody to GRP78 (SPA-850) were obtained from StressGen (York, UK). A goat polyclonal antibody to BiP (N-20) was purchased from Santa Cruz Biotechnology (Mile Elms, Calne, UK). A rabbit polyclonal antibody to calreticulin antibody was purchased from Cambridge Biosciences (Cambridge, UK). A rabbit polyclonal antibody to type III procollagen was prepared as described previously (29). Rabbit polyclonal antibodies to PDI, ERp57, and to calnexin were prepared as described previously (26–28). A mouse monoclonal antibody specific for the α-subunit of prolyl 4-hydroxylase was a gift from Professor Kari Kivirikko (University of Oulu, Finland), and a mouse anti-Myc monoclonal antibody (9E10) was obtained from Calbiochem (Nottingham, UK).

**Plasmid Construction**—The plasmid pI(III)Δ1 containing the sequence encoding the prepro-α(III) chain of type III procollagen had been constructed previously (29). The SpeI/ClaI fragment excised from pI(III)Δ1 containing the prepro-α(III) chain sequence was cloned into the XhoI SalI site of pCI neo (Promega, Madison, WI). The pCI neo vector was then XhoI/ClaI digested to remove the SpeI fragment and ligated with the XhoI SalI fragment from the modified pCI neo vector to create the construct pCI neo αI(III) Δ1. The plasmid HA-trans was constructed previously (7). A Myc-tagged version of pI(III)Δ1 was prepared by introducing the Myc tag between the end of the signal sequence and the beginning of the coding region using standard polymerase chain reaction techniques as described previously (30). A 300-base pair XhoI/KpnI fragment was excised from HA-trans and subcloned into XhoI/SalI cut pI(III)Δ1-Myc to generate HA-trans-Myc. This plasmid was linearized with NotI and subcloned into pIRES2hyg (CLONTECH, Basingstoke, Hampshire, UK) to generate pIRES2hyg-HA-trans-Myc.

**Cell Culture**—All cell lines were maintained in Dulbecco’s modified Eagle’s medium with 110 μg/ml I-glutamine and 10% (v/v) fetal calf serum (complete medium).

**Cell Culture Lines**—HT1080 cells were maintained at 50–80% confluence in complete medium. Cell suspensions were prepared at 50–80% confluence by treatment of cells with trypsin and pelleted by centrifugation. Harvested cells were washed twice in phosphate-buffered saline (PBS) and resuspended in complete medium to an approximate concentration of 1 × 10^6 cell/ml. The cell suspension (80 μl) was placed in a 4-ml electroporation cuvette and mixed 1 min prior to the pulse with 30 μg of pCI neo Δ1 linearized with BgIII or 30 μg of pIRES-hyg-HA-trans linearized with SpeI. Cells were pulsed at 1650 microfarads/250 V using the EasyJect electroporator (Flowgen) and immediately transferred from the cuvettes into a T162-cm2 flask containing complete medium. Cells were incubated for 48 h and subsequently selected by incubation in media containing geneticin at a concentration of 0.5 μg/ml. The resulting G418-resistant colonies were transferred to fresh T162-cm2 flasks and grown to confluence before being transferred to 75-cm2 flasks. The colonies expressing type III procollagen, proteins present in the medium of the clones were precipitated by the addition of PEG-3000 to a concentration of 5% (w/v), harvested by centrifugation at 12,000 × g, separated by SDS-PAGE, and transferred to nitrocellulose. The membrane was probed with a 1:1000 dilution of an antibody against type III procollagen and developed using a chemiluminescence substrate. The expression level of pro-α(III)Δ1 was quantitated by metabolic labeling, and G418 was identified as the cell line with the highest expression level. Similarly, indirect immunofluorescence staining using anti-Myc antibody identified clones expressing HA-trans-Myc at the cell surface and F2 was identified as a high expressing cell line.

**Antibody-Chasing**—All the following procedures were performed at 37 °C. B12 cells were plated to 50–80% confluence in 100-mm2 culture dishes and incubated for 4 h in complete medium. Cells were rinsed twice with 5 ml of PBS, once with 5 ml of methionine- and cysteine-free minimal Eagle’s medium supplemented with 0.3 mg ml^-1 glutamine (starve medium), and incubated for 45 min in 5 ml of the starve medium. Cells were incubated with 5 ml of starve medium containing 0.1 mM cycloheximide and 150 mM NaCl, 0.5% (w/v) NaNO3, and 0.1 mg ml^-1 soybean trypsin inhibitor (SBTI), 0.5 mM phenylmethylsulfonyl fluoride (PMSF). When required, N-ethylmaleimide (NEM) was present in the PBS and the lysis buffer at a concentration of 20 μM. Cells were lysed for 30 min, and the lysate was centrifuged at 12,000 × g for 15 min to remove nuclei and cell debris. Proteins present in the cell lysate and supernatant were precipitated with the addition of PEG as described above.

**Cross-linking with DSP**—B12 cells were radiolabeled for 1 h as described above and pelleted by centrifugation. Harvested cells were washed in PBS and resuspended in 100 μl of PBS. The cell suspension was mixed with 10 μl of freshly prepared 20 μM DSP in MeSO and incubated at 4 °C for 30 min. Cells were washed with 2 ml glycine in PBS to quench the cross-linker, washed once with PBS, and resuspended in 200 μl of Nonidet P-40 lysis buffer (50 mM Tris-HCl, pH 7.4, 2 mM EDTA, 1% (v/v) Triton X-100, 150 mM NaCl, 0.02% (w/v) NaNO3, 0.1 mg ml^-1 soybean trypsin inhibitor (SBTI), 0.5 mM phenylmethylsulfonyl fluoride (PMSF)). When required, N-ethylmaleimide (NEM) was present in the PBS and the lysis buffer at a concentration of 20 μM. Cells were lysed for 30 min, and the lysate was centrifuged at 12,000 × g for 15 min to remove nuclei and cell debris. Proteins present in the lysate and supernatant were precipitated with the addition of PEG as described above.

**Immunoprecipitation**—For the immunoprecipitation of proteins under denaturing conditions, 10% (w/v) SDS was added to the cell lysate supernatant or to PEG precipitates from medium to a final concentration of 0.5%, and the sample was boiled for 5 min. The sample was then diluted 10-fold with immunoprecipitation buffer (50 mM Tris-HCl, pH 7.4, 2 mM EDTA, 1% (v/v) Triton X-100, 150 mM NaCl, 0.02% (w/v) NaNO3 and centrifuged for 15 min to remove non-solubilized material.

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twice with immunoprecipitation buffer, once with high salt buffer (50 mM Tris-HCl, pH 7.4, 2 mM EDTA, 1% (v/v) Triton X-100, 500 mM NaCl, 0.02% (w/v) NaN₃), and once again with immunoprecipitation buffer. The immunoprecipitates were then prepared for analysis by SDS-PAGE.

**Immunoprecipitation**—P2 cells were plated to 80% confluence on 13-mm coverslips and cultured overnight in complete medium supplemented with 50 μg/ml ascorbate if required. The following procedures were performed at room temperature. Cells were washed twice in PBS and then fixed with 4% (w/v) paraformaldehyde in PBS for 15 min. Three washes with PBS were followed by three with NaBH₄ solution (0.5 mg/ml in PBS) to reduce auto-fluorescence, and where appropriate the cells were permeabilized with 0.1% (v/v) Triton X-100, 0.5% (w/v) SDS in PBS for 4 min. The coverslips were rinsed in PBS before incubation with a 1/100 dilution of the anti-Myc mouse monoclonal antibody (9E10) for 1 h. After washing, incubation with a 1/40 dilution of a donkey anti-mouse Cy2 conjugate (Amersham Pharmacia Biotech, Buckinghamshire, UK) was performed for 1 h.

**Poly(I-Proline) Precipitation**—Fractions from sucrose gradients were incubated with gentle agitation for 60 min with 10% (w/v) poly(I-proline)-Sepharose. The poly(I-proline) precipitate was pelleted by centrifugation at 12,000 × g for 15 min and washed four times with P4-H buffer (10 mM Tris, pH 7.8, 0.1 M NaCl, 100 mM glycine, 0.1% Triton X-100). Proteins were eluted from the poly(I-proline)-Sepharose by incubating the precipitate with gentle agitation for 5 min in P4-H buffer containing 3 mg ml⁻¹ poly(L-proline) (3000 kDa). Proteins were precipitated from the eluate by the addition of trichloroacetic acid to 15% (w/v) and acetone to 25% (v/v), and the sample was incubated with gentle agitation for 60 min. Precipitates were harvested by centrifugation at 12,000 × g for 15 min, washed with acetone, and prepared for SDS-PAGE analysis.

**SDS-PAGE**—Immunoprecipitates were resuspended in SDS-PAGE buffer (62.5 mM Tris-HCl, pH 6.8, 1% (w/v) SDS, 10% (v/v) glycerol, bromphenol blue) and boiled for 5 min in either the absence (non-reducing) or presence (reducing) of 50 mM dithiothreitol.

**Results**

The primary purpose of this study was to investigate the molecular mechanism leading to the retention of partially folded procollagen chains within the ER. To facilitate this study we first constructed a cell line expressing a procollagen "mini-chain," designated pro-α1(III)Δ1, that we have previously studied using a semi-permeabilized cell expression system (31). These experiments have shown that procollagen "mini-chains" translated in the presence of SP cells are efficiently translated, modified, and assembled into a correctly aligned triple helix (25). Here we used HT1080 as the host cell line because these cells do not express any fibril-forming procollagens and therefore it is likely that these chains may represent a trimer that is stabilized by the formation of inter-chain disulfide bonds within the C-propeptide. In the presence of ascorbate the chains migrated predominantly with an apparent molecular mass of 260 kDa. We have shown previously that this form represents a trimer that is stabilized by the formation of inter-chain disulfide bonds within the C-propeptide (25). The higher mobility of this trimer relative to the trimer stabilized by inter-chain disulfide bonds within the C-propeptide only is a result of a decrease in the hydrodynamic volume (34). These results demonstrate that the pro-α1(III)Δ1 synthesized in this cell line folds to form a triple helical molecule when expressed in the presence of co-factors for P4H and that we can distinguish between two disulfide-bonded intermediates by carrying out electrophoresis under non-reducing conditions. For simplicity these intermediates will subsequently be referred to as a non-helical or helical trimer.

**Effect of Ascorbate on Procollagen Secretion**—Incubating the B12 cell line in the absence of ascorbate clearly prevents folding of the procollagen molecule leading to a build-up of non-helical trimer. To investigate whether the cells could recognize this intermediate and retain it within the cell, we examined the effect of ascorbate depletion on the secretion of pro-α1(III)Δ1 chains. Proteins synthesized by B12 cells were radiolabeled...
and chased over a 180-min period (Fig. 2). Pro-α1(III)Δ1 chains were immunoprecipitated from the cell lysates (Fig. 2A) and from the medium (Fig. 2B). Immunoprecipitated proteins were subjected to SDS-PAGE under reducing conditions. In the presence of ascorbate, pro-α1(III)Δ1 chains were secreted into the medium after 40 min of chase with a maximal level of secretion of radiolabeled protein at 80 min of chase (Fig. 2B). In the absence of added ascorbate, very little radiolabeled material was secreted, the small amount present in the medium after 80 min of chase probably representing a minimal amount of protein that folds even in the absence of added ascorbate (see Fig. 2B, 40 min). Thus, it would appear that in the absence of added ascorbate the non-helical trimers formed are retained within the cell.

This retention could be a consequence of the ascorbate having a general effect on protein secretion rather than its role as a co-factor of P4H. We investigated this possibility by using an alternative approach to inhibit P4H activity. Along with ascorbate and oxygen, iron is an essential co-factor of this enzyme (10). Chemicals that chelate iron such as α,α′-dipyridyl are inhibitors of this enzyme and have been shown to be effective when added to cells grown in culture leading to the under-hydroxylation of procollagen (35). In the present study, a pulse labeling and chase experiment was carried out using B12 cells incubated in the presence of α,α′-dipyridyl (Fig. 3). Here no pro-α1(III)Δ1 chains were secreted even after 180 min of chase (Fig. 3B, lanes 1–5) with all the radiolabeled chains remaining within the cell (Fig. 3A, lanes 1–5). When these samples were separated under non-reducing conditions the pro-α1(III)Δ1 chains separated as non-helical trimers (results not shown). When B12 cells were labeled in the presence of α,α′-dipyridyl and then incubated in medium containing iron and protein synthesis inhibitor, a reversal of this block in secretion was observed (Fig. 3B, lanes 18–20). After 40 min of chase in the presence of iron, the radiolabeled protein in the cell lysate decreased in electrophoretic mobility when separated under reducing conditions (Fig. 3A, compare lanes 6 and 7). This decrease in mobility is characteristic of proline hydroxylation which is thought to affect SDS binding (36). Pro-α1(III)Δ1 chains were secreted into the medium after 80 min of chase (Fig. 3B, lane 18), indicating that the secretion block is reversible and that the time taken for the non-helical trimers to be hydroxylated, folded, and secreted is between 40 and 80 min. This experiment also demonstrates that the retention of the pro-α1(III)Δ1 chains within the cell is due to a lack of hydroxylation or folding rather than a general effect of ascorbate on the secretory pathway.

Localization of the Block in Secretion of Non-helical Procollagen Trimer—Having established that cells can recognize and retain non-helical procollagen chains, we next addressed the question of where the block in secretion occurs. For this study...
we constructed a procollagen chain where the C-propeptide was replaced with the transmembrane domain from influenza virus hemagglutinin. We have shown that this protein can fold correctly to form a triple helical molecule when expressed in SP cells (7). We also included a Myc epitope at the N terminus of the mature polypeptide to allow visualization by indirect immunofluorescence. As the synthesized polypeptide remains membrane-bound, we could also monitor secretion by visualizing cell-surface expression. A stable cell line expressing a Myc-tagged membrane-bound form of procollagen was grown on coverslips incubated either in the presence or absence of ascorbate (ASC) as indicated. Cells were fixed and either permeasbiliated with Triton X-100 or not permeasbilated before addition of anti-Myc antibody as indicated. The cells were then incubated in the presence of secondary antibody, and immunofluorescence was visualized.

**Fig. 4. Localization of procollagen chains retained within the cell.** A stable cell line (F2) expressing a Myc-tagged membrane-bound form of procollagen was grown on coverslips incubated either in the presence or absence of ascorbate (ASC) as indicated. Cells were fixed and either permeasbilated with Triton X-100 or not permeasbilated before addition of anti-Myc antibody as indicated. The cells were then incubated in the presence of secondary antibody, and immunofluorescence was visualized.

**Fig. 5. Retention of hydroxylated non-helical trimers of proc-α1(III)Δ1.** B12 cells were pulse-labeled for 20 min as described previously in the presence (lanes 1–10) or absence (lane 11) of the proline analogue azetidine-2-carboxylic acid (AZE). Cells were then incubated for the indicated times in the presence (lanes 6–11) or absence (lanes 1–5) of α,α′-dipyridyl (DP). Ascorbate was present in the medium throughout the experiment. The pro-α1(III)Δ1 chains were synthesized were isolated from cell lysates (A and B) or the medium (C) and separated by SDS-PAGE under reducing (A and C) or non-reducing (B) conditions. hyd, hydroxylated; unhyd, unhydroxylated.
in the presence of α,α′-dipyridyl and could reflect release of intracellular material due to prolonged incubation with the proline analogue. This contrasts with the rapid secretion of pro-α1(III)Δ1 chains from B12 cells in the absence of analogue (Fig. 2B). These results indicate that the retention of procollagen within the cell is due to a lack of folding of the collagen triple helix rather than a lack of proline hydroxylation.

Cross-linking of Procollagen to Proteins within the ER—The retention of non-native proteins within the cell has been hypothesized to be mediated by ER resident chaperones that confer their ER residency to such proteins by selectively binding to them (1). Assuming that such a process was responsible for the retention of procollagen chains within the cell, a cross-linking approach was adopted in an attempt to identify ER resident proteins that selectively bound to non-helical pro-α1(III)Δ1 chains. Proteins synthesized by B12 cells were radiolabeled for 1 h and subsequently incubated in either the absence or presence of DSP, a thiol-cleavable homo-bifunctional reagent that is specific for primary amine groups. Ascorbate was omitted in order to minimize the conversion of the non-helical trimer to the helical trimer. Cross-linking complexes containing the following well characterized ER resident proteins PDI, ERP57, calreticulin, calnexin, BiP, GRP94, and Hsp47 were separately immunoprecipitated from the cell lysates before or after prior denaturation of the lysate by boiling in SDS.

The immunoprecipitated complexes were subjected to SDS-PAGE analysis under reducing conditions in order to cleave the cross-linker and allow the identification of the individual components of the cross-linked complexes. Most of the antibodies employed effectively immunoprecipitated their corresponding target proteins from the cross-linked lysates under both native and denaturing conditions with the exception of the BiP antibody which worked best after denaturation of the sample (Fig. 6, lanes 6 and 14). The antibody to the pro-α1(III)Δ1 chain also reacted poorly with its antigen under native conditions (Fig. 6, lane 1).

Several co-immunoprecipitating bands were seen with antibodies to ER proteins when lysates were not denatured prior to immunoprecipitation (Fig. 6, lanes 2–5). These were not covalently cross-linked as they were absent in the immunoprecipitates from denatured lysates (Fig. 6, lanes 10–13). After cross-linking, the pro-α1(III)Δ1 chain was present in the immunoprecipitate of the antibody against PDI, but only when the immunoprecipitation was performed under native conditions (Fig. 6, lanes 1–4). These results indicate that the retention of procollagen within the cell is due to a lack of folding of the collagen triple helix rather than a lack of proline hydroxylation.

Cross-linking of non-helical trimer of pro-α1(III)Δ1 to ER resident proteins. Proteins synthesized by B12 cells were labeled for 1 h and proteins cross-linked with DSP as described under “Material and Methods.” Cells were lysed and immunoprecipitations carried out directly (lanes 1–8) or after denaturation of the proteins (lanes 8–15) with the antibodies indicated. Samples were separated by SDS-PAGE under reducing conditions.

Velocity Centrifugation—The results obtained with cross-linking suggest that non-helical procollagen chains form a complex with P4H. To confirm these results using an alternative approach and to gain some information on the components of the complex, we carried out velocity centrifugation on cell lysates. B12 cells were radiolabeled for 1 h, and proteins present in the cell lysate were separated on a 5–25% sucrose gradient. Ascorbate was omitted from the media in order to minimize the rate of conversion of the non-helical trimer to the helical trimer. After centrifugation the gradients were divided into 14 fractions with fraction 1 being at the bottom of the gradient and fraction 14 being at the top (Fig. 8). To determine the position of conversion of the non-helical trimer to the helical trimer.
within the gradient of individual molecules that are not part of a complex, lysates were first denatured with SDS, and proteins were precipitated with specific antibodies. Alternatively, lysates were separated without denaturation and P4H isolated from fractions by binding to prolyl-Sepharose, an affinity matrix that will specifically bind to free P4H (37). The results show that P4H separated in fractions 5, 6, and 7 (Fig. 8A), and PDI separated in fractions 9–14 (Fig. 8B), whereas pro-

\[ \text{a} \](III)\( \Delta 1 \) separated in fractions 3, 4, and 5 (Fig. 8C). Thus there was a clear separation of these three proteins by velocity centrifugations.

We then separated the cell lysate without prior denaturation and immunoprecipitated the resulting fractions with antibodies to PDI (Fig. 8D). PDI itself was present as expected in fractions 9–14, and co-precipitated P4H \( \alpha \)-subunit was also, as expected, present in fractions 5, 6, and 7. However, PDI and the \( \alpha \)-subunit of P4H were also seen in fractions 2, 3, and 4 along with pro-

\[ \text{a} \](III)\( \Delta 1 \) chains. This clearly demonstrates that a complex between P4H and the procollagen chain was present in the cell lysate and that this complex could be precipitated with antibodies to PDI. The fact that we could isolate such a complex in the absence of cross-linking after sucrose gradient fractionation but not directly from cell lysates by co-immunoprecipitation could be due to a stabilization of the P4H-procollagen interaction in the presence of sucrose. These results confirm the cross-linking experiments demonstrating that P4H interacts and forms a stable complex with procollagen chains.

**Dissociation of P4H from Procollagen Chains following Folding—**If P4H regulates the secretion of procollagen by binding to non-helical trimers then this interaction should persist until the protein folds, whereupon the complex dissociates allowing secretion from the cell. To determine whether procollagen binding to P4H is regulated by the folding status of the proc-

\[ \text{a} \](III)\( \Delta 1 \) chains, B12 cells were pulsed-labeled in media containing \( \alpha,\alpha' \)-dipyridyl. The labeled cells were subsequently chased for 40 min with protein synthesis inhibitors either in the presence of the chelator (Fig. 9A) or excess ferrous ions (Fig. 9B). The 40-min chase period performed in the presence of ferrous ions was shown to be sufficient for the complete conversion of the under-hydroxylated non-helical to the hydroxy-

lated helical trimer (Fig. 3, and results not shown). The proteins present in the cell lysate were separated on a 5–25% sucrose gradient, and complexes containing PDI were immunoprecipitated from the fractions isolated. In the presence of \( \alpha,\alpha' \)-dipyridyl a stable complex was formed as evidenced by the co-immunoprecipitation of P4H and pro-

\[ \text{a} \](III)\( \Delta 1 \) chains (Fig. 9A, fractions 1–5). However, after incubation in the presence of excess iron, no complex was detected (Fig. 9B). This demonstrates that following addition of iron P4H dissociates from the procollagen chain that has folded allowing subsequent secretion from the cell (Fig. 3B).

The complex formed between P4H and procollagen could be regulated not by the folding status but by the extent of hydroxylation of the proline residues within the collagenous domain. Indeed in vitro binding studies have shown that the affinity of P4H for the collagen helical domain is higher for unhydroxylated chains than hydroxylated chains (38). To address this point we incubated cells in the presence of the proline analogue azetidine-2-carboxylic acid. As described in Fig. 5, this does not prevent hydroxylation of the triple helical domain but does prevent folding and subsequent secretion.
ER Retention of Procollagen

Cells were pulse-labeled in the presence of ascorbate, cross-linking agent was added, and cell lysates were immunoprecipitated with antibodies to PDI. In the absence of azetidine-2-carboxylic acid a complex between P4H and the synthesized pro-α1(III)Δ1 chains was only seen when α,α'-dipiridyl was included during the labeling period (Fig. 10, lanes 2 and 4). However, when the proline analogue was present a complex formed between P4H and pro-α1(III)Δ1 chains even in the absence of α,α'-dipiridyl. This demonstrates that non-helical but hydroxylated procollagen chains can still associate with P4H indicating that the folding status of the chains rather than hydroxylation of proline residues regulate the binding of P4H to procollagen chains within the cell.

**DISCUSSION**

It has been known for some time that the secretion of procollagen from mammalian cells is regulated by the extent of hydroxylation of this molecule (9, 22). Under normal physiological conditions, the level of expression of procollagen in the extracellular matrix can be affected dramatically by the availability of co-factors for P4H such as ascorbate, oxygen, and iron. This has led to the hypothesis that there is a mechanism by which the cell can recognize unhydroxylated non-helical molecules and selectively retain them within the cell. The molecular mechanism leading to this retention has been postulated to be mediated either by P4H itself (22, 39) or by the collagen-binding protein Hsp47 (15), which has been shown to bind to procollagen within the ER. Support for a role of P4H in the retention process comes from in vitro binding experiments which demonstrate that formation of a complex between P4H and unhydroxylated procollagen chains occurs and that the affinity of the enzyme for its substrate diminishes upon hydroxylation of the triple helix (38). Indeed unhydroxylated procollagen chains that are allowed to fold to form a triple helix at low temperatures are no longer a substrate for the enzyme (35). Hsp47 on the other hand shows no ability to discriminate between hydroxylated and unhydroxylated forms of procollagen (19), but it is clear that the binding can be regulated by subtle changes in pH (40). Thus, Hsp47 may associate with procollagen within the relatively neutral pH of the ER and then dissociate upon transport to more acidic compartments in the secretory pathway.

To determine the molecular mechanism underlying the retention of procollagen within the cell, we established cell lines expressing a “mini-gene” coding for pro-α1(III) chains containing an “in frame” deletion within the collagen triple helix (29). We established conditions that generated an accumulation of non-helical trimeric molecules within the cell, and we looked for associated ER proteins. To achieve this aim we cross-linked any potential associating proteins and carried out immunoprecipitations with a number of well characterized ER resident proteins. From the results we identified P4H as a potential interacting partner, and this was confirmed by carrying out velocity centrifugation to separate a complex between procollagen and P4H. No binding to Hsp47 was detected even though binding to the full-length pro-α1(III) chain has been reported (41). The reason for the lack of binding of the mini-chain to Hsp47 could be due to the binding site for Hsp47 being contained within the deleted sequence within the triple helical domain. However, this lack of binding did allow us to study the regulation of secretion of a collagenous protein in the absence of any interaction with Hsp47. It is clear from our results that the retention is still tightly regulated and is almost certainly due to the association of P4H with the non-triple helical collagenous domain. Thus, P4H fulfills all the characteristics of a protein required to retain non-helical procollagen within the cell as follows: (i) it forms a stable association with non-helical molecules; (ii) this association is regulated by the folding status rather than hydroxylation; (iii) it is localized in the ER due the -KDEL sequence at the C terminus of its β-subunit (PDI) (42); and (iv) it dissociates rapidly from the protein upon folding of the triple helical domain.

The ability of P4H to interact also with hydroxylated but non-helical procollagen chains also explains how the cell can recognize and retain procollagen molecules containing naturally occurring mutations within the triple helical domain. These mutations give rise to genetic disorders such as osteogenesis imperfecta (14) and Ehlers-Danlos syndrome type IV (43). In these cells, association of wild type and mutant chains occurs normally, but folding of the triple helix is prevented or slowed at the point of the mutation due to replacement of glycine in the Gly-X-Y repeats with a more bulky amino acid side chain. These molecules are retained within the cell resulting in a dramatic reduction in the secretion of procollagen into the extracellular matrix. Thus even though the mutant chains are hydroxylated by P4H they are nevertheless retained due to lack of folding. It is clear that P4H can recognize these chains as non-helical, rather than unhydroxylated, and associates with them to prevent secretion. The association of mutant chains with wild type chains leads to retention of wild type chains within the cell amplifying the severity of the mutation resulting in severe, often lethal phenotypes.

This mechanism is distinct from two other mechanisms for retention of procollagen chains within the cell. Procollagen chains with mutations within the C-propeptide are retained within the cell due to their interaction with BiP (21). Here the mutation prevents folding of the C-propeptide with the resulting unfolded polypeptide interacting with BiP and subsequently being degraded (44). As the mutant chains do not interact with the wild type chains, these mutations generally give rise to less severe phenotypes. Also, during the folding and assembly of type I procollagen, which is composed of one pro-α2(I) and two α1(I) chains, the individual monomeric pro-α2(I) chains bind to PDI. This binding retains them within the ER prior to assembly with pro-α1(I) chains (23). Hence by three separate mechanisms procollagen chains can be retained within the cell. This mechanism of retention depends on whether they have a general folding defect and bind to BiP, are folded into monomers but not formed trimers and bind to PDI, or have formed non-helical trimers and bind to P4H.
Acknowledgments—We thank Dr. Stephen High and Dr. David John for comments on the manuscript.

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