

University of Groningen

**Accumulation of properly folded human type III procollagen molecules in specific intracellular membranous compartments in the yeast *Pichia pastoris***

Keizer-Gunnink, [No Value]; Vuorela, A; Myllyharju, J; Pihlajaniemi, T; Kivirikko, KI; Veenhuis, M; Keizer-Gunnink, Ineke; Kivirikko, Kari I.

*Published in:*  
Matrix Biology

*DOI:*  
[10.1016/S0945-053X\(99\)00059-1](https://doi.org/10.1016/S0945-053X(99)00059-1)

**IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.**

*Document Version*  
Publisher's PDF, also known as Version of record

*Publication date:*  
2000

[Link to publication in University of Groningen/UMCG research database](#)

*Citation for published version (APA):*

Keizer-Gunnink, N. V., Vuorela, A., Myllyharju, J., Pihlajaniemi, T., Kivirikko, KI., Veenhuis, M., Keizer-Gunnink, I., & Kivirikko, K. I. (2000). Accumulation of properly folded human type III procollagen molecules in specific intracellular membranous compartments in the yeast *Pichia pastoris*. *Matrix Biology*, 19(1), 29-36. [https://doi.org/10.1016/S0945-053X\(99\)00059-1](https://doi.org/10.1016/S0945-053X(99)00059-1)

**Copyright**

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: <https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment>.

**Take-down policy**

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

## Accumulation of properly folded human type III procollagen molecules in specific intracellular membranous compartments in the yeast *Pichia pastoris*

Ineke Keizer-Gunnink<sup>a</sup>, Annamari Vuorela<sup>b</sup>, Johanna Myllyharju<sup>b</sup>,  
Taina Pihlajaniemi<sup>b</sup>, Kari I. Kivirikko<sup>b,\*</sup>, Marten Veenhuis<sup>a</sup>

<sup>a</sup>Department of Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute (GBB), Biological Center, University of Groningen, Kerklaan 30, 9751 NN, Haren, Netherlands

<sup>b</sup>Collagen Research Unit, Biocenter and Department of Medical Biochemistry, University of Oulu, P.O. Box 5000, FIN-90401, Oulu, Finland

Received 13 August 1999; received in revised form 25 November 1999; accepted 30 November 1999

### Abstract

It was recently reported that co-expression of the pro $\alpha$ 1(III) chain of human type III procollagen with the subunits of human prolyl 4-hydroxylase in *Pichia pastoris* produces fully hydroxylated and properly folded recombinant type III procollagen molecules (Vuorela, A., Myllyharju, J., Nissi, R., Pihlajaniemi, T., Kivirikko, K.I., 1997. Assembly of human prolyl 4-hydroxylase and type III collagen in the yeast *Pichia pastoris*: formation of a stable enzyme tetramer requires coexpression with collagen and assembly of a stable collagen requires coexpression with prolyl 4-hydroxylase. *EMBO J.* 16, 6702–6712). These properly folded molecules accumulated inside the yeast cell, however, only ~10% were found in the culture medium. We report here that replacement of the authentic signal sequence of the human pro $\alpha$ 1(III) with the *Saccharomyces cerevisiae*  $\alpha$  mating factor prepro sequence led only to a minor increase in the amount secreted. Immunoelectron microscopy studies indicated that the procollagen molecules accumulate in specific membranous vesicular compartments that are closely associated with the nuclear membrane. Prolyl 4-hydroxylase, an endoplasmic reticulum (ER) luminal enzyme, was found to be located in the same compartments. Non-helical pro $\alpha$ 1(III) chains produced by expression without recombinant prolyl 4-hydroxylase likewise accumulated within these compartments. The data indicate that properly folded recombinant procollagen molecules accumulate within the ER and do not proceed further in the secretory pathway. This may be related to the large size of the procollagen molecule. © 2000 Elsevier Science B.V./International Society of Matrix Biology. All rights reserved.

**Keywords:** *Pichia pastoris*; Collagen; Procollagen; Prolyl 4-hydroxylase

**Abbreviations:** 4-PH, prolyl 4-hydroxylase; PDI, protein disulfide isomerase; pro $\alpha$ 1(III) chain, pro $\alpha$ 1 chain of type III procollagen;  $\alpha$  chain,  $\alpha$  chain produced from procollagen by cleavage of the propeptides;  $\alpha$ -MF,  $\alpha$  mating factor prepro sequence

\* Corresponding author. Tel.: +358-8-537-5801; fax: +358-8-537-5810.  
E-mail address: kari.kivirikko@oulu.fi (K.I. Kivirikko)

## 1. Introduction

The collagen superfamily consists of at least 19 proteins formally defined as collagens and more than 10 additional proteins with collagen-like domains. All collagen molecules consist of three identical or non-identical polypeptide chains, called  $\alpha$  chains, that are wrapped around each other into a characteristic triple helix. Collagen types I, II, III, V and XI form extracellular fibrils and are hence known as fibril-forming collagens, while the others form supramolecular aggregates of other kinds. The molecules of the fibril-forming collagens are synthesized in the form of procollagen molecules, which differ from the final collagen molecules in that their pro $\alpha$  chains have propeptide extensions at both their N-terminal and C-terminal ends (for reviews on collagens, see Kielty et al., 1993; Prockop and Kivirikko, 1995; Bateman et al., 1996).

Most recombinant systems now available for the production of proteins cannot be used as such for the expression of recombinant collagens, as bacteria and yeasts have no prolyl 4-hydroxylase activity and insect cells have insufficient amounts of it (Lamberg et al., 1996; Myllyharju et al., 1997). This enzyme plays a crucial role in the synthesis of all collagens, as 4-hydroxyproline residues are essential for the formation of molecules with stable triple helices. The vertebrate prolyl 4-hydroxylases are  $\alpha_2\beta_2$  tetramers in which the  $\beta$  subunit is identical to the enzyme and chaperone protein disulfide isomerase (PDI) (for reviews, see Kivirikko and Myllyharju, 1998; Kivirikko and Pihlajaniemi, 1998).

It was recently reported that co-expression of the  $\alpha$  subunit of human prolyl 4-hydroxylase, the human PDI polypeptide and the pro $\alpha$ 1 chain of human type III procollagen in *Pichia pastoris* produced an active recombinant prolyl 4-hydroxylase tetramer and pro $\alpha$ 1(III) chains in which the appropriate proline residues were hydroxylated to 4-hydroxyproline (Vuorela et al., 1997). The pro $\alpha$ 1(III) chains were of full length and properly disulfide-bonded and formed [pro $\alpha$ 1(III)]<sub>3</sub> procollagen molecules with stable triple helices (Vuorela et al., 1997), but they accumulated predominantly inside the yeast cell, only approximately 10% being found in the culture medium. This finding is surprising, as triple-helical procollagen molecules are rapidly secreted into the extracellular space from various animal cells (see Prockop et al., 1979; Kielty et al., 1993). We therefore studied here the intracellular site of accumulation of the recombinant type III procollagen molecules in *P. pastoris*, and whether replacement of the signal peptide of the pro $\alpha$ 1(III) chain with the *Saccharomyces cerevisiae*  $\alpha$  mating factor ( $\alpha$ MF) prepro sequence will increase the amount of procollagen secreted.

## 2. Results

### 2.1. Analysis of the cellular morphology of the recombinant *P. pastoris* strains

The cellular morphology of the methanol-induced wild-type strain and the recombinant strains  $\alpha$ /PDI $\alpha$ -MF, Pro $\alpha$ 1(III) and  $\alpha$ /PDI $\alpha$ -MF/pro $\alpha$ 1(III) which express human prolyl 4-hydroxylase, human type III procollagen or both proteins, respectively, were studied by electron microscopy using KMnO<sub>4</sub>-fixed cells.

Analysis of the three recombinant strains revealed the usual subcellular organelles such as nuclei, mitochondria, ER, vacuoles and peroxisomes, but additional membranous vesicular structures of irregular shape were also seen in cells of all three recombinant strains (Fig. 1a). In the two strains, Pro $\alpha$ 1(III) and  $\alpha$ /PDI $\alpha$ -MF/pro $\alpha$ 1(III), expressing type III procollagen without, or with, recombinant prolyl 4-hydroxylase, respectively, a few vesicles were considerably larger and less electron-dense (Fig. 1a). Some of the membranes of this cytoplasmic tubular network structure were closely associated with the nuclear membrane (Fig. 1b). These structures were invariably absent from the wild-type cells (Fig. 1c), indicating that specific structures developed in response to the synthesis of the heterologous proteins in the recombinant cells.

### 2.2. Localization of the recombinant human prolyl 4-hydroxylase and type III procollagen using immunoelectron microscopy

In order to study where the triple-helical recombinant human type III procollagen accumulates in the *P. pastoris* cell, the locations of the recombinant prolyl 4-hydroxylase and type III procollagen were studied by immunoelectron microscopy on ultrathin sections of Unicryl-embedded cells. The sections were incubated with polyclonal antibodies to the human PDI polypeptide, the human prolyl 4-hydroxylase  $\alpha$  subunit or the N propeptide of human type III procollagen followed by incubation with a gold-conjugated secondary antibody.

The recombinant human prolyl 4-hydroxylase subunits expressed in *P. pastoris* were expected to be located within the lumen of the ER, as judged by their assembly into an enzyme tetramer that was active both in vitro and inside the cells, the presence of N-linked sugars in the polypeptides, and the cleavage of the signal peptide from the  $\alpha$  subunit and the  $\alpha$ MF-PDI polypeptide (Vuorela et al., 1997). Immunolabeling of cells from the strain co-expressing recombinant human prolyl 4-hydroxylase and type III procollagen with antibodies to the prolyl 4-hydroxyl-

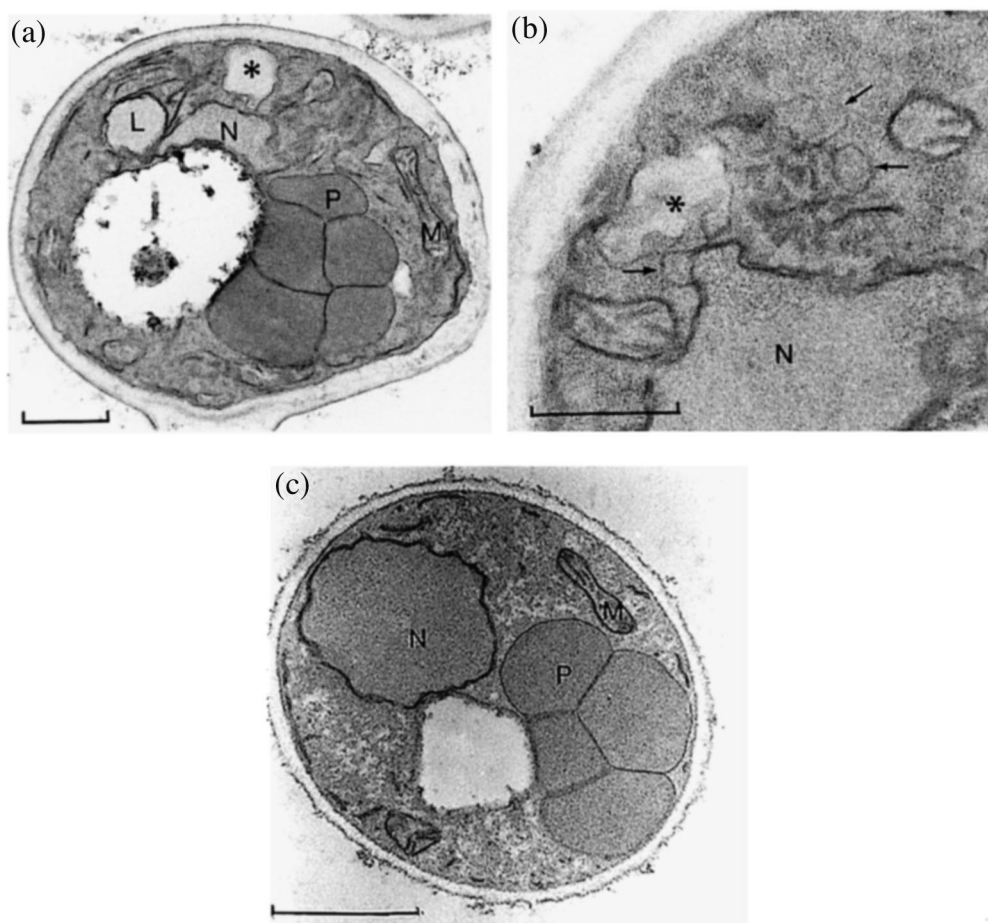


Fig. 1. Analysis of the cellular morphology of the recombinant  $\alpha$ /PDI- $\alpha$ MF/pro $\alpha$ 1(III) (a,b) and wild-type (c) *P. pastoris* strain. The cells were cultured and induced with methanol as described in Section 4, fixed with  $\text{KMnO}_4$ , embedded in Epon 812 and analyzed using a Philips EM400 electron microscope. N, nucleus; P, peroxisome; M, mitochondrion; and L, lipid droplet. The bar represents  $0.5 \mu\text{m}$ . The asterisk (\*) in (a) and (b) indicates a large, less electron-dense vesicle. The arrows in (b) indicate the membranous tubular network structures.

ase  $\alpha$  subunit and PDI polypeptide showed that the enzyme subunits were specifically located in the lumen of the membranes of the proliferating cytoplasmic tubular network (Fig. 2a,b). Labeling was also seen in those regions of the tubular network that were in close contact with the nuclear membrane, and on the nuclear membrane at these contact sites. The antibody to the PDI polypeptide showed an additional extracellular labeling (data not shown), which is in accordance with the previous finding that despite the presence of the -KDEL or -HDEL ER retention signal, some of the  $\alpha$ MF-PDI fusion peptide was secreted into the culture medium (Vuorela et al., 1997). Similar labeling with the antibodies to the prolyl 4-hydroxylase subunits was also seen in the strain expressing recombinant human prolyl 4-hydroxylase without type III procollagen (data not shown).

Immunolabeling of cells from the strain co-expressing recombinant human prolyl 4-hydroxylase and type III procollagen with an antibody to the N

propeptide of human type III procollagen showed that the procollagen was also located in the lumen of the membranes of the tubular networks (Fig. 2c). In order to confirm the co-localization of prolyl 4-hydroxylase and type III procollagen in the same cellular compartments, one side of each cell sections was incubated with the antibody to the PDI polypeptide and a 15-nm gold particle-conjugated secondary antibody, while the other side was incubated with the antibody to the N propeptide of human type III procollagen and a 5-nm gold particle-conjugated secondary antibody. These double-labeling experiments showed that the PDI polypeptide and type III procollagen are located in the same cellular compartments (Fig. 2d). Recombinant human type III procollagen was localized to the lumen of the tubular network structures also in the strain expressing only the type III procollagen molecules (data not shown). No labeling was observed in the extracellular matrix with this antibody.

Control experiments showed that the immunolabel-

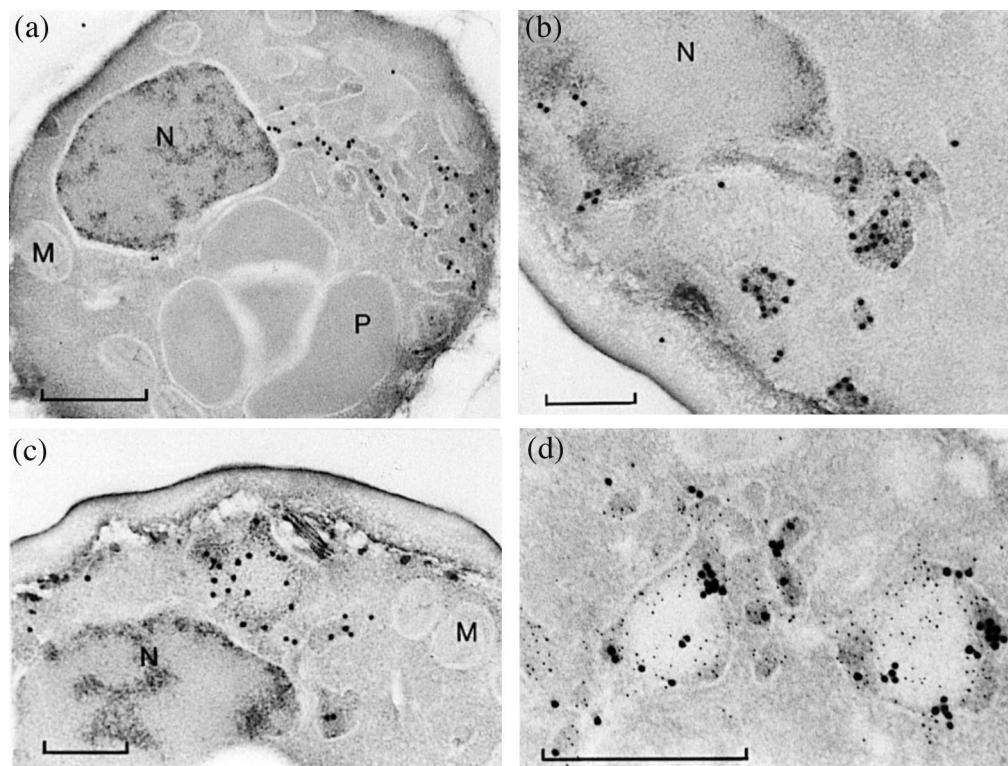


Fig. 2. Cellular localization of the recombinant human prolyl 4-hydroxylase  $\alpha$  subunit (a), PDI polypeptide (b), and type III procollagen (c) by post-embedding immunogold-labeling of ultrathin sections. The recombinant human PDI and type III procollagen were co-labeled using 15 and 5 nm gold particle-conjugated secondary antibodies, respectively (d). The bar represents 0.5  $\mu\text{m}$ .

ing patterns that were seen were specific, as no labeling with any of the antibodies used was seen in the cells from the GS200 strain, no labeling with the antibody to the N propeptide of type III procollagen was seen in the cells from the strain expressing only recombinant human prolyl 4-hydroxylase, and no labeling with the antibodies to the prolyl 4-hydroxylase  $\alpha$  subunit and the PDI polypeptide was seen in cells from the strain expressing only recombinant human type III procollagen.

### 2.3. Secretion of recombinant human type III procollagen with the *S. cerevisiae* $\alpha$ mating factor prepro sequence in *P. pastoris*

In order to study whether the use of the *S. cerevisiae*  $\alpha$ MF prepro sequence would enhance the secretion of recombinant human type III procollagen, a *P. pastoris* strain  $\alpha$ /PDI $\alpha$ -MF/pro $\alpha$ 1(III)- $\alpha$ MF co-expressing human prolyl 4-hydroxylase and type III procollagen was generated. As reported previously, the human PDI polypeptide had the  $\alpha$ MF prepro sequence (Vuorela et al., 1997), and in this new strain the signal sequence of the human pro $\alpha$ 1(III) chain had likewise been replaced with the  $\alpha$ MF prepro sequence. The  $\alpha$ /PDI $\alpha$ -MF/pro $\alpha$ 1(III)- $\alpha$ MF strain was cultured in BMGY medium and recombinant

expression was induced in BMM medium by adding methanol every 12 h to a final concentration of 0.5%. The cells were harvested 72 h after induction, and broken in a 5% glycerol, 1 mM EDTA and 50 mM sodium phosphate buffer (pH 7.4). Aliquots of the soluble fraction of the cell extracts from this strain and the  $\alpha$ /PDI $\alpha$ -MF/pro $\alpha$ 1(III) strain co-expressing prolyl 4-hydroxylase and human type III procollagen with its authentic signal sequence were analyzed by SDS-PAGE under reducing conditions followed by Western blotting with an antibody to the N propeptide of human type III procollagen. Bands corresponding to the full-length pro $\alpha$ 1(III) chains were seen in the immunoblot in both strains, the chain expressed with the  $\alpha$ MF prepro sequence having a slightly slower mobility, apparently due to the additional pro sequence (Fig. 3a). Other aliquots of the cell extracts were digested with pepsin for 2 h at 22°C and analyzed by SDS-PAGE followed by silver staining (Fig. 3b). Pepsin-resistant  $\alpha$ 1(III) chains with an identical mobility were seen in the digested samples from both strains, indicating that the chains formed triple-helical molecules. Concentrated samples of the culture medium from the strains  $\alpha$ /PDI $\alpha$ -MF/pro $\alpha$ 1(III) and  $\alpha$ /PDI $\alpha$ -MF/pro $\alpha$ 1(III)- $\alpha$ MF were analyzed by SDS-PAGE under reducing conditions followed by Western blotting with an antibody to the N

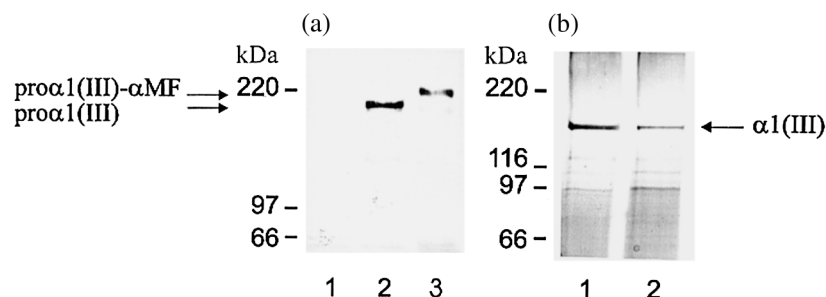


Fig. 3. Expression of human type III procollagen in *P. Pastoris*. (a) The GS200 control strain and the recombinant *P. pastoris* strains were grown for 72 h in BMM containing 0.5% methanol. Aliquots containing 20  $\mu\text{g}$  of total protein from the soluble fractions of the cell extracts obtained from the GS200 (lane 1),  $\alpha$ /PDI- $\alpha$ MF/pro $\alpha$ 1(III) (lane 2) and  $\alpha$ /PDI- $\alpha$ MF/pro $\alpha$ 1(III)- $\alpha$ MF (lane 3) strains were analyzed by 5% SDS-PAGE under reducing conditions followed by an immunoblot analysis using a polyclonal antibody to the N propeptide of human type III procollagen. The position of the pro $\alpha$ 1(III) chain is indicated by an arrow. (b) Aliquots containing approximately 150  $\mu\text{g}$  of total protein from the soluble fractions of the cell extracts obtained from the  $\alpha$ /PDI- $\alpha$ MF/pro $\alpha$ 1(III) (lane 1) and  $\alpha$ /PDI- $\alpha$ MF/pro $\alpha$ 1(III)- $\alpha$ MF (lane 2) strains were digested with pepsin and analyzed by 8% SDS-PAGE followed by silver staining. The position of the  $\alpha$ 1(III) chain is indicated by an arrow.

propeptide of type III procollagen. Only very faint immunostained bands with identical mobilities were seen in the concentrated medium samples from both strains (data not shown). The mobility of these bands was identical to that of the intracellular pro $\alpha$ 1(III) chains expressed with the authentic signal peptide in the strain  $\alpha$ /PDI- $\alpha$ MF/pro $\alpha$ 1(III) (data not shown).

Secretion of type III procollagen was also studied by analyzing aliquots of the soluble fraction of the cell extract and culture medium with a radioimmunoassay for the trimeric N propeptide of human type III procollagen. Most of the recombinant type III procollagen with the  $\alpha$ MF prepro sequence was again found in the soluble fraction of the cell extract, but the amount of type III procollagen with the  $\alpha$ MF prepro sequence in the soluble extracts was approximately 50% of that with the authentic signal peptide (Table 1). The amount of type III procollagen secreted was approximately 10% of the total amount expressed in the  $\alpha$ /PDI- $\alpha$ MF/pro $\alpha$ 1(III) strain and approximately 20% in the  $\alpha$ /PDI- $\alpha$ MF/pro $\alpha$ 1(III)- $\alpha$ MF strain (Table 1).

Thus, secretion of type III procollagen with the  $\alpha$ MF prepro sequence was only slightly improved

relative with its authentic signal sequence (Vuorela et al., 1997). In addition, the total expression level of type III procollagen with the  $\alpha$ MF prepro sequence was lower than that with the authentic signal peptide.

### 3. Discussion

Type III procollagen molecules are assembled in various cells synthesizing collagen within the lumen of the endoplasmic reticulum, and their triple-helical domain is a rigid, rod-like structure with a length of approximately 300 nm and a diameter of approximately 1.5 nm. It is now well established that if triple helix formation is prevented, the non-helical pro $\alpha$  chains accumulate within the lumen of the rough endoplasmic reticulum (see Prockop et al., 1979; Kielty et al., 1993). Triple-helical procollagen molecules are transported from the ER across the Golgi stacks without ever leaving the lumen of the Golgi cisternae (Bonfanti et al., 1998). During transport from the ER to the Golgi, procollagen molecules are found within tubular-saccular structures that have a length greater than 300 nm, and they begin to aggregate laterally

Table 1

Expression and secretion of recombinant human type III procollagen in the *P. pastoris* strain  $\alpha$ /PDI- $\alpha$ MF/pro $\alpha$ 1(III) and four transformants of the strain  $\alpha$ /PDI- $\alpha$ MF/pro $\alpha$ 1(III)- $\alpha$ MF<sup>a</sup>

Strain	Intracellular expression of type III procollagen (ng/100 $\mu\text{g}$ )	Secretion of type III procollagen as a percentage of the total amount of procollagen
$\alpha$ /PDI- $\alpha$ MF/pro $\alpha$ 1(III)	195 $\pm$ 10	7 $\pm$ 1
$\alpha$ /PDI- $\alpha$ MF/pro $\alpha$ 1(III)- $\alpha$ MF 1	80 $\pm$ 5	18 $\pm$ 2
$\alpha$ /PDI- $\alpha$ MF/pro $\alpha$ 1(III)- $\alpha$ MF 2	92 $\pm$ 9	19 $\pm$ 1
$\alpha$ /PDI- $\alpha$ MF/pro $\alpha$ 1(III)- $\alpha$ MF 3	110 $\pm$ 10	17 $\pm$ 4
$\alpha$ /PDI- $\alpha$ MF/pro $\alpha$ 1(III)- $\alpha$ MF 4	103 $\pm$ 7	17 $\pm$ 2

<sup>a</sup> Cells were cultured for 72 h in 25 ml of BMM containing 0.5% methanol. Soluble fractions of the cell extracts and samples of the culture medium were analyzed with a radioimmunoassay for the trimeric N propeptide of human type III procollagen.

within the *cis*-Golgi compartment (Bonfanti et al., 1998). Thus, supramolecular aggregates such as procollagen do not appear to use the conventional vesicle-mediated transport mechanism to traverse the Golgi stacks. The *cis* cisterna evidently progresses through the stack by going through successive maturation stages, during which the procollagen aggregates can be regarded as passive objects lying inside the lumen while the cisternal membrane flows around them in a retrograde direction (Mironov et al., 1997; Bonfanti et al., 1998). During this process the luminal environment changes gradually, leading to increased condensation of the aggregates and resulting in the formation of granules ready for secretion (Bonfanti et al., 1998).

The present data indicate that in the case of *P. pastoris* cells synthesizing recombinant human type III procollagen the secretory process is terminated at an early stage, and properly folded procollagen molecules (Vuorela et al., 1997) accumulate in specific intracellular membranous compartments. The  $\alpha$  subunit of prolyl 4-hydroxylase and the  $\alpha$ MF-PDI polypeptide were found to be located within these same compartments. Prolyl 4-hydroxylase and the free PDI polypeptides have been shown by a variety of techniques to reside within the lumen of the ER, and an active system appears to exist for the transport of 2-oxoglutarate and ascorbate, two co-substrates required by prolyl 4-hydroxylase, into the lumen of the ER through the membranes (see Kivirikko and Pihlajaniemi, 1998). The recombinant human prolyl 4-hydroxylase tetramer is apparently located within the lumen of the ER in *P. pastoris* as well, as the enzyme is active and hydroxylates the pro $\alpha$ 1(III) chains inside the yeast cell (Vuorela et al., 1997). The biochemical data indicating that the signal peptide is cleaved from the  $\alpha$  subunit and the  $\alpha$ MF-PDI polypeptide and that the  $\alpha$  subunit and the  $\alpha$ MF propeptide contain N-linked carbohydrate units agree with this location (Vuorela et al., 1997).

The authentic signal sequence of the human PDI polypeptide has been found to be highly ineffective in transporting the recombinant polypeptide in *P. pastoris* cells into the lumen of the ER, and a marked increase in this transport was obtained when the human signal sequence was replaced by the *S. cerevisiae*  $\alpha$ MF prepro sequence (Vuorela et al., 1997). Interestingly, some of the  $\alpha$ MF-PDI polypeptide was also secreted from the *P. pastoris* cell despite the presence of the -KDEL or -HDEL ER retention signal, as found previously (Vuorela et al., 1997) and confirmed here. We, therefore, studied here whether replacement of the human signal sequence of the pro $\alpha$ 1(III) chain by the  $\alpha$ MF prepro sequence would enhance the secretion of type III procollagen. Only a minor degree of enhancement was found, however.

Pro $\alpha$ 1(III) chains expressed without the prolyl 4-hydroxylase subunits were found to accumulate within the same tubular network structures as the type III procollagen molecules produced by co-expression with the enzyme. Molecules consisting of non-hydroxylated pro $\alpha$  chains have a  $T_m$  of only approximately 24°C (see Prockop et al., 1979; Kivirikko and Pihlajaniemi, 1998), and thus non-hydroxylated chains cannot form triple-helical molecules inside the yeast cell at 30°C. This argument is supported by the finding that the two cysteine residues present at the very end of the collagen domain of the pro $\alpha$ 1(III) chain did not form interchain disulfide bonds when the pro $\alpha$ 1(III) chains were expressed in *P. pastoris* without recombinant prolyl 4-hydroxylase but did form the bonds when expressed with the enzyme (Vuorela et al., 1997). These interchain disulfide bonds are known to be formed only after triple helix formation (Bulleid et al., 1996). As it is very well established that non-helical pro $\alpha$  chains accumulate within the lumen of the ER, our localization of the non-hydroxylated pro $\alpha$ 1(III) chains and the hydroxylated procollagen molecules within the same membranous compartment supports the conclusion that the properly folded molecules accumulate within the ER.

Our data thus indicate that properly folded procollagen molecules accumulate within the ER and do not proceed further in the secretory pathway. The ER-*cis* Golgi transport step in the secretion of procollagen is currently poorly understood. The procollagen molecules that are formed within the ER are too long to enter the COPII-coated vesicles involved in export from this organelle (see Bonfanti et al., 1998), but one possibility is that they might leave the ER inside tubules protruding directly from the ER surface (Bonfanti et al., 1998). The present data indicate that the ER-*cis* Golgi step does not take place in the synthesis of a procollagen in *P. pastoris* and raise the possibility that this defect may be related to the large size of the procollagen molecule.

## 4. Materials and methods

### 4.1. Plasmid construction

The sequence coding for the signal peptide of the pro $\alpha$ 1 chain of human type III procollagen (Tromp et al., 1989) was replaced with that coding for the *S. cerevisiae*  $\alpha$ MF prepro sequence. The 5' end of the cDNA, extending from the codon for the first amino acid after the signal peptide cleavage site to the first internal *Bam*HI site with *Cla*I and *Not*I sites flanking the 5' end, was synthesized by PCR, digested with *Cla*I and *Bam*HI and used first to replace the original 5' end. In order to remove 713 bp of 3'-untranslated

sequence of the cDNA, the 3' end extending from an internal *EcoRI* site to the translation stop codon, with *NotI* and *XbaI* sites following the stop codon, was synthesized by PCR, digested with *EcoRI* and *XbaI* and used to replace the original 3' end. This construct was then digested with *NotI*, and the collagen fragment was cloned into the *NotI* site of pPIC9 (Invitrogen) in frame with the  $\alpha$ MF prepro sequence. From this construct a *BglII*–*NdeI* fragment containing the *AOX1* promoter and the  $\alpha$ MF prepro sequence fused to the 5' end of the pro $\alpha$ 1(III) chain sequence up to an internal *NdeI* site was used to replace the corresponding region in the previously described pPICZ Bpro $\alpha$ 1(III) expression plasmid to generate pPICZ Bpro $\alpha$ 1(III) $\alpha$ -MF.

#### 4.2. *Pichia pastoris* strains and culture conditions

Generation of the strains  $\alpha$ /PDI $\alpha$ -MF expressing human prolyl 4-hydroxylase, Pro $\alpha$ 1(III) expressing the pro $\alpha$ 1 chain of human type III procollagen, and  $\alpha$ /PDI $\alpha$ -MF/pro $\alpha$ 1(III) co-expressing prolyl 4-hydroxylase and the pro $\alpha$ 1(III) chain, has been described recently (Vuorela et al., 1997).

A recombinant strain co-expressing the pro $\alpha$ 1 chain of human type III procollagen with the yeast  $\alpha$ MF prepro sequence and human prolyl 4-hydroxylase [ $\alpha$ /PDI $\alpha$ -MF/pro $\alpha$ 1(III)- $\alpha$ MF] was generated by introducing *PmeI*-linearized pPICZ Bpro $\alpha$ 1(III) $\alpha$ -MF into the  $\alpha$ /PDI $\alpha$ -MF strain by electroporation as described in the *Pichia* Expression Kit Manual, version 3.0 (Invitrogen). The methanol utilization phenotype of the  $\alpha$ /PDI $\alpha$ -MF/pro $\alpha$ 1(III)- $\alpha$ MF strain was confirmed to be methanol utilization plus.

The cells were cultured according to the methods described in version 3.0 of the *Pichia* Expression Kit Manual (Invitrogen) with slight modifications. They were cultured in 25 ml of a buffered glycerol complex medium (BMGY, pH 6.0) containing 1 g/l yeast extract and 2 g/l peptone. Expression of heterologous genes was induced in a buffered minimal methanol medium (BMM, pH 6.0) by adding methanol every 12 or 24 h to a final concentration of 0.5%. Amino acids were added up to 50  $\mu$ g/l as required.

#### 4.3. Analysis and purification of recombinant type III procollagen

The cells were harvested after a 72-h methanol induction at 30°C, washed once and suspended in a 5% glycerol, 1 mM Pefabloc SC, 1 mM EDTA and 50 mM sodium phosphate buffer (pH 7.4). They were then harvested and broken by vortexing with glass beads. The cell extract was centrifuged at 10000  $\times$  g for 20 min and the soluble fraction was collected. The protein concentration in the soluble fraction was de-

termined using the Bio-Rad Protein Assay (Bio-Rad). Aliquots of the soluble fractions were analyzed by SDS-PAGE under reducing conditions followed by Western blotting with a polyclonal antibody to the N propeptide of human type III procollagen (Orion Diagnostica). Samples of the culture medium were precipitated with 66% ethanol, redissolved in 1% SDS and analyzed by SDS-PAGE under reducing conditions followed by Western blotting with the antibody to the N propeptide of human type III procollagen. Further aliquots of the soluble fractions and the culture medium were analyzed with a radioimmunoassay for the trimeric N propeptide of human type III procollagen (Orion Diagnostica). Other aliquots were digested with pepsin (150  $\mu$ g/ml) for 2 h at 22°C and analyzed by SDS-PAGE under reducing conditions followed by silver staining.

#### 4.4. Electron microscopy

The wild-type, GS200,  $\alpha$ /PDI $\alpha$ -MF, Pro $\alpha$ 1(III) and  $\alpha$ /PDI $\alpha$ -MF/pro $\alpha$ 1(III) *P. pastoris* strains were induced as above and harvested 24 h after methanol induction. For morphological studies cells were fixed with  $\text{KMnO}_4$ , embedded in Epon812 (Keizer et al., 1992) and viewed on a Philips EM400 electron microscope. For immunoelectron microscopy the cells were fixed with 3% formaldehyde in sodium cacodylate buffer (pH 7.2) and embedded in Unicryl (JanK). Ultrathin sections were blocked with 0.5% bovine serum albumin in PBS/glycine for 10 min at 4°C and incubated with 1:1000 dilutions of polyclonal antibody K38 to the human PDI polypeptide (Veijola et al., 1996) or K17 to the human prolyl 4-hydroxylase  $\alpha$  subunit (Veijola et al., 1996), or a polyclonal antibody to the N propeptide of human type III procollagen (Orion Diagnostica) for 16 h at 4°C. The sections were then incubated with a 15-nm gold particle-conjugated goat anti-rabbit IgG (Amersham) for 1 h at room temperature and stained with 1% uranyl acetate and 0.2% methylcellulose (Slot and Geuze, 1984). In double-labeling experiments one side of the section was incubated with the K38 antibody and the 15-nm gold particle-conjugated goat anti-rabbit IgG, while the other side was incubated with the polyclonal antibody to the N propeptide of human type III procollagen and a 5-nm gold particle-conjugated goat anti-rabbit IgG (Amersham), followed by staining as above.

#### Acknowledgements

We thank Dr James Cregg of Oregon Graduate Institute of Science and Technology for providing us with the *P. pastoris* GS200 (*arg4*, *his4*) strain and



Minna Siurua and Raija Juntunen for their expert technical assistance. This work was supported by grants from the Health Sciences Council of the Academy of Finland, the European Commission (B104-CT96-0537) and FibroGen Inc. (South San Francisco, CA, USA).

## References

- Bateman, J.F., Lamandè, S.R., Ramshaw, J.A.M., 1996. Collagen superfamily. In: Comper, W.D. (Ed.), *Extracellular Matrix*. Harwood Academic Publisher, Amsterdam, pp. 22–67.
- Bonfanti, L., Mironov Jr, A.A., Martínez-Menárguez, J.A. et al., 1998. Procollagen traverses the Golgi stack without leaving the lumen of cisternae: evidence for cisternal maturation. *Cell* 95, 993–1003.
- Bulleid, N.J., Wilson, R., Lees, J.F., 1996. Type-III procollagen assembly in semi-intact cells: chain association, nucleation and triple-helix folding do not require formation of inter-chain disulphide bonds but triple-helix nucleation does require hydroxylation. *Biochem. J.* 317, 195–202.
- Keizer, I., Roggenkamp, R., Harder, W., Veenhuis, M., 1992. Location of catalase in crystalline peroxisomes of methanol-grown *Hansenula polymorpha*. *FEMS Microbiol. Lett.* 72, 7–11.
- Kielty, C.M., Hopkinson, I., Grant, M.E., 1993. Collagen: the collagen family: structure, assembly, and organization in the extracellular matrix. In: Royce, P.M., Steinmann, B. (Eds.), *Connective Tissue and its Heritable Disorders: Molecular, Genetic and Medical Aspects*. Wiley-Liss, New York, pp. 103–147.
- Kivirikko, K.I., Myllyharju, J., 1998. Prolyl 4-hydroxylases and their protein disulfide isomerase subunit. *Matrix Biol.* 16, 357–368.
- Kivirikko, K.I., Pihlajaniemi, T., 1998. Collagen hydroxylases and the protein disulfide isomerase subunit of prolyl 4-hydroxylases. *Adv. Enzymol. Related Areas Mol. Biol.* 72, 325–398.
- Lamberg, A., Helaakoski, T., Myllyharju, J. et al., 1996. Characterization of human type III collagen expressed in a baculovirus system. Production of a protein with a stable triple helix requires coexpression with the two types of recombinant prolyl 4-hydroxylase subunit. *J. Biol. Chem.* 271, 11988–11995.
- Mironov, A.A., Weidman, P., Luini, A., 1997. Variations on the intracellular transport theme: maturing cisternae and trafficking tubules. *J. Cell Biol.* 138, 481–484.
- Myllyharju, J., Lamberg, A., Notbohm, H., Fietzek, P.P., Pihlajaniemi, T., Kivirikko, K.I., 1997. Expression of wild-type and modified pro $\alpha$  chains of human type I procollagen in insect cells leads to the formation of stable  $[\alpha 1(I)]_2\alpha 2(I)$  collagen heterotrimers and  $[\alpha 1(I)]_3$  homotrimers but not  $[\alpha 2(I)]_3$  homotrimers. *J. Biol. Chem.* 272, 21824–21830.
- Prockop, D.J., Kivirikko, K.I., Tuderman, L., Guzman, N.A., 1979. The biosynthesis of collagen and its disorders. *N. Engl. J. Med.* 310, 13–23.
- Prockop, D.J., Kivirikko, K.I., 1995. Collagens: molecular biology, diseases, and potentials for therapy. *Annu. Rev. Biochem.* 64, 403–434.
- Slot, J.W., Geuze, H.J., 1984. Gold markers for single and double immunolabeling of ultrathin cryosections. In: Polak, J.M., Varnell, I.M. (Eds.), *Immunolabeling for Electron Microscopy*. Elsevier Scientific Publisher, Amsterdam, pp. 129–142.
- Tromp, G., Kuivaniemi, H., Shikata, H., Prockop, D.J., 1989. A single base mutation that substitutes serine for glycine 790 of the  $\alpha 1(III)$  chain of type III procollagen exposes an arginine and causes Ehlers–Danlos syndrome IV. *J. Biol. Chem.* 264, 1349–1352.
- Veijola, J., Pihlajaniemi, T., Kivirikko, K.I., 1996. Co-expression of the  $\alpha$  subunit of human prolyl 4-hydroxylase with BiP polypeptide in insect cells leads to the formation of soluble and insoluble complexes. Soluble  $\alpha$ -subunit-BiP complexes have no prolyl 4-hydroxylase activity. *Biochem. J.* 315, 613–618.
- Vuorela, A., Myllyharju, J., Nissi, R., Pihlajaniemi, T., Kivirikko, K.I., 1997. Assembly of human prolyl 4-hydroxylase and type III collagen in the yeast *Pichia pastoris*: formation of a stable enzyme tetramer requires coexpression with collagen and assembly of a stable collagen requires coexpression with prolyl 4-hydroxylase. *EMBO J.* 16, 6702–6712.