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

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Abstract

It was recently reported that co-expression of the pro\textalpha\textsubscript{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 $\sim 10\%$ were found in the culture medium. We report here that replacement of the authentic signal sequence of the human pro\textalpha\textsubscript{1} chain with the Saccharomyces cerevisiae 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\textalpha\textsubscript{1} 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

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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 α 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 proα 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 hydroxylated to 4-hydroxyproline w. full length and properly disulfide-bonded and formed prorecombinant prolyl 4-hydroxylase tetramer and Pichia pastoris III procollagen in PDI polypeptide and the pro subunit of human prolyl 4-hydroxylase, the human type III procollagen or both proteins, respectively, were studied by electron microscopy using KMnO₄-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, Proα1(III) and α/PDIα-MF/proα1-III which express human prolyl 4-hydroxylase, human type III procollagen or both proteins, respectively, were studied by electron microscopy using immunoelectron microscopy on ultrathin sections of Unicryl-embedded cells. The sections were immunolabeling of cells from the strain co-expressing 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 α 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 active 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 α subunit and the α-MF-PDI polypeptide (Vuorela et al., 1997). Immunofluorescence of cells from the strain co-expressing recombinant human prolyl 4-hydroxylase and type III procollagen with antibodies to the prolyl 4-hydroxyl-
ase α 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 αMF-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 section 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 α 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 μm.

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 α 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 α mating factor prepro sequence in P. pastoris

In order to study whether the use of the S. cerevisiae α-MF prepro sequence would enhance the secretion of recombinant human type III procollagen, a P. pastoris strain α/PDIα-MF/proα1(III)-α-MF co-expressing human prolyl 4-hydroxylase and type III procollagen was generated. As reported previously, the human PDI polypeptide had the α-MF prepro sequence (Vuorela et al., 1997), and in this new strain the signal sequence of the human proα1(III) chain had likewise been replaced with the α-MF prepro sequence. The α/PDIα-MF/proα1(III)-α-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 α/PDIα-MF/proα1(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 proα1(III) chains were seen in the immunoblot in both strains, the chain expressed with the α-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 α1(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 α/PDIα-MF/proα1(III) and α/PDIα-MF/proα1(III)-α-MF were analyzed by SDS-PAGE under reducing conditions followed by Western blotting with an antibody to the N propeptide.
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 proα1(III) chains expressed with the authentic signal peptide in the strain α/PDI-α-MF/proα1(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. The amount of type III procollagen secreted was approximately 10% of the total amount expressed in the soluble extracts was approximately 50% of that with the authentic signal peptide in the strain α/PDI-α-MF/proα1(III) (data not shown).

Table 1

<table>
<thead>
<tr>
<th>Strain</th>
<th>Intracellular expression of type III procollagen (ng/100 μg)</th>
<th>Secretion of type III procollagen as a percentage of the total amount of procollagen</th>
</tr>
</thead>
<tbody>
<tr>
<td>α/PDI-α-MF/proα1(III)</td>
<td>195 ± 10</td>
<td>7 ± 1</td>
</tr>
<tr>
<td>α/PDI-α-MF/proα1(III)-α MF 1</td>
<td>80 ± 5</td>
<td>18 ± 2</td>
</tr>
<tr>
<td>α/PDI-α-MF/proα1(III)-α MF 2</td>
<td>92 ± 9</td>
<td>19 ± 1</td>
</tr>
<tr>
<td>α/PDI-α-MF/proα1(III)-α MF 3</td>
<td>110 ± 10</td>
<td>17 ± 4</td>
</tr>
<tr>
<td>α/PDI-α-MF/proα1(III)-α MF 4</td>
<td>103 ± 7</td>
<td>17 ± 2</td>
</tr>
</tbody>
</table>

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.

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 proα 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 relative with its authentic signal sequence (Vuorela et al., 1997). In addition, the total expression level of type III procollagen with the α MF prepro sequence was lower than that with the authentic signal peptide.
Interestingly, some of the also secreted from the presence of the pro replacement of the human signal sequence of the confirmed here. We, therefore, studied here whether signal, as found previously Vuorela et al., 1997 and minor degree of enhancement was found, however.

ProαI(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 proα 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 proαI(III) chain did not form interchain disulfide bonds when the proαI(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 proα chains accumulate within the lumen of the ER, our localization of the non-hydroxylated proαI(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 proα1 chain of human type III procollagen (Tromp et al., 1989) was replaced with that coding for the S. cerevisiae αMF 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 BamHI site with ClaI and NorI sites flanking the 5′ end, was synthesized by PCR, digested with ClaI and BamHI 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 NorI 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 NorI, and the collagen fragment was cloned into the NorI site of pPIC9 (Invitrogen) in frame with the αMF prepro sequence. From this construct a BglII–NdeI fragment containing the AOX1 promoter and the αMF prepro sequence fused to the 5’ end of the proα1(III) chain sequence up to an internal NdeI site was used to replace the corresponding region in the previously described pPICZ Bproα1(III) expression plasmid to generate pPICZ Bproα1(III)α-MF.

4.2. Pichia pastoris strains and culture conditions

Generation of the strains α/PDIα-MF expressing human prolyl 4-hydroxylase, Proα1(III) expressing the proα1 chain of human type III procollagen, and α/PDIα-MF/prooα1(III) co-expressing prolyl 4-hydroxylase and the proo1(III) chain, has been described recently (Vuorela et al., 1997).

A recombinant strain co-expressing the proα1 chain of human type III procollagen with the yeast αMF prepro sequence and human prolyl 4-hydroxylase [α/PDIα-MF/prooα1(III)-αMF] was generated by introducing PmeI-linearized pPICZ Bproo1(III)α-MF into the α/PDIα-MF strain by electroporation as described in the Pichia Expression Kit Manual, version 3.0 (Invitrogen). The methanol utilization phenotype of the α/PDIα-MF/prooα1(III)-α-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 μg/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 10,000 × g for 20 min and the soluble fraction was collected. The protein concentration in the soluble fraction was determined 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 radioimmununoassay for the trimERIC N propeptide of human type III procollagen (Orion Diagnostica). Other aliquots were digested with pepsin (150 μg/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, α/PDIα-MF, Proα1(III) and α/PDIα-MF/prooα1(III) P. pastoris strains were induced as above and harvested 24 h after methanol induction. For morphological studies cells were fixed with KMnO₄, 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).

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References


