Cloning of a Novel Prolyl 4-Hydroxylase Subunit Expressed in the Fibrous Cap of Human Atherosclerotic Plaque

Caroline Van Den Diepstraten, MSc; Karen Papay, MSc; Zuzana Bolender, MSc; Arthur Brown, PhD; J. Geoffrey Pickering, MD, PhD

Background—The production of collagen is fundamental to atherosclerosis and critically dependent on posttranslational modification by prolyl 4-hydroxylase.

Methods and Results—We report the cloning of a novel prolyl 4-hydroxylase catalytic (α)-subunit from human vascular smooth muscle cells. The peptide displayed conservation of critical residues for interacting with Fe²⁺ and 2-oxoglutarate, essential cosubstrates for prolyl 4-hydroxylase activity. Furthermore, when the recombinant protein was expressed in cells, it associated with the β-subunit of prolyl 4-hydroxylase and could catalyze prolyl 4-hydroxylation of a collagen-like peptide. The tissue distribution was dissimilar from that of the 2 previously cloned α-subunits, suggesting a role beyond redundancy. Importantly, the novel gene was expressed in the fibrous cap of human carotid atherosclerotic lesions.

Conclusions—The discovery of a novel prolyl 4-hydroxylase α-subunit, here termed the α(III)-subunit, suggests a new participant in collagen synthesis that, in view of the expression findings, may be relevant to atherosclerotic disease. (Circulation. 2003;108:508-511.)

Key Words: atherosclerosis • muscle, smooth • collagen • genes

The production of type I collagen by smooth muscle cells (SMCs) is vital to vascular structure and function. Central to collagen elaboration is the winding of 3 pro-α collagen chains within the lumen of the endoplasmic reticulum to form a long triple-helical structure. This folding reaction depends critically on the activity of prolyl 4-hydroxylase. Central to collagen elaboration is the winding of 3 pro-α collagen chains within the lumen of the endoplasmic reticulum to form a long triple-helical structure. This folding reaction depends critically on the activity of prolyl 4-hydroxylase. Central to collagen elaboration is the winding of 3 pro-α collagen chains within the lumen of the endoplasmic reticulum to form a long triple-helical structure. This folding reaction depends critically on the activity of prolyl 4-hydroxylase.
4-hydroxylase α(I)- and α(II)-subunits were similarly generated. Human embryonic 293 cells were transfected with constructs by using calcium-phosphate precipitation. Cellular PDI was then immunoprecipitated from transient transfectants by using a polyclonal antibody (StressGen) and protein A–agarose. Immunoprecipitates were washed extensively with RIPA buffer (1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS in PBS), resolved on a 7.5% polyacrylamide gel, and transferred to a nitrocellulose membrane. Membranes were incubated with monoclonal antibody to c-myc (Pharmingen), and bound primary antibody was detected by antimouse peroxidase-conjugated Fab fragments and chemiluminescence (Promega Corp).

**Enzyme Activity Assay**

To assess enzyme activity, 293 or HT1080 fibrosarcoma cells were cotransfected with cDNA encoding PDI and either prolyl 4-hydroxylase c-myc–tagged α(I)- or α(III)-subunits. Protein was immunoprecipitated in modified RIPA buffer, containing 1% NP-40, 0.25% deoxycholate, and no SDS, using anti–c-myc antibody and capture ligand, which was then bound reversibly to a column resin. The washed immune complex was then released in soluble form in elution buffer (Capture and Release, Upstate). Prolyl 4-hydroxylase of the collagen-like peptide (Pro-Pro-Gly), was then assayed, based on the hydroxylylation-coupled decarboxylation of 2-oxo[1-14C]glutamate (Perkin Elmer).8

**In Situ Hybridization**

Human carotid endarterectomy samples were fixed in 4% paraformaldehyde overnight and frozen in OCT. Nonradioactive RNA in situ hybridization was performed on 6-μm sections by using a digoxigenin-labeled antisense riboprobe transcribed from a 488-bp fragment of Clone 36. Incubation of artery tissue with a sense probe transcribed from the same template, as well as incubation of brain tissue with the antisense probe, confirmed the absence of nonspecific signals. Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI, 0.5 μg/mL) (Sigma).

**Results**

While screening for differentially expressed transcripts in maturing HITB5 SMCs, we identified a 728-bp reverse transcription (RT)-PCR product that was noteworthy because the predicted amino acid sequence of the first 133 nucleotides displayed homology with the human prolyl 4-hydroxylase α(I)-subunit.6 The strategy used for cloning the gene, as well as a representation of some of the cDNA clones isolated, is illustrated in Figure 1A. RT-PCR using HITB5 SMC RNA was performed as a final step, generating a 1885-bp cDNA clone that consisted of 33 bp of 5’UTR, 1632 bp of coding sequence, and 220 bp of 3’UTR.

Sequence alignment with the 2 currently known human prolyl 4-hydroxylase α-subunits, α(I) and α(II), showed homology throughout the length of the novel gene (Figure 1B) which, based on subsequent enzyme analysis, we termed the prolyl 4-hydroxylase α(III)-subunit (sequence deposited in GenBank, accession numberAY327887). The Table compares details of the 3 genes showing similarities in protein size; the presence of an N-terminal signal peptide, deduced using the computational parameters of von Heijne; and the absence of an ER retention signal, which for collagen prolyl 4-hydroxylases is afforded by the β-subunit.1 The region of least homology with the α(I)- and α(II)-subunits occurs at a variable interdomain region,8 whereas close homology exists in functionally critical regions. There are 5 conserved cysteine residues, 4 of which have been shown in the α(I)-subunit to form intrachain disulfide bonds essential to maintaining the structure required for tetramer assembly.1 As well, homology is greatest at the catalytically important C-terminus. Within this terminus, there is conservation of 5 key residues essential for cosubstrate binding; 3 residues critical for binding Fe2+ (which in the α(III)-subunit correspond to His-421, Asp-423, and His-491), a lysine required to bind the C-5 carboxyl group of 2-oxoglutarate (Lys-501), and a histidine involved in binding the C-1 carboxyl group of 2-oxoglutarate to Fe2+ and the decarboxylation of this cosubstrate (His-509).1 Collectively, these data strongly suggest that the cloned gene is a prolyl 4-hydroxylase catalytic subunit. The sequence data also place it in the family of prolyl 4-hydroxylases that act on collagen rather than on hypoxia-inducible factor.9 This was further supported by expression data. Transcripts for the prolyl 4-hydroxylase α(III) subunit were detected in type 1 collagen-producing SMCs but not in 2 different cell lines that did not express type I collagen (Figure 2A).

To constitute mammalian collagen prolyl 4-hydroxylase, an α-subunit must associate with the β-subunit, PDI. To determined if the α(III)-subunit associated with PDI, a c-myc–tagged α(III)-construct was expressed in 293 cells from which PDI was immunoprecipitated. As shown in Figure 2B, the α(III)-subunit coimmunoprecipitated with PDI, as did the epitope-tagged α(I)- and α(II)-subunits of prolyl 4-hydroxylase, whereas there was no immunoreactive band in sham-transfected cells. Reversing the antibodies used for immunoprecipitation and immunoblotting gave similar results (data not shown).

To test for enzymatic activity of the α(III)-subunit–containing protein, c-myc–tagged α(III)-subunit was coexpressed with PDI in HT1080 fibrosarcoma cells. Protein was immunoprecipitated by using anti–c-myc antibody, released from binding resin in a soluble state, and assayed for prolyl 4-hydroxylase of a collagen-like peptide. As shown in Figure 2C, the recovered protein displayed enzymatic activity, similar to that of the α(I)–subunit–containing protein.

Prolyl 4-hydroxylase α(III)-subunit transcripts were found to be expressed in human placenta, liver, and skeletal muscle (Figure 2D). We also compared the expression profile of the α(III)-subunit in maturing HITB5 SMCs with that of the α(I)- and α(II)-subunits. Interestingly, all 3 transcripts were expressed in HITB5 SMCs, but only the α(II)-subunit mRNA increased as SMCs matured (Figure 2E).

The fidelity of collagen production is central to atheroma- tosous disease. As illustrated in Figure 2F through 2H, we identified expression of the α(III)-subunit, by in situ hybridization, in atherosclerotic carotid artery samples harvested from 5 of 10 patients. The strongest and most prevalent staining was in SMC-like cells within the fibrous cap of lipid-rich lesions. Cells within the necrotic lipid core did not express the gene. As well, the media of nonatherosclerotic artery samples (internal thoracic artery, n = 2) showed little if any expression of the α(III)-subunit transcript.

**Discussion**

Until recently, the collagen prolyl 4-hydroxylase was believed to be an isolated enzyme that was not part of a family. An isoform of the catalytic subunit, designated α(II), however, was recently cloned from humans.10 Because the β-subunit (PDI) is invariant, it was established that the α-subunit functionally defines the collagen prolyl 4-hydroxylase enzyme. We have discovered, cloned, and characterized a gene encoding a new prolyl 4-hydroxylase α-subunit, termed the α(III)-subunit. This gene is
expressed by SMCs and found, among other tissues, in the
fibrous cap of atherosclerotic human carotid arteries.

Key features of the α(III)-subunit include (1) sequence
similarity with the α(I)- and α(II)-subunits of prolyl
4-hydroxylase, (2) conservation of cysteines required in
maintenance of structure of the α(I)-subunit.

Comparison of Prolyl 4-Hydroxylase α-Subunits

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<td>10q</td>
<td>11q</td>
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*Denotes conserved residues critical for cosubstrate binding.

Figure 1. A, Cloning strategy. The original cDNA fragment (clone 31-1) contained 133 bp of putative coding sequence, 3′UTR, and a poly A tail. 5′RACE reactions generated clones 36, 224, and 371, but none of 15 clones sequenced extended to the putative translational start site. GenBank and clone sequences were used to design primers P3 and P4, from which the full-length coding sequence was generated by RT-PCR from RNA harvested from HITBS SMCs. B, Peptide sequence aligned with that of prolyl 4-hydroxylase α(I)- and α(II)-subunits. A 19-amino-acid signal peptide sequence also exists at the N-terminus (MGPGARLAALLAVGLG).

Comparison of Prolyl 4-Hydroxylase α-Subunits
ferentiation that has also been noted with osteoblasts. The possibility of differential participation of prolyl 4-hydroxylase isoforms in vascular restructuring therefore warrants study. In addition, because the α(III)-subunit was expressed in other tissues, a broader role is also likely.

In summary, discovery of the prolyl 4-hydroxylase α(III)-subunit suggests a new molecular participant in collagen production that may be relevant to development or repair of a number of tissues, including the atheromatous artery wall.

Acknowledgments

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References


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