Identification and Characterization of a Third Human, Rat, and Mouse Collagen Prolyl 4-Hydroxylase Isoenzyme*

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Collagen prolyl 4-hydroxylases (C-P4Hs) catalyze the formation of 4-hydroxyproline by the hydroxylation of proline in -X-Pro-Gly- triplets. The vertebrate enzymes are αβ2 tetratrams, the β-subunit being identical to protein-disulfide isomerase (PDI). Two isoforms of the catalytic α-subunit, which combine with PDI to form [α(I)]β2 and [α(II)]β2 tetratrams, have been known up to now. We report here on the cloning and characterization of a third vertebrate C-P4H α-subunit isoform, α(III). The processed human, rat and mouse α(III) polypeptides consist of 520–525 residues, all three having signal peptides of 19–22 additional residues. The sequence of the processed human α(III) polypeptide is 35–37% identical to those of human α(I) and α(II), the highest identity being found within the catalytically important C-terminal domain. Other than the C-terminal residues at the substrate binding sites being conserved, the sequence within a region corresponding to the peptide-substrate binding domain is less conserved, but all five α helices constituting this domain can be predicted to be located in identical positions in α(I), α(II), and α(III) and to have essentially identical lengths. The α(III) mRNA is expressed in many human tissues, but at much lower levels than the α(I) and α(II) mRNAs. In contrast to α(I) and α(II), no evidence was found for alternative splicing of the α(III) transcripts. Coexpression of a recombinant human α(III) polypeptide with PDI in human embryonic kidney cells led to the formation of an active enzyme that hydroxylated collagen chains and a collagen-like peptide and appeared to be an [α(III)]β2 tetraramer. The catalytic properties of the recombinant enzyme were very similar to those of the type I and II C-P4Hs, with the exception that its peptide binding properties were intermediate between those of the type I and type II enzymes.

Collagen prolyl 4-hydroxylases (C-P4Hs, EC 1.14.11.2), enzymes residing within the lumen of the endoplasmic reticulum (ER), catalyze the formation of 4-hydroxyproline by the hydroxylation of proline in -X-Pro-Gly- triplets. The vertebrate enzymes are αβ2 tetratrams, the β-subunit being identical to protein-disulfide isomerase (PDI). Two isoforms of the catalytic α-subunit, which combine with PDI to form [α(I)]β2 and [α(II)]β2 tetratrams, have been known up to now. We report here on the cloning and characterization of a third vertebrate C-P4H α-subunit isoform, α(III). The processed human, rat and mouse α(III) polypeptides consist of 520–525 residues, all three having signal peptides of 19–22 additional residues. The sequence of the processed human α(III) polypeptide is 35–37% identical to those of human α(I) and α(II), the highest identity being found within the catalytically important C-terminal domain. Other than the C-terminal residues at the substrate binding sites being conserved, the sequence within a region corresponding to the peptide-substrate binding domain is less conserved, but all five α helices constituting this domain can be predicted to be located in identical positions in α(I), α(II), and α(III) and to have essentially identical lengths. The α(III) mRNA is expressed in many human tissues, but at much lower levels than the α(I) and α(II) mRNAs. In contrast to α(I) and α(II), no evidence was found for alternative splicing of the α(III) transcripts. Coexpression of a recombinant human α(III) polypeptide with PDI in human embryonic kidney cells led to the formation of an active enzyme that hydroxylated collagen chains and a collagen-like peptide and appeared to be an [α(III)]β2 tetraramer. The catalytic properties of the recombinant enzyme were very similar to those of the type I and II C-P4Hs, with the exception that its peptide binding properties were intermediate between those of the type I and type II enzymes.

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EXPERIMENTAL PROCEDURES

Isolation of cDNA Clones—PCR primers 1-α(III) (5'-TTAGGAGATG-CAGACCGTTGGT-3') and 2-α(III) (5'-CCTGGAGCTCAGCGTGCGT-3') were used to obtain a 162-bp product from a Human Fetal Umbilical Vein Endothelial Cell cDNA library (BD Biosciences), which was then used to screen a human umbilical vein endothelial cell (HUVEC) lambda cDNA library (Stratagene), 13 positive clones being obtained. The human α(III) cDNA was also analyzed by consecutive overlapping 5'- and 3'-RACE reactions in a Marathon-Ready human total fetal DNA pool (BD Biosciences), and its 5'-end was defined by 5'-RACE of HUVEC cDNA using SMART-GE 2 polymerase (BD Biosciences). HUVEC cDNA was generated using the SMART RACE cDNA amplification kit (BD Biosciences), and total RNA isolated from cultured HUVEC cells with the RNeasy Midi kit (Qiagen). The full-length human α(III) cDNA was cloned into pUC18 in two steps. An α(III) fragment extending from nucleotide 184 to the poly(A) tail was first cloned into pUC18 using the SureClone ligation kit (Amersham Biosciences), after which this construct was digested with BamHI-Apal and a similarly digested α(III) fragment covering bp 1–418 was ligated to it. The rat and mouse α(III) cDNAs were assembled by consecutive overlapping 5'- and 3'-RACE reactions using various rat and mouse Marathon-Ready cDNA pools (BD Biosciences) as templates and initial 5'- and 5'-RACE primers based on a 169 bp rat sequence (GenBank™ accession number AW921407). DNA sequencing was performed on ABI Prism 377 (Applied Biosystems). Nucleotide and amino acid sequence homology comparisons were made using the ClustalW service at the European Bioinformatics Institute.

Analysis of the Expression of Human α(III) Subunit mRNA—Human MTN Blot (BD Clontech) and Real™ Human Fetal mRNA Blot I and II (Invitrogen) ( hybridizing 2 μg poly(A) RNA per lane were hybridized using ULTRAbth™ solution under the conditions specified by the manufacturer, with 0.5 μg of a 250-bp α(III) or a 1440-bp α(I) cDNA fragment as the probe, the blots being exposed for 72 and 4 h, respectively. PCR analysis of the Human MTC Panel I and Human Fetal MTC Panel (BD Clontech) was performed according to the manufacturer's protocol with the primer pairs α(I)panel-3’5’-RACE (GAAGCGGCTGTCCATCCATGATAAAATGTC) and α(I)panel-3’5’-RACE (CTTCTGAGCTGGATCGATTGGAATGAGAC), and α(III)panel-5’ (GACATGGGGGATTATTACCATGC- GAAATCAACAGAAGTTGACTG). The amounts of the α(I) and α(III) primers were optimized prior to the analyses to produce similar amounts of DNA from equal amounts of the α(I) and α(III) plasmids under the same PCR conditions. The preincubation was at 95 °C for 1 min, followed by 25, 30, 34, 38, and 42 cycles at 95 °C for 30 s and 68 °C for 1 min, 5-μl aliquots being taken at each point and analyzed on 1% agarose gels.

Total RNA from human fetal epiphyseal cartilage was a gift from Dr. E. Vuento, University of Turku, Finland, while total RNA from human fibroblasts was isolated using the RNeasy Midi kit (Qiagen). RT-PCR was performed using a SMART RACE cDNA amplification kit (BD Clontech), and PCR analysis as for the MTC panels, using the α(I) and α(III) primers described above and the α(I) primers α(I)panel-5’ (GAGGGACCGAGATTCATCATAGATAAAGTGTC) and α(I)panel-3’ (GAAGCGGCTGTCCATCCATGATAAAATGTC) and α(III)panel-3’ (GAGGGACCGAGATTCATCATAGATAAAGTGTC).

Alternative splicing of the α(III) mRNA was analyzed by PCR with various human MTC panel and Marathon-Ready cDNAs (BD Biosciences) as templates. The primer pairs used were: α(I)-17 (CAGCCTGACCTCAGTATCTCTACACT) and α(I)-19 (CTTCTGAGCTGGATCGATTGGAATGAGAC) from exon 9, and α(III)-20 (CTTCTAGCTTGCTATGACGACAACTA) from exon 13. Expression and analysis of a Recombinant α(III) Polyepitope in Insect and Mammalian Cells—The full-length human α(III) cDNA was cut into two pieces from pUC18 with BamHI-EcoRI digestion and cloned into a similarly digested baculovirus vector pVL1393 (BD Pharmingen). The α(I) cDNA lacking sequences coding for the signal peptide was generated by PCR and cloned into pACPG67A (BD Pharmingen) in-frame with the GP67 signal sequence. The recombinant baculoviruses were generated and the SF9 and High Five insect cells were infected with viruses encoding the α(I) and PDI polyepitopes and analyzed as described previously (17, 18).

Mammalian expression vectors were generated by cloning the full-length PDI cDNA into pCDNA3.1 (+) (Invitrogen) and the full-length α(III) and α(I) cDNAs into pCDNA3.1 (+) PDI vector (Invitrogen). The pCDNA3.1 (+) PDI construct was linearized with BglII, transfected into human embryonic kidney HEK-293 cells using PolyFect (Qiagen), and stably transfected cells were selected with 400 μg/ml of Geneticin (Invitrogen). The HEK-293 cells were cultured in Dulbecco's modified Eagle's medium (Biochrom) supplemented with 10% fetal calf se-

run (BioClear) and 50 μg/ml of ascorbic acid at 37 °C. The pCDNA3.1 (+) Hygro vectors encoding the α(III) and α(I) polyepitopes were linearized with BglII and FspI, respectively, transfected into the stable HEK-293 cell line expressing recombinant human PDI, and stable cell lines were selected with 25 μg/ml of hygromycin (Invitrogen). A control cell line was established by stable transfection of pCDNA3.1 (+) HygroLaCZ (Invitrogen). The cells were harvested at confluence and homogenized in a Triton X-100 buffer (17, 18).

Soluble and insoluble fractions of the insect and mammalian cell homogenates were analyzed by 8% SDS-PAGE under reducing or non-reducing conditions and 8% nondenaturing PAGE followed by Coomasie Blue staining or Western blotting with a PDI antibody (Dako), a polyclonal α(I) antibody K7 (19) or a monoclonal antibody VTT1081 against the α(III) subunit generated at the Technical Research Centre of Finland by immunizing mice with a recombinant α(III) polyepitope (amino acids 177–501) expressed in Escherichia coli using the pET expression system (Novagen) and purified from the inclusion bodies (20). P4H activity was assayed by methods based on the formation of 4-hydroxy(Lys)polypeptide in a [14C]polypeptide-labeled substrate consisting of nonhydroxylated procollagen polypeptide chains or the hydroxylation-coupled decarboxylation of 2-oxo-1,4C]glutarate (21). K<sub>m</sub> and K<sub>v</sub> values were determined as described (17). Native immunoprecipitation was performed with Protein G Sepharose 4 Fast Flow according to the instructions provided by the manufacturer (Amersham Biosciences). The cell lysates were precleared, immunoprecipitated with 1–5 μg of the antibodies VTT1081 against the α(III) subunit or anti-FLAG (Sigma) as a negative control, and the Sepharose and the bound antibody-protein complexes were washed three times with a Triton X-100 buffer (17, 18). The samples were then boiled in SDS-PAGE sample buffer and analyzed by SDS-PAGE followed by Western blotting. N-glycosidase F treatment was carried out according to the instructions provided by the manufacturer (Roche Applied Science). Gel filtration was performed in a calibrated Superdex 200 column (Amersham Biosciences). Recombinant human type I and II C-P4Hs were expressed in insect cells, and the type I enzyme was purified as described (6, 18).

RESULTS AND DISCUSSION

Cloning of the Human, Rat, and Mouse α(III) Subunits—A sequence homology search identified several human ESTs representing a gene product with similarity to the conserved C-terminal regions of the C-P4H (α(I) and (α) subunits. EST AA116081 was used to design primers for the initial 5'- and 3'-RACE reactions and amplification of a 162-bp probe, which was then used to screen a human umbilical vein endothelial cell cDNA library, the 5'-end of the cDNA being obtained by 5'-RACE reactions. The cDNA clones cover 43 bp of the 5'-untranslated sequence, a 1635-bp open reading frame, and 591 bp of the 3'-untranslated sequence, with a canonical polyadenylation signal ATTAAA followed 15 bp downstream by a poly(A) tail.

A sequence homology search with the human α(III) cDNA identified a 169-bp rat sequence that is 89% identical to the coding nucleotides at the 3'-end of the human sequence. The rat sequence information was used to design primers for 5'-RACE reactions in rat cDNA pools, and 5'- and 3'-RACE reactions in mouse cDNA pools. The cDNA fragments obtained cover the full-length coding sequences of the rat and mouse α(III) subunit cDNAs, including 25 and 13 bp of the 5'-untranslated region, respectively.

The human, rat, and mouse α(III) cDNA sequences have been deposited in the GenBank™ with accession numbers AY313448, AY313450, and AY313449, respectively.

Amino Acid Sequences of the Human, Rat, and Mouse α(III) Subunits—The human and rat α(III) cDNAs encode polypeptides of 544 amino acids, the mouse polypeptide being two residues shorter. Putative signal peptides (22) of 19, 19, and 22 residues are present in the N termini of the human, rat, and mouse α(III) polypeptides, respectively, the lengths of the processed human and rat α(III) subunits thus being 525 amino acids and that of the mouse α(III) 520 amino acids (Fig. 1). The processed human α(III) subunit is slightly longer than the processed human α(I) and α(II), which consist of 517 and 514
The human α(III) sequence shows 91 and 94% identity to the rat and mouse sequences, respectively, the latter two being 95% identical. The overall sequence identity between the processed human α(III) and α(I) subunits is 35%, and that between α(III) and α(II) 37% (Fig. 1), while the identity between α(I) and α(II) is higher, 65%. The identity is highest within the catalytically important C-terminal region (1–3, 17), the 120 C-terminal residues of the human α(III) subunit being 56–57% identical to those of human α(I) and α(II) (Fig. 1), while the identity between α(I) and α(II) in this region is 80%. All four critical residues at the catalytic site, the two histidines and one aspartate that bind the Fe$^{2+}$ atom and the lysine that binds the C-5 carboxyl group of 2-oxoglutarate in position +10 with respect to the second iron-binding histidine (1–3, 17), are conserved in all these α-subunits (Fig. 1). The residue that binds the C-5 carboxyl group of 2-oxoglutarate in the HIF asparaginyl hydroxylase (FIH) is also a lysine, but it is present in position +15 with respect to the first iron-binding histidine (23, 24). The other 2-oxoglutarate-dependent dioxygenases, including the HIF-P4Hs and lysyl hydroxylases, differ from the HIF-P4Hs and lysyl hydroxylases, differ from the FIH–prolyl 4-hydroxylases, and the decarboxylation of this cosubstrate (17) are indicated by asterisks. The positions of cysteine residues (•) and potential N-glycosylation sites (–) in α(III) are indicated above the human α(III) sequence. Residues that are identical between an α(III) subunit and α(I) or α(II) are shown by white letters on a black background. The five α helices of the α(I) peptide–substrate binding domain (28) are indicated below the α(I) amino acids 144–244 with black cylinders and the predicted (29) α helices of the corresponding α(III) and α(II) regions with white and gray cylinders, respectively.
(Fig. 2B). The expression levels of the α(III) mRNA were again much lower than those of the α(I) mRNA in all the tissues studied, since no α(III) mRNA was detected in these samples after 34 cycles, whereas the α(I) mRNA was already clearly seen in many tissues after 30 cycles (Fig. 2B). Furthermore, PCR analysis of human fibroblast and fetal epiphyseal cartilage cDNA pools indicated that the α(III) mRNA was expressed at much lower levels than the α(I) and α(II) mRNAs in both samples (Fig. 2C). Our data thus demonstrate that the α(III) mRNA is expressed at low levels in many fetal and adult tissues, and presumably also in many cell types. Further studies will be needed to demonstrate whether there are cells that express this mRNA at levels higher than those of the other C-P4H α-subunit mRNAs.

Organization of the Human α(III) Subunit Gene—A search of High Throughput Genomic Sequences from the GenBankTM data base indicated that the human α(III) subunit gene is present on chromosome 11q12 (accession number AC006595), while the human α(I) and α(II) genes are located on chromosomes 10q21.3–23.1 (I) and 5q31 (30), respectively. The exon-intron organization of the human α(III) gene is very similar to those of the α(I) and α(II) genes (30, 31). However, a number of 5′-RACE reactions carried out with various human, rat, and mouse cDNAs (details not shown) indicated that the α(III) gene lacks an exon corresponding to the first exon of the α(I) and α(II) genes (30, 31) and that only one α(III) exon, number 1, corresponds to exons 2 and 3 in the α(I) and α(II) genes (Fig. 3A). The subsequent exon-intron boundaries are mainly conserved, except that the α(III) exon 3, corresponding to exon 5 in α(I) and α(II), is 86 bp longer and that the region corresponding to exon 6 in α(I) and α(II) is split into two exons, 4 and 5, in the α(III) gene, while the α(III) sequence corresponding to exons 11 and 12 in α(I) and exons 10 and 11 in α(II) is fused into one exon, number 9 (Fig. 3A). Small differences were also found in the lengths of some of the exons (Fig. 3A).

Two forms of α(I) and α(II) mRNA resulting from mutually exclusive alternative splicing of the homologous exons 9 and 10 in the human α(I) gene and exons 12a and 12b in the human α(II) gene have previously been identified (30, 31). Careful sequence homology analysis of all introns in the human α(III) GenBankTM clone AC006595 indicated that none of them contained any sequences with similarity to those in the preceding or following exon, thus excluding the presence of consecutive homologous exons that might be subject to mutually exclusive alternative splicing in the α(III) gene (details not shown).

The possible presence of alternatively spliced exons was studied further by PCR analysis of human multitissue cDNA panels and sequencing of the products. The primer pairs used in Fig. 3, panel B, were from the boundary between exons 1 and 2 and from exon 9, while those used in Fig. 3, panel C, were from exons 7 and 13. PCR products of the expected lengths of 1041 (Fig. 3B) and 667 bp (Fig. 3C) were obtained from all the tissues studied. An additional product of about 650 bp was obtained with the primers used in panel B, but sequencing indicated that it was a nonspecific product not related to any α(III) transcript. Our data thus exclude the presence of mRNA
The 650-bp product had no activity in panel B, however, and no association between cells together with a virus encoding human PDI (18). No P4H signal peptide GP67, were generated and used to infect insect cell lines (Fig. 4). PDI was found in both stably transfected polypeptides (Fig. 4). To study the assembly of type III C-P4H in mammalian cells, two polypeptides was immunoprecipitated with an antibody to the Type III C-P4H is probably an α2β2 tetramer, in which PDI Serves as the β-Subunit—To analyze whether the α(III) and PDI polypeptides are present in the same molecule, the Triton X-100 buffer-soluble fraction from cells coexpressing the two polypeptides was immunoprecipitated with an antibody to the α(III) polypeptide and the precipitate was analyzed by SDS-PAGE under reducing conditions followed by Western blotting. The antibody was found to precipitate both the α(III) and PDI polypeptides from the soluble fraction from cells coexpressing these two polypeptides (Fig. 5, lanes 1 and 4), but not the α(I) or PDI polypeptide from cells coexpressing these two (Fig. 5, lanes 2 and 5). An anti-FLAG antibody used as a negative control precipitated neither of the two polypeptides from cells coexpressing α(III) and PDI (Fig. 5, lanes 3 and 6).

Fig. 3. Comparison of the C-P4H α(I), α(II), and α(III) gene structures (A), and PCR analysis of possible alternative splicing of the α(III) mRNA (B and C). A, a schematic representation of the exon-intron organization of the C-P4H α(I), α(II), and α(III) subunit genes. The exons are represented by numbered boxes and their lengths are given in bp below them. The coding sequences are indicated in black. The introns are shown by lines that are not drawn to scale. B and C, possible alternative splicing of the α(III) mRNA was analyzed by PCR using primer pairs from the boundary between exons 1 and 2 and from exon 9 (B) and from exon 7 and exon 13 (C). The tissue sources are indicated on top of the panels. In panel B an additional product of about 650 bp was obtained besides the product of the expected size of 1041 bp, but sequencing indicated that the 650-bp product had no α(III) sequence.
Table I

| Polypeptide expressed | P4H activity (dpm/mg) |
|-----------------------|-----------------------|
| β-galactosidase       | 17,000                |
| α(III) + PDI          | 57,000                |
| α(I) + PDI            | 104,000               |

Values are given as dpm/mg for three independent experiments.

The data thus indicate that the α(III) and PDI polypeptides are very likely to be present in the same molecule.

The subunit composition of the type III enzyme was studied further by gel filtration of soluble fractions from cells coexpressing recombinant polypeptides in a calibrated Superdex 200 column. The elution position of the P4H activity was found to be identical for cell lines coexpressing either the α(III) and PDI or the α(I) and PDI polypeptides (Fig. 6A). This elution position also corresponded to that of a purified recombinant type I C-P4H tetramer, and could be stained with the α(III) and PDI antibodies (Figs. 6D and F), but not with the α(I) antibody (Fig. 6D). Similarly, non-denaturing PAGE of the fractions from cells coexpressing α(I) and PDI showed a band reactive with the α(I) and PDI antibodies (Fig. 6E), but not with the α(III) antibody (data not shown). It thus seems very likely that the type III C-P4H has a similar subunit composition to the type I and type II C-P4Hs (1–3) and is an [α(III)]β2 tetramer in which PDI serves as the β-subunit.

Previous coexpression studies in insect cells argue against the presence of a mixed tetramer containing the α(I) and α(II) subunits from vertebrate species, whereas no bands were detected with the α(III) and PDI antibodies (Fig. 6F). This elution position also corresponded to that of a purified recombinant type III C-P4H tetramer in which PDI serves as the β-subunit.

The Additional Cysteine Residue Present in the Human α(III) Subunit Is Not Involved in Intrachain or Intermolecular Disulfide Bonding—The human α(III) subunit contains six conserved cysteines in positions identical to those of cysteines 1–6 in α(I), while the α(II) subunit having an additional cysteine between the α(III) cysteines 5 and 6 (Fig. 1). Site-directed mutagenesis studies of human α(III) have indicated that intrachain disulfide bonds are essential for tetramer assembly, and are formed between cysteines corresponding to the third and fourth and the fifth and sixth cysteines in α(III), while the cysteine is not involved in any disulfide bonding (39, 40). The additional cysteine (residue 226) present in human α(III) is located in helix 4 of the peptide-substrate binding domain, while the first cysteine (residue 165) is located in helix 1 (Fig. 1). The α(I) peptide-binding domain has recently been crystallized (41) and its structure solved. Inspection of the crystal structure indicated that the α(I) residues corresponding to α(III) Cys-165 and Cys-226 are far from each other. As the structures of the α(I) and α(III) peptide-substrate binding domains are likely to be highly similar (see above), it seems evident that the α(III) Cys-165 and Cys-226 cannot form any intrachain disulfide bond.

The possibility was not excluded, however, that Cys-226 might form an interchain disulfide bond, which is not found in the type I or type II C-P4H (1). This possibility was studied by analyzing the soluble fraction from cells coexpressing the α(III) and PDI polypeptides by SDS-PAGE under nonreducing condit
The mobility of \( \alpha(III) \) was found to be identical under nonreducing and reducing conditions and to correspond to that of the polypeptide monomer (Fig. 7A, lanes 1 and 3). It is thus evident that the additional cysteine present in the human \( \alpha(III) \) polypeptide is not involved in either intrachain or interchain disulfide bonding. This cysteine is not likely to be involved in the catalytic mechanism, either, as the mechanism of 2-oxoglutarate dioxygenases is currently well understood and does not involve any cysteine residue (1–3), and as the type I C-P4H, which has very similar catalytic properties (see below), contains only one cysteine not involved in disulfide bonding (corresponding to Cys-165 in \( \alpha(III) \)) and this can be mutated to serine with no loss of catalytic activity (40). The rat and mouse \( \alpha(III) \) polypeptides contain one additional cysteine at position \( \alpha(III) \) 8 with respect to Cys-226 in the human polypeptide (Fig. 1), but its role was not studied further.

The Two N-Glycosylation Sites Have No Role in the Activity of the Type III C-P4H—The human, rat, and mouse \( \alpha(III) \) polypeptides contain two N-glycosylation sites that are conserved between species but not in \( \alpha(I) \) and \( \alpha(II) \) (Fig. 1), which also have two N-glycosylation sites but in different positions (4–6). Site-directed mutagenesis studies of the human \( \alpha(I) \) subunit have shown that glycosylation has no role in the assembly of the type I C-P4H tetramer or its catalytic activity (40). To study whether N-glycosylation has any role in the catalytic activity of the human type III C-P4H, the soluble fraction from cells coexpressing the \( \alpha(III) \) and PDI polypeptides was treated with N-glycosidase F and analyzed by SDS-PAGE under reducing conditions (B and C) and by nondenaturing PAGE (D and E) followed by Western blotting with the antibodies indicated on top of the lanes, the expressed polypeptides being indicated below the panels. Panel F shows Coomassie Blue-stained nondenaturing PAGE of purified recombinant type I C-P4H (18).
CONCLUSIONS

C-P4H was long believed to be of one type only, until an isof orm of the α-subunit, α(I), was cloned and characterized from mouse and human sources (5, 6). The present study reports on the identification of a further vertebrate α-subunit isof orm, the α(III) subunit, the human polypeptide showing 35–37% sequence identity to the human C-P4H α(I) and α(II) subunits (4, 6) but no distinct sequence similarity to the HIF-P4Hs (9, 10). This degree of identity is considerably lower than the 65% observed between the human α(I) and α(II) subunits (4, 6). The α(III) polypeptide also resembles the C-P4H α-subunits rather than the HIF-P4Hs in that its residue that binds the C-5 carboxyl group of the 2-oxoglutarate is a lysine (17) rather than an arginine (9–11) and that the IC_{50} of the type III enzyme for pyridine 2,5-dicarboxylate is about 7 μM, whereas it is much more than 300 μM for all three human HIF-P4Hs (42). The α(III) polypeptide, like the α(I) and α(II) polypeptides, appears to form with PDI an α₂β₂ tetramer that hydroxylates collagen chains and collagen-like peptides, its elution position in gel filtration and mobility in nondenaturing PAGE excluding the presence of any active dimer or monomer. The α(III) mRNA was found to be expressed in many human tissues, but at much lower levels than the α(I) and α(II) mRNAs, no general trend being found for any distinct difference between embryonic and adult tissues. Our data do not, however, exclude the possibility that some cell types may express the α(III) mRNA at levels higher than those of the other C-P4H α-subunit mRNAs.

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