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Cloning, baculovirus expression, and characterization of a second mouse prolyl 4-hydroxylase α-subunit isoform: Formation of an $\alpha_2\beta_2$ tetramer with the protein disulfide-isomerase/β subunit

TARJA HELAAKOSKI*, PIA ANNUNEN*, KRISTIINA VUORI*, IAN A. MACNEIL†, TAINA PIHLAJANIEMI*, AND KARI I. KIVIRIKKO*‡

*Collagen Research Unit, Biocenter and Department of Medical Biochemistry, University of Oulu, FIN-90220 Oulu, Finland; and †DNAX Research Institute of Molecular and Cellular Biology, Palo Alto, CA 94304

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ABSTRACT **Prolyl 4-hydroxylase (EC 1.14.11.2) catalyzes the posttranslational formation of 4-hydroxyproline in collagens. The vertebrate enzyme is an $\alpha_2\beta_2$ tetramer, the β subunit of which is a highly unusual multifunctional polypeptide, being identical to protein disulfide-isomerase (EC 5.3.4.1). We report here the cloning of a second mouse α subunit isoform, termed the α(II) subunit. This polypeptide consists of 518 aa and a signal peptide of 19 aa. The processed polypeptide is one residue longer than the mouse α(I) subunit (the previously known type), the cloning of which is also reported here. The overall amino acid sequence identity between the mouse α(II) and α(I) subunits is 63%. The mRNA for the α(II) subunit was found to be expressed in a variety of mouse tissues. When the α(II) subunit was expressed together with the human protein disulfide-isomerase/β subunit in insect cells by baculovirus vectors, an active prolyl 4-hydroxylase was formed, and this protein appeared to be an $\alpha(II)_2\beta_2$ tetramer. The activity of this enzyme was very similar to that of the human $\alpha(I)_2\beta_2$ tetramer, and most of its catalytic properties were also highly similar, but it differed distinctly from the latter in that it was inhibited by poly(L-proline) only at very high concentrations. This property may explain why the type II enzyme was not recognized earlier, as an early step in the standard purification procedure for prolyl 4-hydroxylase is affinity chromatography on a poly(L-proline) column.**

Prolyl 4-hydroxylase (EC 1.14.11.2) catalyzes the formation of 4-hydroxyproline in collagens and related proteins by the hydroxylation of proline residues in -Xaa-Pro-Gly- sequences. The reaction requires Fe^{2+}, 2-oxoglutarate, O_2, and ascorbate. The vertebrate enzyme is an $\alpha_2\beta_2$ tetramer in which the α subunits contribute to most parts of the catalytic sites (for recent reviews, see refs. 1–3). The β subunit has been cloned from many sources (1–5) and found to be a highly unusual multifunctional polypeptide that is identical to the enzyme protein disulfide-isomerase (PDI; EC 5.3.4.1) (6, 7), a cellular thyroid hormone-binding protein (8), the smaller subunit of the microsomal triacylglycerol transfer protein (9), and an endoplasmic reticulum luminal polypeptide uniquely binding various peptides (5, 10, 11). The catalytically important α subunit has been cloned from human (12), chicken (13) and *Caenorhabditis elegans* (14), and its RNA transcripts have been shown to undergo alternative splicing involving sequences encoded by two consecutive, homologous 71-bp exons (12, 15).

Prolyl 4-hydroxylase plays a crucial role in the synthesis of all collagens, as the 4-hydroxyproline residues are essential for the folding of the newly synthesized collagen polypeptide chains into triple-helical molecules. The enzyme has been purified and characterized from many sources, with no definite evidence for the existence of any isoenzymes (1–3). The extent of glycosylation of the two potential attachment sites for asparagine-linked carbohydrate units varies, however, leading to heterogeneity in the size of the polypeptide (16, 17). We report here the cloning and characterization of a second mouse α-subunit isoform, which we term the α(II) subunit, which appears to be expressed in a variety of tissues. The amino acid sequence of the mouse α(II) subunit is 63% identical to that of the already known α subunit, termed here the α(I) subunit, the cloning of which from mouse tissues is also reported here. We further report that the α(II) subunit, like the α(I) subunit, forms an $\alpha_2\beta_2$ tetramer with the multifunctional PDI/β subunit polypeptide; this tetramer has some unique properties.§

MATERIALS AND METHODS

Isolation of cDNA Clones. The first cDNA clone for the mouse α(II) subunit, BT14.1, was obtained from a BALB/c mouse brain cDNA library in λgt10 (Clontech) by using a cDNA encoding the thymic shared antigen 1 (18) as a probe. The BT14.1 clone had a high degree of homology to the human and chicken prolyl 4-hydroxylase α subunit. Rescreening of the mouse brain library with BT14.1 as a probe gave four positive clones out of 600,000 recombinants. One of them, M1, was used to screen 1.6×10^6 plaques of a mouse skeletal muscle cDNA library in λgt10 (Clontech), and one positive clone, M6, was obtained.

To isolate cDNA clones for the mouse prolyl 4-hydroxylase α(I) subunit, a mouse 3T3 fibroblast λgt11 cDNA library (Clontech) was screened with the human α(I) subunit cDNA clone PA-49 (12) as a probe. Eight positive clones were obtained out of 600,000 plaques. Three of them, MA3, MA4, and MA7, were characterized further.

Nucleotide Sequencing, Sequence Analysis, and Northern Blot Analysis. The nucleotide sequences were determined by the dideoxynucleotide chain-termination method (19) with T7 DNA polymerase (Pharmacia). Vector-specific or sequence-specific primers synthesized in an Applied Biosystems DNA synthesizer (Department of Biochemistry, University of Oulu) were used. The DNASIS and PROSIS version 6.00 sequence-analysis software (Pharmacia), ANTHEPROT (20), the Wisconsin Genetics Computer Group package version 8 (September 1994), and BOXSHADE (Kay Hofmann, Bioinformatics Group, Institut Suisse de Recherches Experimentales sur le Cancer, Lausanne, Switzerland) were used to compile the sequence data.

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Abbreviation: PDI, protein disulfide-isomerase.

‡To whom reprint requests should be addressed at: Department of Medical Biochemistry, University of Oulu, Kajaanintie 52 A, FIN-90220 Oulu, Finland.

§The sequences reported in this paper have been deposited in the GenBank data base (accession nos. U16162 and U16163).

A mouse multitissue Northern blot (Clontech) containing 2 μg of poly(A)$^+$ RNA per sample isolated from various mouse tissues was hybridized under the stringent conditions suggested in the manufacture's instructions. The probe used was ^{32}P-labeled cDNA clone BT14.1 or MA7.

Cell Cultures and Generation of Recombinant Baculoviruses. *Spodoptera frugiperda* Sf9 insect cells were cultured at 27°C in TNM-FH medium (Sigma) supplemented with 10% fetal bovine serum (GIBCO). To construct an α(II)-subunit cDNA for expression, the clone BT14.1 was digested with the *Bam*HI and *Eco*RI restriction enzymes, giving a fragment encompassing bp 592–2168. The 5′ fragment was amplified from the λ DNA of M6. The primers used were cDNA specific, M3PH (5′-AAGTTGCGGCCGCGAGCATCAGCAAGGTACTGC-3′), containing an artificial *Not* I site, and M65′PCR (5′-TGTCCGGATCCAGTTTGTACGTGTC-3′), containing a natural *Bam*HI site. PCR was performed under the conditions recommended by the supplier of the *Taq* polymerase (Promega), and the reactions were cycled 27 times as follows: denaturation at 94°C for 1 min, annealing at 66°C for 1 min, and extension at 72°C for 3 min. The product was digested with *Not* I and *Bam*HI restriction enzymes to give a fragment that extended from bp 120 to 591. The two *Not* I–*Bam*HI and *Bam*HI–*Eco*RI fragments were then cloned into the pBluescript vector (Stratagene), the construct was digested with *Not* I and *Eco*RV, and the resulting fragment was ligated into a *Not* I–*Sma* I site of the baculovirus transfer vector pVL1392 (21). The pVL construct was cotransfected into Sf9 insect cells with a modified *Autographa californica* nuclear polyhedrosis virus DNA by using the BaculoGold transfection kit (PharMingen). The resultant viral pool was collected 4 days later, amplified, and plaque purified. The recombinant virus was checked by PCR-based methods (22).

Expression and Analysis of Recombinant Proteins. Insect cells were infected at a multiplicity of 5. For production of an enzyme tetramer, the human α59(I) (17) or mouse α(II) viruses and the PDI/β viruses (17) were used in a 1:1 or 2:1 ratio. The cells were harvested 72 hr after infection, homogenized in 0.01 M Tris, pH 7.8/0.1 M NaCl/0.1 M glycine/10 μM dithiothreitol/0.1% Triton X-100, and centrifuged. The resulting supernatants were analyzed by SDS/8% PAGE or nondenaturing 7.5% PAGE and assayed for enzyme activities. The cell pellets were further solubilized in 1% SDS and analyzed by SDS/8% PAGE. Western blot analysis was performed with a polyclonal antibody to the β subunit of human prolyl 4-hydroxylase.

Enzyme Activity Assays. Prolyl 4-hydroxylase activity was assayed by a method based on the decarboxylation of 2-oxo[1-^{14}C]glutarate (23). The K_m values were determined by varying the concentration of one substrate in the presence of fixed concentrations of the second while the concentrations of the other substrates were kept constant (24). The 0.1% Triton X-100 extracts from cell homogenates were used as the sources of the enzymes. The quantity and radioactivity of hydroxyproline were determined as described (23).

RESULTS AND DISCUSSION

Isolation of cDNA Clones for Mouse α(II) and α(I) Subunits. While screening cDNA clones for a mouse brain thy-

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   1  GCAGTTTCAGAGACCGGTGGCGATTGGCTGACTGATTCAACAAATAGAGCATTCTCTGTGCCTGGAGACTTTCAAGGACTGAGGCAGGCAGAAGGGAAGACTCAGAAAGTTCAGGTCCAG
                                                                                                     ▼
 121  AGCATCAGCAAGGTACTGCCCTTTCCAGTTATGAAGCTCCAGGTGTTGGTGTTGGTGTTGCTGATGTCCTGGTTCGGTGTCCTGAGCTGGGTGCAGGCAGAATTCTTCACCTCCATTGGG
 -19                                  M  K  L  Q  V  L  V  L  V  L  L  M  S  W  F  G  V  L  S  W  V  Q  A  E  F  F  T  S  I  G

 241  CACATGACCGATCTGATTTACGCAGAGAAGGACCTGGTACAGTCTCTGAAGGAGTACATCCTTGTGGAGGAAGCCAAGCTCGCCAAGATTAAGAGCTGGGCCAGCAAGATGGAAGCCCTG
  12   H  M  T  D  L  I  Y  A  E  K  D  L  V  Q  S  L  K  E  Y  I  L  V  E  E  A  K  L  A  K  I  K  S  W  A  S  K  M  E  A  L

 361  ACCAGCAGATCAGCTGCCGACCCCGAGGGCTACCTGGCTCATCCTGTGAATGCCTACAAGCTGGTGAAGCGGTTGAACACAGACTGGCCTGCACTGGGGGACCTTGTCCTTCAGGATGCT
  52   T  S  R  S  A  A  D  P  E  G  Y  L  A  H  P  V  N  A  Y  K  L  V  K  R  L  N  T  D  W  P  A  L  G  D  L  V  L  Q  D  A

 481  TCGGCAGGTTTTGTCGCTAACCTCTCAGTTCAGCGGCAATTCTTCCCCACTGATGAGGACGAGTCTGGAGCTGCCAGAGCCCTGATGAGACTTCAGGACACGTACAAACTGGATCCGGAC
  92   S  A  G  F  V  A [N  L  S] V  Q  R  Q  F  F  P  T  D  E  D  E  S  G  A  A  R  A  L  M  R  L  Q  D  T  Y  K  L  D  P  D

 601  ACGATTTCCAGAGGGGAACTTCCAGGCACAAAGTACCAGGCCATGCTGAGTGTGGACGACTGCTTTGGGCTGGGCCGCTCAGCTTACAATGAAGGAGACTATTACCATACTGTGCTGTGG
 132   T  I  S  R  G  E  L  P  G  T  K  Y  Q  A  M  L  S  V  D  D (C) F  G  L  G  R  S  A  Y  N  E  G  D  Y  Y  H  T  V  L  W

 721  ATGGAGCAGGTACTGAAGCAGCTCGATGCTGGGGAGGAGGCCACTGTTACCAAGTCCCTGGTGCTGGACTACCTGAGCTATGCTGTCTTCCAACTGGGTGACCTGCACCGTGCTGTGGAA
 172   M  E  Q  V  L  K  Q  L  D  A  G  E  E  A  T  V  T  K  S  L  V  L  D  Y  L  S  Y  A  V  F  Q  L  G  D  L  H  R  A  V  E

 841  CTCACCCGCCGCCTGCTCTCTCTTGACCCAAGCCACGAACGAGCTGGAGGGAATCTGCGGTACTTTGAACGGTTGTTAGAGGAAGAAAGAGGGAAATCACTGTCAAATCAGACAGACGCC
 212   L  T  R  R  L  L  S  L  D  P  S  H  E  R  A  G  G  N  L  R  Y  F  E  R  L  L  E  E  E  R  G  K  S  L  S [N  Q  T] D  A

 961  GGACTGGCCACCCAGGAAAACTTGTACGAGAGGCCCACGGACTACCTGCCTGAGAGGGATGTGTACGAGAGCCTGTGTCGAGGGGAGGGCGTGAAACTGACACCCCGGAGGCAGAAGAAG
 252   G  L  A  T  Q  E  N  L  Y  E  R  P  T  D  Y  L  P  E  R  D  V  Y  E  S  L (C) R  G  E  G  V  K  L  T  P  R  R  Q  K  K

1081  CTTTTCTGTAGGTACCATCATGGAAACAGAGTGCCACAGCTCCTCATCGCCCCCTTCAAAGAGGAAGACGAGTGGGACAGCCCACACATCGTCAGGTACTATGATGTGATGTCCGACGAA
 292   L  F (C) R  Y  H  H  G  N  R  V  P  Q  L  L  I  A  P  F  K  E  E  D  E  W  D  S  P  H  I  V  R  Y  Y  D  V  M  S  D  E

1201  GAAATCGAGAGGATCAAGGAGATTGCTAAGCCCAAACTTGCACGAGCCACTGTGCGTGACCCCAAGACAGGTGTCCTCACTGTTGCCAGCTACAGAGTTTCCAAAAGCTCCTGGCTAGAG
 332   E  I  E  R  I  K  E  I  A  K  P  K  L  A  R  A  T  V  R  D  P  K  T  G  V  L  T  V  A  S  Y  R  V  S  K  S  S  W  L  E

1321  GAGGATGACGACCCTGTTGTGGCCCGGGTCAACCGGCGGATGCAACATATCACCGGGCTAACGGTGAAGACTGCAGAGCTATTGCAGGTCGCAAACTACGGAATGGGGGGACAGTACGAA
 372   E  D  D  D  P  V  V  A  R  V  N  R  R  M  Q  H  I  T  G  L  T  V  K  T  A  E  L  L  Q  V  A  N  Y  G  M  G  G  Q  Y  E

1441  CCACACTTTGACTTCTCAAGGAGCGATGACGAAGATGCTTTCAAGCGTTTAGGGACTGGGAACCGTGTGGCCACGTTTCTAAACTACATGAGCGATGTCGAAGCTGGTGGTGCCACCGTC
 412   P  H  F  D  F  S  R  S  D  D  E  D  A  F  K  R  L  G  T  G  N  R  V  A  T  F  L  N  Y  M  S  D  V  E  A  G  G  A  T  V

1561  TTTCCTGACTTGGGAGCTGCTATTTGGCCCAAGAAGGGCACAGCTGTATTCTGGTACAACCTTCTTCGCAGTGGGGAAGGTGATTATCGGACGAGACATGCAGCCTGCCCTGTGCTTGTG
 452   F  P  D  L  G  A  A  I  W  P  K  K  G  T  A  V  F  W  Y  N  L  L  R  S  G  E  G  D  Y  R  T  R  H  A  A (C) P  V  L  V

1681  GGCTGCAAGTGGGTCTCCAACAAGTGGTTCCATGAGCGAGGACAGGAGTTCTTAAGACCTTGTGGAACAACGGAAGTTGATTGACGTCCTTTTCTCTCCGCTCCTCCCTGGCCCATAGTC
 492   G (C) K  W  V  S  N  K  W  F  H  E  R  G  Q  E  F  L  R  P (C) G  T  T  E  V  D  ***

1801  CAAATCATCTTCAAGTTCAACATGACAGCTTCCTTTTTTATGTCCCAGCTCCTGTCAGGCAGGTCATTGGAGGAGCCAGTGTTTGACTGAATTGAGAGAGTATATCCTGAGCCTAGTCCT

1921  GGGTGACCTGGGCCCCAGACTCTGACCAGCTTACACCTGCCCTGGCTCTGGGGGTGTCTTGGCATGGCTGCGGTAGAGCCAGACTATAGCACCCGGCACGGTCGCTTTGTACCTCAGATA

2041  TTTCAGGTAGAAGATGTCTCAGTGAAACCAAAGTTCTGATGCTGTTTACATGTGTGTTTTTATCACATTTCTATTTGTTGTGGCTTTAACCAAAAAATAAAAATGTTCCTGCCAAAAAAA

2161  AAAAAAAA
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FIG. 1. Nucleotide and deduced amino acid sequence for the α(II) subunit of mouse prolyl 4-hydroxylase. The amino acid sequence is shown in one-letter symbols. ▼, Putative N-terminal end of the processed α(II) subunit; ***, stop codon of translation. The six cysteine residues are circled, and the potential attachment sites for asparagine-linked oligosaccharides are boxed. A putative polyadenylylation signal, AATAAA, and a poly(A) tail are underlined. Numbering of the nucleotides begins with the extreme 5′ nucleotide of the cDNA, and that of the amino acids with the first residue of the mature protein as +1.

mocyte surface protein, we found a clone, BT14.1, which did not show any sequence similarity to the thymocyte protein. Instead it was homologous but not identical to the α subunit of human and chick prolyl 4-hydroxylase and was at first named an α subunit-like polypeptide and later the α(II) subunit. Since the cDNA clone BT14.1 did not contain sequences coding for the N-terminal region of the polypeptide, it was used as a probe to screen mouse brain and skeletal muscle cDNA libraries. Among 600,000 recombinants, 4 positive clones were obtained. Two of them, M1 and M4, were found to be identical, while M2 had a deletion and M3 contained two nonrelated inserts. The clone M1 was characterized further and was found to be included in BT14.1. The 5′ ends of M1 and BT14.1 were at the same internal *Eco*RI site (at nt 220 of the sequence shown in Fig. 1). The extreme 5′ clone was isolated by using M1 to screen a mouse skeletal muscle cDNA library, and one positive clone was obtained, M6. Together the cDNA clones cover the whole coding region of the mouse α(II) subunit.

cDNA clones for the mouse α(I) subunit were isolated by screening a 3T3 fibroblast cDNA library with the human cDNA clone PA-49 for the α(I) subunit (12), and eight positive clones were obtained. Three of them, MA3, MA4, and MA7, were isolated and sequenced. The nucleotide and predicted amino acid sequences of the clones showed a significant similarity to those of the human and chick prolyl 4-hydroxylase α subunit. Two of the clones, MA3 and MA4, were found to represent the mouse counterparts of human mRNA containing the alternatively spliced exon 10 sequences (12, 15), whereas MA7 contained exon 9 sequences. The cDNA clones did not contain the extreme 5′ end of the mRNA. Comparison of the cDNA-derived amino acid sequences with those of the human and chick α(I) subunits (12, 13) suggests that the cDNA clones cover the whole processed polypeptide but do not cover the 5′ untranslated region or the sequences corresponding to the N-terminal half of the signal peptide (these cDNA sequences are not shown, but they have been deposited in the GenBank data base, accession no. U16162).

Nucleotide and Derived Amino Acid Sequence of the Mouse α(II) Subunit cDNA. The cDNA clones cover 2168 nt of the corresponding mRNA and encode a 537-aa polypeptide (Fig. 1). A putative signal peptide is present at the N terminus of the deduced polypeptide, the most likely first amino acid of the mature α(II) subunit being tryptophan, based on the computational parameters of von Hejne (25), which means that the size of the signal sequence would be 19 aa and that of the processed α(II) subunit 518 aa. The molecular weight of the processed polypeptide is 59,000. The cDNA clones also cover 150 bp and 407 bp of the 5′ and 3′ untranslated sequences, respectively (Fig. 1). The 3′ untranslated sequence contains a canonical polyadenylylation signal, which is accompanied 12 nt downstream by a poly(A) tail of 15 nt.

Comparison of the Amino Acid Sequence of the Mouse α(II) Subunit with Those of the Mouse α(I) and *C. elegans* α Subunits. The mouse α(II) and mouse α(I) polypeptides are of similar sizes, α(II) being 518 and α(I) 517 aa, if we assume that the α(II) polypeptide begins with a tryptophan residue and α(I) with a histidine residue, as does the human α(I) polypeptide (Fig. 2). The processed human α(I) subunit contains 517 aa (12) and the chick α(I) subunit 516 aa (13), whereas the processed *C. elegans* α subunit is longer, 542 aa (14), the difference being mainly due to a 32-aa extension present in the C terminus of the polypeptide (Fig. 2).

The mouse α(II) and α(I) subunits contain two potential attachment sites for asparagine-linked oligosaccharides; the positions of the -Asn-Leu-Ser- and -Asn-Glu-Thr- sequences of the α(II) subunit are indicated in Fig. 1. The positions of the five cysteine residues present in the human, mouse, and chicken α(I) subunits and the *C. elegans* α subunit are all conserved in the α(II) subunit, but the latter contains an additional cysteine between the fourth and fifth cysteines of the α(I) subunits (Figs. 1 and 2). Interestingly, this is located at a site where the conserved stretch of amino acids is also interrupted in the mouse α(I) and *C. elegans* α subunits (Fig. 2).

The overall amino acid sequence identity and similarity between the mouse α(II) and mouse α(I) subunits are 63% and 83%, respectively, and those between the mouse α(II) and *C. elegans* α subunits are 41% and 67%, respectively, which are almost the same as between the mouse α(I) and *C. elegans* α

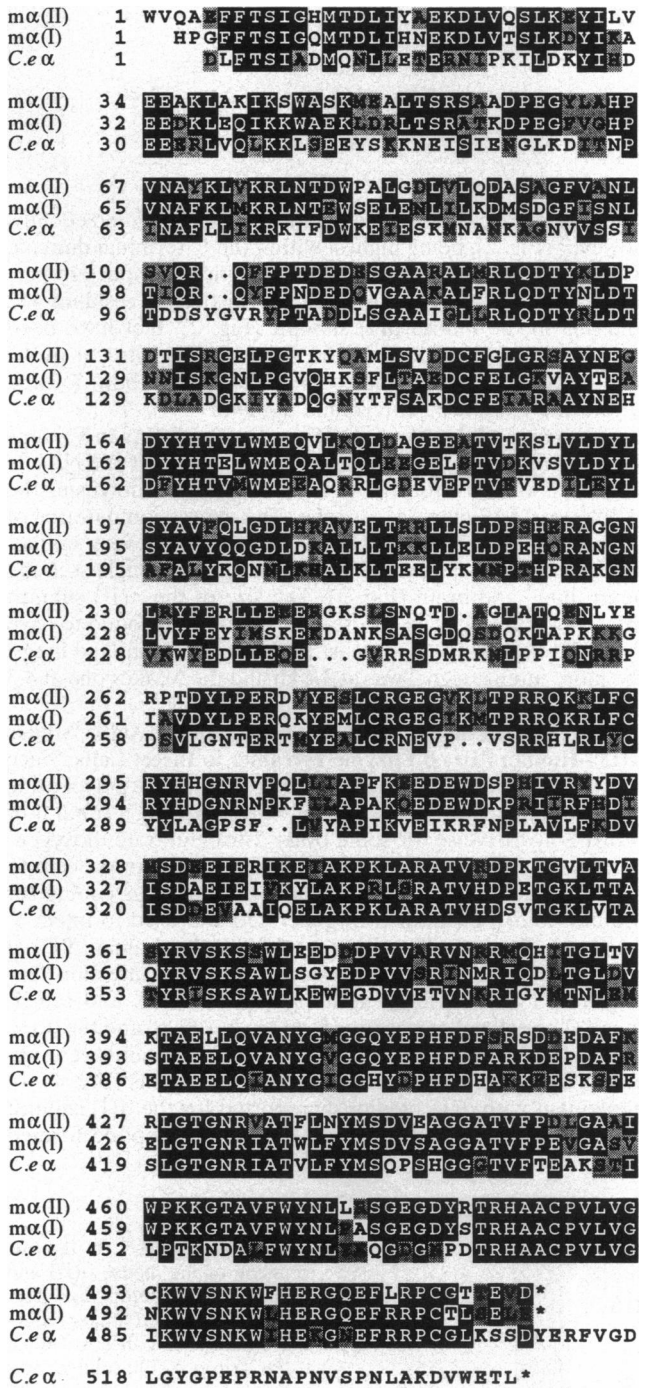

FIG. 2. Alignment of the amino acid residues of the mouse α(II) [mα(II)], mouse α(I) [mα(I)], and *C. elegans* α (*C.e*α) subunits of prolyl 4-hydroxylase. *, Stop codon of translation. Gaps (·) were introduced for maximal alignment of the polypeptides. White letters on a black background indicate identity and black letters on a gray background indicate similarity. Similar amino acids: G, A, S; A, V; V, I, L, M; I, L, M, F, Y, W; L, K, H; D, E, Q, N; S, T, E, N.

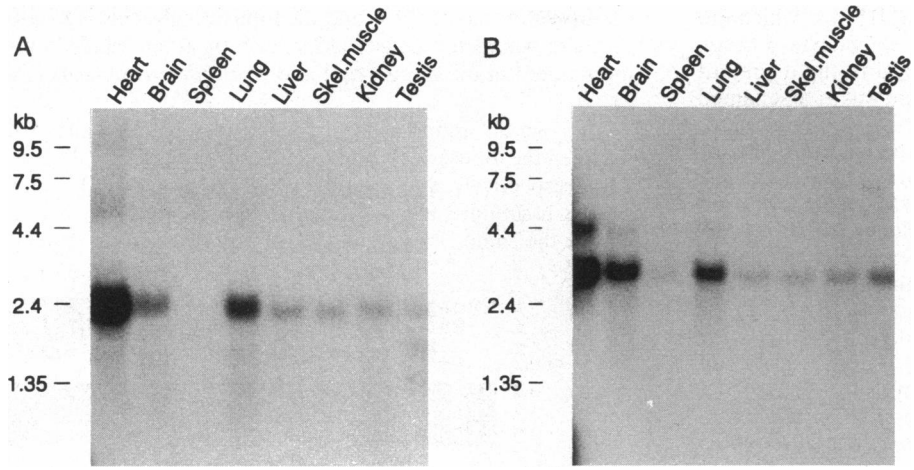

FIG. 3. Northern blot analysis of the prolyl 4-hydroxylase α(II)- and α(I)-subunit mRNAs in mouse tissues. Each lane contained 2 μg of poly(A)$^+$ RNA from the adult mouse tissue indicated. The blot was hybridized to cDNA clone BT14.1 (*A*) or MA7 (*B*). The autoradiography time was 2 days.

subunits, 43% and 67%. The identity is not distributed equally, however (Fig. 2), being highest within the C-terminal domain, which is believed to represent the catalytically important part of the α(I) subunit (14, 26). The two histidines, residues 412 and 483 in the mouse α(I) subunit (Fig. 2), that have been suggested to be involved in the Fe^{2+} binding sites of prolyl 4-hydroxylase (26) are both conserved and are both located within the conserved C-terminal domain.

Expression of the Mouse α(II) and α(I) mRNAs in Various Tissues. Expression of the two mouse α-subunit mRNAs in various tissues was studied by Northern hybridization using the BT14.1 or MA7 clone as a probe. The expression patterns of both types of α-subunit mRNA were found to be very similar, the intensities of the hybridization signals being highest in the heart, lung, and brain (Fig. 3). The size of the α(II) subunit mRNA was 2.4 kb. The mouse α(I) subunit was found to have two mRNA transcripts, at least in the heart, brain, and lungs: the more intense signal was at 3.4 kb and the weaker one at 4.3 kb.

Expression of the Mouse α(II) Subunit and an Active Mouse α(II)–Human PDI/β Enzyme Tetramer in Insect Cells. Since it was not known initially whether the α(II) polypeptide represented an α subunit of prolyl 4-hydroxylase, a subunit of prolyl 3-hydroxylase, or some other 2-oxoglutarate dioxygenase, a recombinant polypeptide was expressed in insect cells to elucidate its function. A recombinant baculovirus coding for the mouse α(II) subunit was generated and used to infect *S. frugiperda* insect cells with or without the human PDI/β subunit. The cells were harvested 72 hr after infection, homogenized in a buffer containing Triton X-100, and centrifuged. The cell pellet was solubilized in 1% SDS, and the 0.1% Triton X-100-soluble and 1% SDS-soluble proteins were analyzed by SDS/PAGE under reducing conditions (Fig. 4). In agreement with data previously reported for the α(I) subunit of prolyl 4-hydroxylase (14, 17, 27), the polypeptide formed insoluble aggregates, and efficient extraction of the recombinant mouse α(II) subunit from the cell homogenates required the use of 1% SDS (Fig. 4).

In order to study whether an association between the mouse α(II) subunit and the human PDI/β subunit could be achieved, insect cells were coinfected with two recombinant viruses coding for the two polypeptides. A hybrid protein was indeed formed and was soluble in a buffer containing 0.1% Triton X-100, as shown by PAGE performed under nondenaturing conditions (Fig. 5*A*, lane 3). The mouse α(II) subunit expressed alone did not give any extractable recombinant protein under the same conditions (Fig. 5*A*, lane 1). The mobility of the hybrid protein was identical to that of the human $\alpha(I)_2\beta_2$ enzyme tetramer (Fig. 5*A*, lane 4), termed here the type I tetramer, indicating that the hybrid protein is likely to be an $\alpha(II)_2\beta_2$ tetramer, termed the type II tetramer. No difference was found in the association of the α(II) and α(I) subunits with the PDI/β subunit into the tetramer. To show that the hybrid protein formed contains the human PDI/β subunit, Western blotting was performed with a polyclonal antibody specific for the human PDI/β subunit. When the mouse α(II) subunit was expressed together with the human PDI/β subunit, the protein complex formed could be stained with the antibody, indicating that the complex contained the PDI/β subunit (Fig. 5*B*).

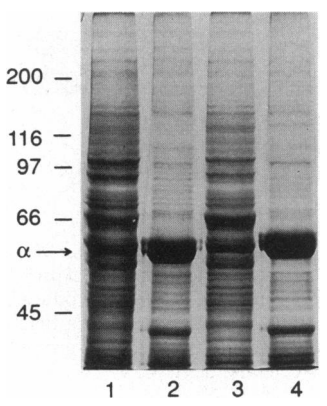

FIG. 4. Analysis of the expression of the mouse α(II) and human α(I) subunits in insect cells by SDS/PAGE under reducing conditions. Lanes 1 and 3, 0.1% Triton X-100-soluble proteins from cells infected with mouse α(II)- and human α(I)-subunit viruses, respectively; lanes 2 and 4, remaining cell pellets solubilized with 1% SDS in the same order. The gel was stained with Coomassie brilliant blue.

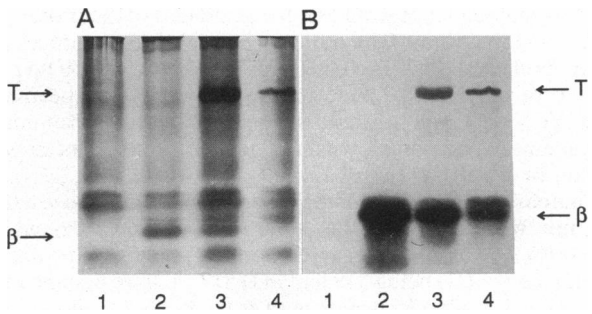

FIG. 5. Nondenaturing PAGE analysis of enzyme tetramer formation from mouse α(II) and human α(I) subunits and human PDI/β subunits expressed in insect cells. The samples were extracted with a buffer containing 0.1% Triton X-100 and electrophoresed in a 7.5% polyacrylamide gel. The samples were analyzed by Coomassie blue staining (*A*) and by Western blotting using an anti-PDI/β human antibody (*B*). Lanes 1 and 2, extracts from cells infected with the mouse α(II) virus and the human PDI/β virus, respectively; lanes 3 and 4, extracts from cells coinfected either with the mouse α(II) virus (lane 3) or human α(I) virus (lane 4) and the human PDI/β virus. Arrows T indicate the enzyme tetramers formed from the mouse α(II) and human PDI/β subunits (lanes 3) and from the human α(I) and PDI/β subunits (lanes 4). Arrows β indicate the nonassociated PDI/β subunit. As reported previously (17), considerable amounts of the PDI/β subunit always remain unassociated.

Table 1. K_m values for cosubstrates and the peptide substrate and K_i values for certain inhibitors of the human type I and mouse/human type II prolyl 4-hydroxylase tetramers

Cosubstrate, substrate, or inhibitor	Constant	K_m or K_i, μM	
		$\alpha(I)_2\beta_2$	$\alpha(II)_2\beta_2$
Fe^{2+}	K_m	4	4
2-Oxoglutatrate	K_m	22	12
Ascorbate	K_m	330	340
$(Pro\text{-}Pro\text{-}Gly)_{10}$	K_m	18	45
Poly(L-proline), M_r 7000	K_i	0.5	300*
Poly(L-proline), M_r 44,000	K_i	0.02	30*
Pyridine-2,4-dicarboxylate	K_i	2	1

*Values determined as IC_{50}.

Activity of the Mouse–Human Type II Enzyme Tetramer. The 0.1% Triton X-100 extracts from cell homogenates containing either the mouse–human type II or the human type I enzyme were analyzed for prolyl 4-hydroxylase activity with an assay based on the hydroxylation-coupled decarboxylation of 2-oxo[1-^{14}C]glutarate (23). The activities were very similar for both (data not shown). To show that the activity of the mouse/human type II enzyme was indeed prolyl 4-hydroxylase activity, the amount of 4-hydroxyproline in a $(Pro\text{-}Pro\text{-}Gly)_{10}$ substrate was determined after the reaction. The values indicated that the type II and type I enzymes behaved very similarly (data not shown) and that the activity of the type II enzyme was indeed prolyl 4-hydroxylase activity. The K_m values for Fe^{2+}, 2-oxoglutarate, and ascorbate and the K_i value for pyridine-2,4,-dicarboxylate, which acts as a competitive inhibitor with respect to 2-oxoglutarate, were likewise highly similar for the two enzymes (Table 1). Nevertheless, they differed distinctly in that the type II enzyme was inhibited by poly(L-proline) only at very high concentrations. This finding is highly surprising, as poly(L-proline) is a well-recognized, effective competitive inhibitor of type I prolyl 4-hydroxylase from all vertebrate sources studied and as poly(L-proline) is an effective polypeptide substrate for all plant prolyl 4-hydroxylases studied (1–3). However, an early report on crude preparations of prolyl 4-hydroxylase from *Ascaris lumbricoides* did suggest that this enzyme was not inhibited by poly(L-proline) (28), and a recent study on a recombinant *C. elegans* α subunit–human PDI/β subunit prolyl 4-hydroxylase demonstrated that this enzyme was not inhibited by poly(L-proline) at concentrations 60-fold higher than the K_i for the vertebrate enzyme (14). Distinct differences thus appear to exist in the structures of the peptide binding sites of various prolyl 4-hydroxylases, but no detailed data are currently available on this aspect.

Comparison of the amino acid sequence for the *C. elegans* α subunit with the mouse α(I) and α(II) subunit sequences (Fig. 2) indicated that the former has features characteristic of both types of mouse α subunit. The lack of the extra cysteine in the *C. elegans* α subunit is a feature that is especially characteristic of the α(I) subunit, whereas the lack of inhibition of the *C. elegans* enzyme by poly(L-proline) appears to be characteristic of the α(II) subunit. Preliminary Western blot experiments with antibodies to the α(II) subunit indicate that the mRNA studied here is translated to the corresponding polypeptide, and we have also found that the existence of the α(II) subunit is not limited to the mouse, as an mRNA for this subunit is expressed in human tissues (P.A., T.H., J. Veijola, T.P., and K.I.K., unpublished observations).

The finding that the type II prolyl 4-hydroxylase tetramer is only very poorly inhibited by poly(L-proline) may explain why this enzyme has remained unrecognized until now. The standard procedure for the purification of prolyl 4-hydroxylase includes as an early step an affinity chromatography on a poly(L-proline) column (29), and it is thus possible that the type II enzyme has always been discarded together with other proteins that do not bind to this column.

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