The Novel Type II Prolyl 4-Hydroxylase Is the Main Enzyme Form in Chondrocytes and Capillary Endothelial Cells, whereas the Type I Enzyme Predominates in Most Cells* 

(Received for publication, December 4, 1997, and in revised form, January 16, 1998) 

Pia Annunen‡, Helena Autio-Harmainen§, and Kari I. Kivirikko‡

From the §Collagen Research Unit, Biocenter and Department of Medical Biochemistry, and ¶Department of Pathology, University of Oulu, FIN-90220 Oulu, Finland

Procollagen-proline dioxygenase (EC 1.14.11.2), an \( \alpha_{1} \beta_{2} \) tetramer in vertebrates, plays a central role in the synthesis of all collagens. Recently an isoform of the \( \alpha \) subunit, the \( \alpha(II) \) subunit, was characterized in man and mouse and found to form a tetramer with the same \( \beta \) subunit as the previously known \( \alpha(I) \) subunit. We report here that the \( \alpha(I) \beta_{2} \) type I tetramer is the main enzyme form in most cell types and tissues and that its contribution to total prolyl 4-hydroxylase activity in cultured cells increases in confluence. Surprisingly, however, the \( \alpha(II) \beta_{2} \) type II enzyme was found to represent at least about 70% of the total prolyl 4-hydroxylase activity in cultured mouse chondrocytes and about 80% in mouse cartilage, the corresponding percentage in mouse bone being about 45% and that in many other mouse tissues about 10% or less. Immunofluorescence studies on samples from a fetal human foot confirmed these data and additionally indicated that the type II enzyme represents the main or only enzyme form in capillary endothelial cells. Thus the type II prolyl 4-hydroxylase is likely to play a major role in the development of cartilages and cartilaginous bones and also of capillaries.

Procollagen-proline dioxygenase (EC 1.14.11.2) plays a central role in the synthesis of all collagens, as the 4-hydroxyproline residues formed in its reaction are essential for the formation of the collagen triple helix at body temperature. The line residues formed in its reaction are essential for the formation of the collagen triple helix. The enzyme requires Fe\(^{2+}\), 2-oxoglutarate, \( \text{O}_{2} \), and ascorbate and acts on proline residues in -Xaa-Pro-Gly- sequences. The vertebrate enzyme is an \( \alpha_{1} \beta_{2} \) tetramer in which sequences contributing to the two catalytic sites are located in the \( \alpha \) subunit, and the \( \beta \) subunit is identical to protein disulfide-isomerase (EC 5.3.4.1) (for reviews, see Refs. 1–3).

Prolyl 4-hydroxylase had long been assumed to be of one type only, but an isoform of the \( \alpha \) subunit termed the \( \alpha(II) \) subunit, has recently been cloned and characterized from mouse (4) and human (5) tissues. This \( \alpha \) subunit was found to form a \( \alpha(II)\beta_{2} \) tetramer, the type II enzyme, with the protein disulfide-isomerase polypeptide (4, 5). The previously known \( \alpha \) subunit (6) and enzyme form are now correspondingly called the \( \alpha(I) \) subunit and the type I enzyme (4). Data on coexpression in insect cells strongly argue against the existence of a mixed \( \alpha(I)\alpha(II)\beta_{2} \) tetramer (5). The properties of the type II enzyme are very similar to those of the type I enzyme, with the distinct difference that it is inhibited by poly-L-proline only at very high concentrations and does not become bound to poly-L-proline affinity columns (4, 5).

The type II enzyme was recently shown to represent about 30% of the total prolyl 4-hydroxylase activity in cultured human WI-38 lung fibroblasts and HT-1080 fibrosarcoma cells (5). No other data are currently available on the contribution of the two enzyme forms to the total prolyl 4-hydroxylase activity in various cells. We report here that the type I enzyme is the main form in most cell types and tissues and that its proportion of the total prolyl 4-hydroxylase activity in cultured cells increases in confluence. Surprisingly, however, the type II prolyl 4-hydroxylase was found to be the main enzyme form in cultured chondrocytes and in cartilage and also in capillary endothelial cells.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures**—The cell lines used here were fetal human skin fibroblasts (ATCC CRL-1475), adult human skin fibroblasts (ATCC CRL-1987), human embryonic lung fibroblasts (WI-38, ATCC CCL-75), simian virus 40-transformed WI-38 cells (V13/WI-38, ATCC CCL 75.1), human embryonal rhabdomyosarcoma cells (RD, ATCC CCL 136), mouse embryonal fibroblasts (3T3, ATCC CCL-92), and mouse chondrocytes, which were obtained from the heads of the ribs of 7-day-old mice. The rib cartilage was minced with a knife and digested with collagenase in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% newborn calf serum (Life Technologies, Inc.) and 50 \( \mu \)g of ascorbic acid/ml at 37 °C. The chondrocytes were used in the second passage.

**Measurement of Type I and Type II Prolyl 4-Hydroxylase Activity in Cells and Mouse Tissues**—The cells of logarithmic phase or confluent cultures were harvested and washed with PBS, pH 7.4. Cells from 1–10 10-cm plates (diameter) were pooled, homogenized in a solution of 0.1 M glycine, 0.2 M NaCl, 50 \( \mu \)M dithiothreitol, 0.1% Triton X-100, 0.01% soybean trypsin inhibitor, and 20 \( \mu \)g Tris-HCl, pH adjusted to 7.5 at 4 °C, and centrifuged at 10,000 × g for 30 min. Kidney, heart, liver, skeletal muscle, skin, bone (femur and tibia), and rib cartilage tissues from 1–2-month-old mice were homogenized and centrifuged as above. Tissue from one to three mice (depending on the tissue) was pooled to form one sample. Total prolyl 4-hydroxylase activity was measured in aliquots of the supernatants, and other aliquots were passed through small poly(L-proline) columns as described (5). The type II enzyme activity was then measured in samples of the column effluents, and the type I enzyme activity was calculated as the difference between the total activity and the type II activity after correction for dilution (5).

**Immunofluorescence Staining**—A foot specimen from an apparently healthy 17-week-old gestational male human fetus (described in Ref. 7) was available for indirect immunofluorescence studies. This tissue had been immediately frozen in liquid nitrogen and stored at −70 °C. Sam-
Type II Prolyl 4-Hydroxylase

Results and Discussion

Contribution of the Two Isoenzymes to Total Prolyl 4-Hydroxylase Activity in Cultured Cells—It has recently been demonstrated that the recombinant human type I prolyl 4-hydroxylase tetramer present in a crude protein extract from insect cells becomes completely bound to a poly(L-proline) affinity column, whereas all the type II enzyme is found in the column effluent (5). This allowed the contribution of the type II enzyme activity to total prolyl 4-hydroxylase activity in crude cell extracts to be determined in a sample of column effluent, while the type I enzyme activity could be calculated by subtracting the type II enzyme activity from the total activity determined before the column (5). The values obtained by this method for the ratios of the two isoenzymes in cultured WI-38 and HT-1080 cells were in complete agreement with those obtained for the proportions of the α(I) and α(II) subunits in extracts from these cells by Western blotting (5). In the present work the poly(L-proline) column method was used to measure the proportions of the type I and II enzyme activities in various samples.

The type I prolyl 4-hydroxylase was found to be the main enzyme form in cultured adult and fetal human skin fibroblasts, human WI-38 lung fibroblasts, mouse 3T3 cells, and the two malignantly transformed human cell lines, i.e. embryonal rhabdomyosarcoma cells (RD) and SV40-transformed WI-38 cells (Va-13) (Fig. 1). In all these cell types the proportion of the type I enzyme increased with higher confluent cells than in the logarithmic phase of growth, the highest proportion being about 92%, seen in confluent adult human skin fibroblasts (Fig. 1). The proportion of type I enzyme activity measured here for confluent WI-38 cells (Fig. 1) is higher than that reported previously (5), probably because the cells in the previous study were subconfluent rather than confluent.

No major difference in the ratio of type I to type II enzyme activity was found between malignantly transformed and non-transformed cells (Fig. 1), even though the levels of total prolyl 4-hydroxylase activity in the confluent RD and Va-13 cells were only about 19% and 17% of that in the confluent adult human skin fibroblasts (details not shown). Thus, the marked decrease in the amount of prolyl 4-hydroxylase activity previously reported in malignantly transformed cells (9, 10) is due to a similar decrease in the amounts of both isoenzymes.

Cultured mouse chondrocytes differed distinctly from all the other cell types studied in that the type II enzyme was their main prolyl 4-hydroxylase form, representing at least about 70% of the total enzyme activity in confluent cells (Fig. 1). The actual proportion is likely to be even higher, as chondrocytes dedifferentiate in monolayer cultures and reduce the synthesis of cartilage-specific macromolecules such as type II collagen, beginning to synthesize type I and type III collagens (11).

Contributions of the Two Isoenzymes to Total Prolyl 4-Hydroxylase Activity in Mouse Tissues—In agreement with data obtained in cultured cells, the type I prolyl 4-hydroxylase was found to be the main enzyme form in most mouse tissues studied (Fig. 2). This isoenzyme represented about 90% or more of the total prolyl 4-hydroxylase activity in the kidneys, heart, liver, skeletal muscle, and skin. Bone and cartilage differed distinctly from the other tissues studied, however, in that the type I enzyme contributed only slightly more than half of the total activity in bone and only about 20% of that in cartilage (Fig. 2).

Immunofluorescence Staining for the Two Isoenzymes in a Fetal Human Foot—The development of the bones in the foot begins by condensation of undifferentiated mesenchymal cells into tightly packed cell islands prior to enchondral bone formation. These undifferentiated mesenchymal cells became strongly stained by a monoclonal antibody to the prolyl 4-hydroxylase α(I) subunit (Fig. 3A) but showed only a very weak staining with a monoclonal antibody to the α(II) subunit (Fig. 3B). The chondrocytic cells in the center of the cell islands, corresponding to the advancement of enchondral ossification, showed an intense staining with both the α(I) and α(II) subunit antibodies (Fig. 3, C and D). The cells at the periphery of such
islands, representing cells of the developing perichondrium and synovial membrane, gave a strong staining with the α(I) subunit antibody but a distinctly weaker staining with the α(II) subunit antibody (Fig. 3, C and D). The osteoblasts of a small ossification center in the phalangeal shaft also expressed a strong immunoreaction to the α(I) subunit (Fig. 3E) and a weaker signal for the α(II) subunit (Fig. 3F).

Epidermal cells and dermal fibroblasts showed strong staining with the antibody to the α(I) subunit (Fig. 3G) but were essentially negative with the α(II) subunit antibody (Fig. 3H). In contrast, the endothelial cells of the capillaries were negative with the α(I) subunit antibody (Fig. 3H). Smooth muscle cells around the larger arteries showed a weak signal for the α(I) subunit and were negative for that of the α(II) subunit (not shown). A weak signal for the α(II) subunit was also seen in the basement membrane zone below the epidermis (Fig. 3H).

Conclusions—The type I prolyl 4-hydroxylase was found to be the main enzyme form in most cell types and tissues studied. However, although it represented at least about 90% of total enzyme activity in the kidneys, heart, liver, skeletal muscle, and skin, the data do not exclude the possibility that these tissues may also contain cell types in which the type II enzyme is the predominant prolyl 4-hydroxylase form. One such example was found here in the case of capillary endothelial cells, which gave strong immunofluorescence with the antibody to the α(I) subunit but were negative in the staining for the α(II) subunit.

The immunofluorescence studies on developing bone indicate temporal differences in the expression of the two prolyl 4-hydroxylase forms, in that the type I enzyme appears to play a central role in the earliest phases of bone morphogenesis. The type II enzyme appears to be expressed particularly in the more differentiated cell types such as chondrocytes, in which it is the predominant form, and osteoblasts, in which the type I enzyme may represent a slightly more abundant form. The immunofluorescence data for skin indicate that there are not only temporal but also other differences in the expression of the two enzyme forms, in that type I appears to be the major form in the epidermal cells and dermal fibroblasts while type II is the main or only form in the capillary endothelial cells. Further research will be needed to demonstrate whether there are other cell types that also possess the type II enzyme as their main or only form of prolyl 4-hydroxylase.

The data as a whole indicate that the type II enzyme probably plays a major role in the development of cartilages and cartilaginous bones and of capillaries. Abnormalities in these tissues may thus be associated with mutations in the gene for the α(II) subunit.

Acknowledgments—We thank Riitta Polojärvi, Jaana Träskelin, and Annikki Huhtela for their expert technical assistance and Mirka Vuoristo and Janna Saarela for help with the preparation of the mouse specimens.

REFERENCES
1. Kivirikko, K. I., Myllylä, R., and Pihlajaniemi, T. (1989) FASEB J. 3, 1609–1617
2. Kivirikko, K. I., Myllylä, R., and Pihlajaniemi, T. (1992) in Post-Translational Modifications of Proteins (Harding, J. J., and Crabbe, M. J. C., eds) pp. 357–368
3. Kivirikko, K. I., and Myllyharju, J. (1998) Matrix Biol. 16, 357–368
4. Helaakoski, T., Annunen, P., MacNeil, I. A., Vuori, K., Pihlajaniemi, T., and Kivirikko, K. I. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 4427–4431
Type II Prolyl 4-Hydroxylase

5. Annunen, P., Helaakoski, T., Myllyharju, J., Veijola, J., Pihlajaniemi, T., and Kivirikko, K. I. (1997) J. Biol. Chem. 272, 17342–17348
6. Helaakoski, T., Vuori, K., Myllyla, R., Kivirikko, K. I., and Pihlajaniemi, T. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 4392–4396
7. Hagg, P. M., Hagg, P. O., Peltonen, S., Autio-Harjanne, H., and Pihlajaniemi, T. (1997) Am. J. Pathol. 138, 415–420
8. Kivirikko, K. I., and Myllyla, R. (1982) Methods Enzymol. 82, 245–304
9. Myllyla, R., Alitalo, K., Vaheri, A., and Kivirikko, K. I. (1981) Biochem. J. 196, 683–692
10. Myllyla, R., Keiru, J., Pihlajaniemi, T., and Kivirikko, K. I. (1983) Eur. J. Biochem. 134, 7–11
11. Benya, P. D., and Brown, P. D. (1986) in Articular Cartilage Biochemistry (Kuettner, K., and Schleyerbach, R., eds) pp. 219–233, Raven Press, New York
The Novel Type II Prolyl 4-Hydroxylase Is the Main Enzyme Form in Chondrocytes and Capillary Endothelial Cells, whereas the Type I Enzyme Predominates in Most Cells

Pia Annunen, Helena Autio-Harmainen and Kari I. Kivirikko

*J. Biol. Chem.* 1998, 273:5989-5992.

doi: 10.1074/jbc.273.11.5989

Access the most updated version of this article at [http://www.jbc.org/content/273/11/5989](http://www.jbc.org/content/273/11/5989)

Alerts:
- When this article is cited
- When a correction for this article is posted

[Click here](http://www.jbc.org/content/273/11/5989.full.html#ref-list-1) to choose from all of JBC's e-mail alerts

This article cites 9 references, 3 of which can be accessed free at [http://www.jbc.org/content/273/11/5989.full.html#ref-list-1](http://www.jbc.org/content/273/11/5989.full.html#ref-list-1)