Regulation of proline 3-hydroxylation and prolyl 3-hydroxylase and 4-hydroxylase activities in transformed cells

Kari MAJAMAA,* Raili MYLLYLÄ,* Kari ALITALO† and Antti VAHERI†

*Department of Medical Biochemistry, University of Oulu, Oulu, Finland, and †Department of Virology, University of Helsinki, Helsinki, Finland

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Prolyl 3-hydroxylase activity and the extent of collagen proline 3-hydroxylation were studied in six transformed and three control human cell lines. In the transformed cell lines, the enzyme activity was markedly high in two, similar to that in control cells in two and significantly low in two. The extent of proline 3-hydroxylation was markedly high in cell lines with high enzyme activity, but it was also significantly high in some transformed cell lines with enzyme activities similar to those in the controls. The results thus suggest that, in addition to the amount of enzyme activity present, the rate of collagen synthesis also affects the extent of proline 3-hydroxylation in the newly synthesized collagen. The effect of acute cell transformation on prolyl 3-hydroxylase and 4-hydroxylase activities was studied by infecting chick-embryo fibroblasts with Rous sarcoma virus mutant NY68, temperature-sensitive for transformation. At the permissive temperature prolyl 3-hydroxylase activity showed a more rapid increase and decrease than did prolyl 4-hydroxylase activity, the maximal activity for both enzymes being about 2.5 times that in the control chick fibroblasts. When the transformed cells were shifted to the non-permissive temperature the decays in the elevated enzyme activities were similar, suggesting identical half-lives.

Prolyl 3-hydroxylase (EC 1.14.11.7) and prolyl 4-hydroxylase (EC 1.14.11.2) catalyse the post-translational modification of prolyl residues in the biosynthesis of collagen. The genetically different collagen types show relatively small but distinct differences in the extent of their proline 4-hydroxylation, but show marked variation in the extent of their proline 3-hydroxylation. An additional variation is found within the same collagen type from different tissues and even from the same tissue in many physiological and pathological states. Factors involved in the regulation of the extent of the post-translational modifications in collagen synthesis probably include the rate of polypeptide synthesis, the amount of active enzymes required in the modification reactions, the amounts of co-substrates and cofactors and the rate of triple-helix formation (for references, see Prockop *et al.*, 1976; Kivirikko & Myllylä, 1979, 1980, 1982a).

Malignantly transformed cells provide a good tool for studying the effect of the rate of procollagen synthesis on the extent of the polypeptide-chain modifications and on the regulation of the enzyme activities. Procollagen synthesis is clearly decreased in such cells (Arbogast *et al.*, 1977; Hata &

Peterkofsky, 1977; Kamine & Rubin, 1977), whereas the four intracellular enzyme activities of collagen biosynthesis so far studied show quite variable behaviour, only prolyl 4-hydroxylase activity being constantly decreased (Myllylä *et al.*, 1981). In freshly transformed chick-embryo cells, however, the activity of prolyl 4-hydroxylase paradoxically increases by 100% while procollagen synthesis decreases by 85% (Myllylä *et al.*, 1981). No data are currently available on the regulation of prolyl 3-hydroxylase activity in transformed cells.

In the present study prolyl 3-hydroxylase activity and the extent of collagen proline 3-hydroxylation were assayed in a number of transformed cell lines. The objective was to gain information on both the effect of enzyme activity and the effect of the rate of procollagen synthesis on the extent of proline 3-hydroxylation. In addition, the prolyl 3-hydroxylase and 4-hydroxylase activities were assayed in chick-embryo cells transformed by a temperature-sensitive mutant of the Rous sarcoma virus in order to study the regulation of prolyl 3-hydroxylase activity under conditions of acute cell transformation and to study further the paradoxical behaviour of prolyl 4-hydroxylase activity in this situation.

Experimental

Human embryonic and adult skin fibroblasts were established locally, and embryonic lung fibroblasts (WI-38, A.T.C.C. CCL 75) were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). The human sarcoma cell lines were simian-virus-40-transformed WI-38 cells (Va-13/WI-38; A.T.C.C. CCL 75.1), MG-63 osteosarcoma cells (A.T.C.C. CRL 1427), RD embryonal (A.T.C.C. CCL 136) and A-204 adult rhabdomyosarcoma cells, 8387 fibrosarcoma cells and A3048 leiomyosarcoma cells (provided by Dr. J. E. DeLarco and Dr. G. J. Todaro, Frederick Cancer Research Center, Frederick, MD, U.S.A.). The cells were grown as described previously (Myllylä *et al.*, 1981). All these cells synthesize mainly types I and III collagens, except RD cells, which synthesized mainly type III, and A-204 cells, mainly type V collagen (Krieg *et al.*, 1979; Alitalo, 1980; Alitalo *et al.*, 1981). Tertiary cultures of chick-embryo fibroblasts were infected with a T-class mutant NY68 (Kawai & Hanafusa, 1971) of Rous sarcoma virus (RSV), Schmidt–Ruppin strain, subgroup A (Vaheri *et al.*, 1978).

The cell cultures were washed three times with proline-free medium before labelling for 24 h with 5 μ Ci of [14 C]proline/ml. The labelling was stopped by adding a solution containing unlabelled proline and proteinase inhibitors, and the cells plus medium were exhaustively dialysed against water (Myllylä *et al.*, 1981). The dialysed material was then digested with 13 μ g of highly purified bacterial collagenase (type VI from *Clostridium histolyticum*; Sigma Chemical Co., St. Louis, MO, U.S.A.)/ml at 37°C for 3 h (see Myllylä *et al.*, 1981). For the assay of 3-hydroxy[14 C]proline, 4-hydroxy[14 C]proline and [14 C]proline the samples were hydrolysed in 6 M-HCl at 120°C for 16 h, and the amino acids were separated in an amino acid analyser and counted for radioactivity (Risteli *et al.*, 1977).

For the enzyme assays, the cells (5.0×10^6) were homogenized with a Teflon/glass homogenizer (1200 rev./min, 50 strokes) in 0.5 ml of a cold solution containing 0.2 M-NaCl, 0.1 M-glycine, 1 mM- β -mercaptoethanol, 0.01% soya-bean trypsin inhibitor, 0.1% Triton X-100 and 0.05 M-Tris/HCl buffer adjusted to pH 7.5 at 4°C, and samples of the supernatants were used for the assays. Prolyl 3-hydroxylase activity was assayed by using a method based on the release of $^3\text{H}_2\text{O}$ during 3-hydroxylation of a [2,3- ^3H]proline-labelled biologically prepared polypeptide substrate in which all the prolyl residues recognized by prolyl 4-hydroxylase had been converted into 4-hydroxyprolyl residues (Risteli *et al.*, 1978a). Prolyl 4-hydroxylase activity was assayed by measuring the formation of 4-hydroxyproline in an unhydroxylated procollagen

substrate (see Kivirikko & Myllylä, 1982b). The extractable cell protein was assayed by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

The statistical significances of the differences between two means were calculated by Student's *t* test.

Results

Prolyl 3-hydroxylase activity and the extent of collagen proline 3-hydroxylation in human sarcoma cells

Prolyl 3-hydroxylase activity was assayed in three control and in six transformed cell lines (Table 1). The activities are given per mg of soluble cell protein, there being only minor differences between the extractable cell protein of the control and transformed cells.

The prolyl 3-hydroxylase activity was markedly high in the MG-63 osteosarcoma cells and in the RD cells, the embryonal origin of rhabdomyosarcoma cells. Simian-virus-40-transformed WI-38 cells and A-204 cells, the adult origin of rhabdomyosarcoma cells, contained a similar amount of prolyl 3-hydroxylase activity to that in the control cell lines. Significantly low enzyme activities were found only in the fibrosarcoma (8387) and in the leiomyosarcoma (A3048) cell line.

An attempt was made to discover whether the various cell samples could release $^3\text{H}_2\text{O}$ in the enzyme assay from the substrate artificially. The residue after distillation of an ordinary enzyme assay obtained with different cell samples was therefore subjected to amino acid analysis. A good correlation ($r = 0.985$) was found between the $^3\text{H}_2\text{O}$ released and the 3-hydroxy[^3H]proline/total hydroxy[^3H]proline ratio (Fig. 1).

The extent of collagen proline 3-hydroxylation was studied by incubating the cells with [14 C]proline as described in the Experimental section. The collagenase-digestible fraction of non-dialysed radioactivity in the human sarcoma cells was about 10% or even less of the value for the adult skin fibroblasts (Table 1). A markedly high proline 3-hydroxylation value was found in the human sarcoma cells (Table 1), the highest extent of proline 3-hydroxylation being in the MG-63 and RD cell lines. The WI-38 control cells had a slightly higher degree of proline 3-hydroxylation than the adult skin fibroblasts, an observation which agrees with previous results concerning the other modifications of collagen (Myllylä *et al.*, 1981). Proline 3-hydroxylation in the simian-virus-40-transformed WI-38 cells (Va-13/WI-38) was 2–3 times that in the untransformed WI-38 cells even though the enzyme activity was not high.

Table 1. *Prolyl 3-hydroxylase activity, relative collagen synthesis and the extent of proline 3-hydroxylation in human sarcoma cells*

Prolyl 3-hydroxylase activity is expressed as d.p.m./mg of protein extractable in the assay described in the Experimental section. The numbers of samples are indicated in parentheses. Relative collagen synthesis was calculated in terms of collagenase-sensitive [^{14}C]proline-labelled protein as a percentage of total [^{14}C]proline-labelled protein. The extent of proline 3-hydroxylation is expressed as 3-hydroxy[^{14}C]proline/total hydroxy[^{14}C]proline calculated from the collagenase-sensitive protein. The values are means $\pm$ S.D. for three samples. Statistical significances were calculated versus the values for adult skin fibroblasts (first superscript) and for WI-38 cells (second superscript): ^a $P < 0.001$, ^b $P < 0.01$, ^c $P < 0.05$, ⁿnot significant ($P > 0.05$). Abbreviation: n.d., not determined.

Cell line		Prolyl 3-hydroxylase activity (d.p.m./mg of protein)	Collagenase-sensitive [^{14}C]proline (%)	$10^2 \times$ 3-Hydroxy[^{14}C]proline/total hydroxy[^{14}C]proline
Controls				
Adult skin fibroblasts	(8)	2370 ± 840	39.6 ± 5.2	1.7 ± 0.2
Embryonic lung fibroblasts (WI-38)	(3)	$3390 \pm 990^{\text{n}}$	$20.8 \pm 1.5^{\text{b}}$	$2.4 \pm 0.4^{\text{b}}$
Embryonic skin fibroblasts	(7)	$3280 \pm 1060^{\text{n}, \text{n}}$	n.d.	n.d.
Sarcoma cells				
Va-13/WI-38	(4)	$2930 \pm 790^{\text{n}, \text{n}}$	$3.4 \pm 0.6^{\text{a}, \text{a}}$	$6.0 \pm 1.4^{\text{c}, \text{c}}$
RD	(4)	$6500 \pm 680^{\text{b}, \text{b}}$	$5.2 \pm 0.6^{\text{a}, \text{a}}$	$7.0 \pm 0.8^{\text{b}, \text{b}}$
A-204	(4)	$2330 \pm 410^{\text{n}, \text{c}}$	$2.3 \pm 0.3^{\text{a}, \text{a}}$	$2.0 \pm 1.0^{\text{n}, \text{n}}$
MG-63	(4)	$12830 \pm 3900^{\text{c}, \text{c}}$	$5.7 \pm 0.4^{\text{b}, \text{b}}$	$8.7 \pm 0.6^{\text{a}, \text{a}}$
A3048	(10)	$1510 \pm 640^{\text{b}, \text{a}}$	n.d.	n.d.
8387	(10)	$1660 \pm 240^{\text{a}, \text{a}}$	n.d.	n.d.

Prolyl 3- and 4-hydroxylase activities in chick-embryo fibroblasts after transformation with a temperature-sensitive mutant of the Rous sarcoma virus

Chick-embryo fibroblasts infected with the temperature-sensitive Rous sarcoma virus NY68 were shifted to the higher (41°C, non-permissive) or lower (35°C, permissive) temperature so that both the effect of an appearing cell transformation on the enzyme activities and that of a disappearing transformation could be studied. Prolyl 3-hydroxylase activity increased rapidly in the transformed cells to 2.5-fold (Fig. 2a), thereafter decreasing linearly with time, and the control cell value was reached 96 h after temperature shift. Prolyl 4-hydroxylase activity increased more slowly and the maximal ratio of 2.4 was obtained 72 h after transformation. After this point prolyl 4-hydroxylase activity declined, although it was still quite high at 96 h (Fig. 2a).

When infected cells were shifted from a permissive to a non-permissive temperature, the prolyl 3-hydroxylase and 4-hydroxylase activities behaved quite similarly (Fig. 2b), remaining high, 1.8–1.9 times the control values, during the first 24 h, and then starting to decline. Activities equal to the control values were obtained 72 h after the shift to the non-permissive temperature, the decline being similar for both enzyme activities.

Discussion

The pattern of prolyl 3-hydroxylase activity did not resemble those of the four other intracellular enzymes of collagen biosynthesis. The activity was

Fig. 1. *Correlation between prolyl 3-hydroxylase activity assayed by the release of $^3\text{H}_2\text{O}$ and proline 3-hydroxylation of the substrate*

Cell samples [●, WI-38; □, A-204; ■, HT-1080 (A.T.C.C. CCL 121) and ○, adult skin fibroblasts] were homogenized and assayed for prolyl 3-hydroxylase activity as described in the Experimental section. After distillation of the $^3\text{H}_2\text{O}$, the substrate residue was suspended in 6 M-HCl, hydrolysed and analysed for radioactive 3-hydroxyproline, 4-hydroxyproline and proline. The abscissa shows the $^3\text{H}_2\text{O}$ released in the prolyl 3-hydroxylase assay and the ordinate shows the ratio of 3-hydroxy[^3H]proline to total hydroxy[^3H]proline in the substrate. The line was fitted by linear regression analysis. △, Control substrate incubated without enzyme addition.

not significantly decreased in the simian-virus-40-transformed WI-38 cells, was high in the RD cell, and was about the same as in the control cell lines in

Fig. 2. Relative activities of prolyl 3-hydroxylase (O) and 4-hydroxylase (●) in NY68-infected chick cells at permissive and non-permissive temperatures

Cultured chick-embryo fibroblasts were infected with a temperature-sensitive mutant NY68 of the Rous sarcoma virus and the transformation was monitored microscopically. Either (a) the infected cells were maintained at 41°C and then shifted to 35°C, the temperature permissive for transformation, or (b) the cells were maintained at 35°C and then shifted to the non-permissive temperature, 41°C. Uninfected cultured chick-embryo fibroblasts were used as controls. Samples were taken for the assay of enzyme activities at the time points indicated after the shift. The results are expressed as a ratio of enzyme activity/mg of extractable cell protein in the infected cells to that in the control cells.

the A-204 cell line. The findings thus differed clearly from those found for prolyl 4-hydroxylase and also for the other collagen enzymes studied in these cells (Myllylä *et al.*, 1981). The highest prolyl 3-hydroxylase activity was found in the osteosarcoma cell line, and a significantly low enzyme activity was found only in two sarcoma cell lines.

The highest degree of proline 3-hydroxylation was found in the MG-63 and RD cells, which agrees well with the high enzyme activities. The simian-virus-40-transformed WI-38 cells also had a high extent of proline 3-hydroxylation even though the enzyme activity was not high. This suggests that the low rate

of collagen synthesis is probably the main factor affecting the extent of modification in this instance. A comparable situation has been found in the HT-1080 cell line, where the high extent of proline 3-hydroxylation seemed, at least in part, to be due to a high ratio of enzyme to substrate caused by the low rate of collagen synthesis in these cells (Pihlajaniemi *et al.*, 1981). The rhabdomyosarcoma cell line A-204 had the same amount of enzyme activity as that of adult skin fibroblasts, but, owing to the low rate of collagen synthesis, the ratio of enzyme to substrate was in the same range as in the other transformed cells. The extent of proline 3-hydroxylation was low, however, for an unknown reason.

Prolyl 4-hydroxylase activity has previously been found to be twice that in control cells 120h after transformation with the wild-type Rous sarcoma virus (Myllylä *et al.*, 1981), whereas the rate of procollagen synthesis is clearly decreased (Sandmeyer & Bornstein, 1979). The present results on the temperature-sensitive mutant of Rous sarcoma virus show that the activity is increased even at 12h after temperature shift and reaches its maximal value at 72 h. Prolyl 3-hydroxylase activity behaves differently, however, and the extrapolation of the present data suggests that it resembles the other three intracellular enzymes of collagen biosynthesis (Myllylä *et al.*, 1981). The synthesis of both enzymes seems to decrease substantially after the peak values, but, owing to the long half-lives (see below), it takes a considerable time for the enzyme to reach the new activity value found in the established transformed cells. When transformed cells were allowed to acquire a normal phenotype after shift to the non-permissive temperature, the prolyl 3-hydroxylase and 4-hydroxylase activities behaved identically, suggesting similar half-lives for the enzymes.

The two prolyl hydroxylase activities are not always regulated identically. These activities show similar changes both when cells are grown from the early- and the late-exponential phase (results not shown; Risteli *et al.*, 1979) and as a function of age in animal tissues (Tryggvason *et al.*, 1979), but they differ in liver tissue after chemically induced liver injury (Risteli *et al.*, 1978b). In the present study the two prolyl hydroxylase activities are also seen to differ not only in sarcoma cells but also in freshly transformed cells.

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