Posttranslational Modifications in the Biosynthesis of Type IV Collagen by a Human Tumor Cell Line†

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ABSTRACT: Factors responsible for the high extent of intracellular posttranslational modifications in type IV collagens were studied in a cultured human tumor cell line, HT-1080. These cells do not synthesize any detectable amounts of interstitial collagens but produce type IV collagen at a high rate, corresponding to about one-third of the production of interstitial collagens by cultured human skin fibroblasts. Prolyl 4-hydroxylase activity was lower in the HT-1080 cells than in human skin fibroblasts, there being a rough correlation between this enzyme activity and the rate of 4-hydroxyproline formation in these two cell types. The differing extents of the respective modifications could largely be explained by differences in the activities of lysyl hydroxylase and the hydroxylysyl glycosyltransferases between the two cell types. No difference was found in prolyl 3-hydroxylase activity, however, even though the extent of 3-hydroxylation of proline residues was about 6-fold in the type IV collagens. In experiments where the HT-1080 cells were studied in suspension, a lag of about 100 min was found before the secretion of type IV collagen from the cells became linear. Pulse–chase experiments in suspension indicated that all the intracellular enzyme reactions proceeded for about 40 min, presumably due to the slow triple-helix formation in type IV collagens. This slow helix formation apparently contributed to the high extent of all the intracellular modifications but was not a major factor.

Collagen biosynthesis involves a large number of enzyme-catalyzed posttranslational modifications, many of which are unique to collagens and a few other proteins with collagen-like amino acid sequences [for recent reviews, see Fessler & Fessler (1978), Prockop et al. (1979a,b), and Eyre (1980)]. The intracellular modifications of the collagen domains of the pro α chains consist of the hydroxylation of appropriate proline and lysine residues to 4-hydroxyproline, 3-hydroxyproline, and hydroxylysine and the glycosylation of certain hydroxylysine residues to galactosylhydroxylysine and glucosylgalactosylhydroxylysine. These reactions are catalyzed by five distinct enzymes [for reviews, see Kivirikko & Myllylä (1979, 1980)].

The extent of the intracellular modifications varies markedly between the genetically distinct collagen types, being highest in type IV collagens from basement membranes and lowest in type I collagen [see Kivirikko & Myllylä (1979, 1980) and Bornstein & Sage (1980)]. Additional variations are found within the same collagen type from different tissues and even the same tissue in many physiological and pathological states. Factors affecting the extent of these reactions probably include the intracellular concentrations of the active enzymes, the concentrations of the cofactors and cosubstrates required by these enzymes, the rate of procollagen synthesis, and the rate at which the newly synthesized collagenous polypeptide chains fold into the triple-helical conformation [see Prockop et al. (1979a,b) and Kivirikko & Myllylä (1979, 1980)]. The degree to which these various factors contribute to the markedly high extent of the modifications in type IV collagens is unknown, however. Enzyme activity assays carried out at the level of homogenized tissue samples do not indicate the activity values in the cells responsible for the synthesis of individual collagen types. Furthermore, it is not possible to carry out short pulse–chase experiments with radioactive amino acids in intact tissue specimens.

The cultured human tumor cell line HT-1080 synthesizes type IV procollagen as one of its major secreted proteins but does not produce any detectable amounts of interstitial collagens (Alitalo et al., 1980b). The type IV procollagen synthesized by these cells is similar to that produced by a cultured mouse tumor (EHS sarcoma) tissue (Tryggvason et al., 1980), by epithelial cells from human amniotic fluid (Crouch & Bornstein, 1979; Crouch et al., 1980) and membranes (Alitalo et al., 1980a), and by cultured rat lenses (Heathcote et al., 1980), in that it consists of two types of polypeptide chains, pro α1(IV) and pro α2(IV), with apparent molecular weights of about 180 000 and 165 000. This cell line was used here to identify factors responsible for the high extent of the posttranslational modifications in type IV collagens and to determine whether there is any conversion of the newly synthesized polypeptide chains to shorter chains during a period of 3 days in culture.

Experimental Procedures

Materials. L-[U-^{14}C]Proline (>225 Ci/mol) and L-[U-^{14}C]lysine (>270 Ci/mol) were purchased from the Radiochemical Centre and uridine diphosphate D-[U-^{14}C]glucose (229 Ci/mol) and uridine diphosphate D-[U-^{14}C]galactose (274 Ci/mol) from New England Nuclear. Nonradioactive UDP-glucose and UDP-galactose were from Sigma Chemical Co., and chromatographically purified bacterial collagenase (form III) was from Advance Biofactures Corp. HT-1080 human fibrosarcoma cells (ATCC CCL 121) were purchased from the American Type Culture Collection, and parietal yolk sac (PYS) cells were obtained from Dr. Ilmo Leivo, Department of Pathology, University of Helsinki. Dulbecco's modified Eagle's medium (DMEM), fetal calf serum, and phosphate-buffered saline (PBS) were purchased from Grand Island Biological Co. [^{14}C]Proline-labeled and [^{14}C]lysine-labeled protocollagen (unhydroxylated procollagen) substrates were prepared in freshly isolated chick-embryo tendon cells as described previously (Risteli & Kivirikko, 1976). Gelatinized calf skin collagen was prepared as described elsewhere (Myllylä et al., 1975b).

Cell Cultures and Metabolic Labeling. HT-1080 cells and locally established adult skin fibroblasts (HSF) were grown in DMEM supplemented with 10% (v/v) fetal calf serum, 50 μg/mL ascorbic acid, 100 U/mL penicillin, and 100 μg/mL streptomycin. For radioactive labeling, the cell cultures were

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washed 3 times with PBS and incubated with [^{14}C]proline or [^{14}C]lysine at the concentrations and conditions indicated. Labeling was stopped by adding a final concentration of 0.33 mg/mL unlabeled amino acid, 0.1 mg/mL cycloheximide, 1 mM α,α'-dipyridyl, 1 mM EDTA, 0.9 mM *N*-ethylmaleimide, and 0.2 mM phenylmethanesulfonyl fluoride. The cells and medium were treated as described in the legends to the corresponding tables and figures. In some experiments the HT-1080 cells were incubated in suspension. The cultured cells were harvested by trypsinization and washed twice with modified Krebs medium supplemented with 20% fetal calf serum and once with the same medium without serum. The cells were suspended (about 10^7 cells/mL) in modified Krebs medium (Dehm & Prockop, 1971, 1972) and incubated with labeled amino acid as indicated. In the pulse–chase experiments the pulse with the radioactive amino acid was stopped by adding a final concentration of 0.33 mg/mL of the corresponding unlabeled amino acid, and the incubation was continued for an additional 5–60 min as indicated. The chase was terminated by providing a final concentration of 0.1 mg/mL cycloheximide, 1 mM α,α'-dipyridyl, 1 mM EDTA, 0.9 mM *N*-ethylmaleimide, and 0.2 mM phenylmethanesulfonyl fluoride.

Enzyme Assays. The cells were grown to subconfluency, harvested by trypsinization, and stored in the form of a pellet at −70 °C for up to 1 month (Myllylä et al., 1981). After thawing, they were homogenized with a Teflon–glass homogenizer (1200 rpm; 50 strokes) in a cold solution containing 0.2 M NaCl, 0.1 M glycine, 0.1% (w/v) Triton X-100, 0.01% (w/v) soybean trypsin inhibitor, and 0.02 M Tris-HCl buffer, pH adjusted to 7.5 at 4 °C [(50–70) × 10^6 cells/mL]. The homogenates were centrifuged at 15000*g* for 30 min at 4 °C, and aliquots of the supernatants were taken for the assays.

Prolyl 4-hydroxylase activity was assayed by measuring the formation of radioactive 4-hydroxyproline in a [^{14}C]proline-labeled type I protocollagen substrate [see Tuderman et al. (1975)] and lysyl hydroxylase activity from the formation of radioactive hydroxylysine in a [^{14}C]lysine-labeled type I protocollagen substrate (Kivirikko & Prockop, 1972). Prolyl 3-hydroxylase activity was assayed by measuring the tritiated water formed during hydroxylation of [2,3-^{3}H]proline in a fully 4-hydroxylated type I protocollagen substrate (Risteli et al., 1978). Hydroxylysyl galactosyltransferase and galactosylhydroxylysyl glucosyltransferase activities were assayed by determining the radioactive galactosylhydroxylysine and glucosylgalactosylhydroxylysine formed in a gelatinized calf skin collagen substrate (Myllylä et al., 1975a, 1976). The UDP-glycoside concentrations used were 37 μM UDP-galactose (33.9 Ci/mol) in the galactosyltransferase assay and 67 μM UDP-glucose (19.3 Ci/mol) in the glucosyltransferase assay.

Measurement of Radioactive Amino Acids. Radioactive hydroxyproline was determined in protein secreted by HT-1080 cells by the procedure of Juva & Prockop (1966). The peptides prepared by collagenase digestion were assayed either for [^{14}C]lysine, hydroxy[^{14}C]lysine, glucosylgalactosylhydroxy-[^{14}C]lysine, and galactosylhydroxy[^{14}C]lysine after hydrolysis in 2 M NaOH at 105 °C for 24 h or for 3-hydroxy[^{14}C]-proline, 4-hydroxy[^{14}C]proline, and [^{14}C]proline after hydrolysis in 6 M HCl at 120 °C for 16 h. Further purification of the products, separation of the products in an amino acid analyzer, and radioactivity assays were carried out as described previously (Oikarinen et al., 1976; Risteli et al., 1977).

Measurement of the Specific Activity of Free Intracellular [^{14}C]Proline. This was carried out as described earlier (Breul et al., 1980). The labeled cells were cooled on ice, rinsed 8 times with PBS, and harvested by suspension in 5 mL of 1% picric acid. The proteins were allowed to precipitate overnight at 4 °C, the precipitate was removed by centrifugation (1000*g*; 10 min), and the supernatant containing the free intracellular proline pool was passed through a small column of Dowex 1-X8 (100–200 mesh) to remove the picric acid. The column was washed with 5 mL of H_2O, and the combined eluates were lyophilized. The amount of proline and its radioactivity were determined in an amino acid analyzer connected to a fraction collector.

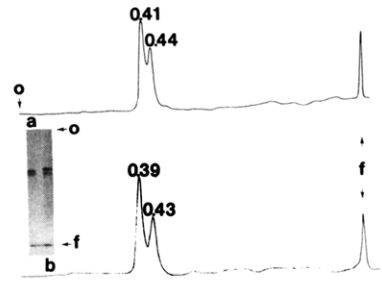

FIGURE 1: NaDoDSO$_4$–polyacrylamide gel electrophoresis of radioactively labeled proteins precipitated by specific antibodies to type IV collagen from the culture medium of HT-1080 (a) and mouse parietal yolk sac cells (b) incubated with [^{14}C]proline. The immunoprecipitation was carried out in the presence of 1 mM phenylmethanesulfonyl fluoride as described under Experimental Procedures. The origins (o), dye fronts (f), and relative mobilities of the two protein bands (in the densitometric tracings) are indicated.

Other Assays. Collagenase digestion was carried out at 37 °C for 3 h with 1.5 units of highly purified bacterial collagenase/10^6 cells [see Myllylä et al. (1981)].

In some experiments the protein produced by the HT-1080 cells, EHS mouse sarcoma tissue, or mouse parietal yolk sac cells was immunoprecipitated from the labeled culture medium in the presence of 1 mM phenylmethanesulfonyl fluoride by adding 10–20 μg of specific antibodies to type IV collagen (Alitalo et al., 1980b). After incubation for 1 h at room temperature, carrier rabbit serum and antirabbit IgG serum were added to precipitate the antibodies during an overnight incubation at 4 °C. The precipitates were collected by centrifugation, washed several times, and dissolved in the electrophoresis buffer before slab gel electrophoresis.

Polyacrylamide slab gel electrophoresis was performed with a Tris–glycine buffer system containing NaDodSO$_4$[1] as described by Laemmli (1970). Samples were run on 5% gels after reduction with dithiothreitol. Fluorography of the gels was performed as described by Bonner & Laskey (1974).

Results

Rate of Collagen Production by Cultured HT-1080 Cells. As reported previously (Alitalo et al., 1980b), the cultured HT-1080 cells did not produce any detectable amounts of the interstitial collagens. The two collagenous polypeptide chains, pro α1(IV) and pro α2(IV), showed mobilities in NaDodSO$_4$–polyacrylamide gel electrophoresis very similar to, but not identical with, the two pro α chains of type IV collagen synthesized by cultured mouse parietal yolk sac cells (Figure 1) or organ cultures of the EHS sarcoma (not shown).

The rate of collagen production was quantified as recently described for cultured human lung fibroblasts (Breul et al., 1980), based on measurement of the specific activity of the free intracellular labeled amino acid precursor, the amount of hydroxy[^{14}C]proline synthesized, and the extent of 4-

[1] Abbreviation used: NaDodSO$_4$, sodium dodecyl sulfate.

Table I: Rate of Collagen Production in Cultured HT-1080 Cells and Human Skin Fibroblasts (HSF) [a]

cell line	[^{14}C]Pro incorpn (dpm/10^6 cells)	[^{14}C]Pro incorpn (pmol/10^3 cells)	procollagen prodn (%)	10^{-5}(no. of procollagen chains cell^{-1} h^{-1})
HSF	11 690	9.64	5.50	11.12
	13 240	10.92	5.48	12.57
HT-1080	39 500	20.78	0.66	2.95
	46 510	24.48	0.76	3.94

[a] The cells (4×10^6) were grown to subconfluency in conditions described under Experimental Procedures and labeled for 4 h with 4 μCi/mL [^{14}C]proline. Proline incorporation (picomoles per 10^3 cells) and procollagen production (number of procollagen chains per cell per hour) were quantified from the specific activity of the free intracellular proline pool, the amount of hydroxy-[^{14}C]proline synthesized, and the extent of 4-hydroxylation of proline residues in collagenase-susceptible regions of the pro α chains [see Breul et al. (1980)]. Procollagen production is also expressed as a percentage of total protein by correcting the data for the number of imino acids in collagen relative to those in noncollagen protein [see Breul et al. (1980)].

hydroxylation of proline residues in the collagenase-susceptible regions of the pro α chains. The amount of collagen produced by the HT-1080 cells was compared to that from cultured human skin fibroblasts, which synthesize only interstitial type I and III collagens [see Bornstein & Sage (1980)]. The HT-1080 cells were found to have a higher rate of [^{14}C]proline incorporation into protein, but the rate of procollagen chain production was only about 30% of that of the human skin fibroblasts (Table I).

Extent of Modification of Prolyl and Lysyl Residues. Cultured HT-1080 cells and human skin fibroblasts were incubated with [^{14}C]proline or [^{14}C]lysine for 4 h as described under Experimental Procedures, and the radioactivity of 3-hydroxy[^{14}C]proline, 4-hydroxy[^{14}C]proline, and [^{14}C]proline or hydroxy[^{14}C]lysine, galactosylhydroxy[^{14}C]lysine, glucosylgalactosylhydroxy[^{14}C]lysine, and [^{14}C]lysine was measured in the dialyzable peptides produced from the total labeled protein in the cultures (cells plus medium) by exhaustive digestion with highly purified bacterial collagenase. This procedure was used since the enzyme activities given below were assayed in the cells responsible for the synthesis of all the protein, and thus any purification procedure that might involve a selective loss of some of the labeled collagenous material would lead to an erroneous relationship between the enzyme activity and the overall extent of modification of the products formed.

The collagenous protein synthesized by the HT-1080 cells differed from that synthesized by human skin fibroblasts, especially in the extent of proline 3-hydroxylation, lysine hydroxylation, and hydroxylysine galactosylation (Table II). The extent of proline 4-hydroxylation in the collagen synthesized by the HT-1080 cells was also distinctly higher, whereas the extent of galactosylhydroxylysine glucosylation was only slightly higher, even though the amount of glucosylgalactosylhydroxylysine residues per total lysine residues was more than 10-fold. This difference is therefore mainly due to a difference in the extents of the preceding enzyme reactions.

The interstitial collagens synthesized by the cultured human skin fibroblasts (Table II) were modified more extensively than those found in adult human tissues in vivo [see Kivirikko & Myllylä (1979, 1980)]. The values are similar, however, to those previously reported for human fibroblasts in culture (Lembach et al., 1977; Schwartz et al., 1979; Myllylä et al., 1981).

Intracellular Enzyme Activities. The enzyme activities are expressed both per milligram of extractable cell protein and in relation to prolyl 4-hydroxylase activity, as it has been found that changes in this enzyme activity roughly parallel those in the rate of collagen synthesis in many situations [see Kivirikko & Myllylä (1980)]. No difference in the amount of extractable protein per cell was found between the HT-1080 cells and the cultured human fibroblasts, and hence the magnitudes of all the differences in enzyme activities are identical whether expressed per cell or per extractable cell protein.

The prolyl 4-hydroxylase activity in the HT-1080 cells was about 66% of that in the human skin fibroblasts (Table III). This ratio is not as low as that for the rate of procollagen chain production, about 30% (Table I). It should be noted, however, that the newly synthesized polypeptide chains of type IV collagen are about 20% larger than those of the interstitial collagens [see Bornstein & Sage (1980)] and have a higher extent of 4-hydroxylation of proline (Table II). Accordingly, the magnitude of the difference in prolyl 4-hydroxylase activity between the two cell types is roughly similar to that in the amout of 4-hydroxyproline residues synthesized.

Prolyl 3-hydroxylase activity was also lower in the HT-1080 cells than in the skin fibroblasts, there being no significant difference in the ratio of prolyl 3-hydroxylase to 4-hydroxylase activity between these two cell types (Table III). In contrast, lysyl hydroxylase activity was markedly higher in the HT-1080 cells, about 2.1-fold when expressed per unit of cell protein or about 3.1-fold when expressed per unit of prolyl 4-hydroxylase activity (Table III). Hydroxylysyl galactosyltransferase activity was also higher in the HT-1080 cells, about 1.8-fold when expressed per unit of prolyl 4-hydroxylase activity, whereas galactosylhydroxylysyl glucosyltransferase activity was slightly lower when expressed per unit of cell

Table II: Comparison of the Extents of Posttranslational Modifications in Collagens Synthesized by Subconfluent Cultures of HT-1080 Cells and Human Skin Fibroblasts (HSF) [a]

cell line	3-Hyp/ total Hyp (%)	3-Hyp/ total Pro (%)	4-Hyp/ total Pro (%)	total Hyl/ total Lys (%)	total Gal-Hyl/ total Hyl (%)	total Gal-Hyl/ total Lys (%)	Glc-Gal-Hyl/ total Gal-Hyl (%)	Glc-Gal-Hyl/ total Lys (%)
HSF	2.0	0.9	45	32	25	8.0	73	5.8
HT-1080	8.4	5.8	64	80	91	73	91	66

[a] Cultured subconfluent HT-1080 cells and human skin fibroblasts were labeled for 4 h with 4 μCi/mL [^{14}C]proline or 4 μCi/mL [^{14}C]lysine. The degrees of 3-hydroxylation and 4-hydroxylation of proline, hydroxylation of lysine, and glycosylation of hydroxylysine were calculated from the amounts of radioactivity in proline, 3-hydroxyproline, and 4-hydroxyproline or lysine, hydroxylysine, galactosylhydroxylysine, and glucosylgalactosylhydroxylysine in collagenase-sensitive protein. The term "total proline" is used for the sum of proline and the two hydroxyprolines, "total lysine" for the sum of lysine, hydroxylysine, and its glycosides, "total hydroxyproline" for the sum of the two hydroxyprolines, "total hydroxylysine" for the sum of hydroxylysine and its glycosides, and "total galactosylhydroxylysine" for the sum of galactosylhydroxylysine and glucosylgalactosylhydroxylysine.

Table III: Comparison of the Hydroxylase Activities of Subconfluent HT-1080 Cells with Those of Subconfluent Human Skin Fibroblasts (HSF) [a]

cell line	prolyl 4-hydroxylase act. 10^{-3}(dpm/mg of protein)	prolyl 3-hydroxylase act. 10^{-1}(dpm/mg of protein)	prolyl 3-hydroxylase act. 10^{2} per prolyl 4-hydroxylase	lysyl hydroxylase act. 10^{-3}(dpm/mg of protein)	lysyl hydroxylase act. per prolyl 4-hydroxylase
HSF	134 ± 6	145 ± 49	1.12 ± 0.44	47.3 ± 11.3	0.36 ± 0.10
HT-1080	89 ± 8	89 ± 27	1.00 ± 0.30	98.0 ± 21.5	1.11 ± 0.26
	$P < 0.001$	$P < 0.05$	n.s.	$P < 0.001$	$P < 0.001$

[a] Enzyme activities were assayed as described under Experimental Procedures and are expressed per milligram of extractable cell protein and in relation to prolyl 4-hydroxylase activity. The results are given as means ± SD for eight samples of each cell type in all cases, except for prolyl 3-hydroxylase (five samples).

Table IV: Comparison of Hydroxylysyl Glycosyltransferase Activities of Subconfluent HT-1080 Cells with Those of Subconfluent Human Skin Fibroblasts (HSF) [a]

cell line	galactosyltransferase act. 10^{-3}(dpm/mg of protein)	galactosyltransferase act. per prolyl 4-hydroxylase	glucosyltransferase act. 10^{-3}(dpm/mg of protein)	glucosyltransferase act. per prolyl 4-hydroxylase
HSF	19.4 ± 1.9	0.14 ± 0.02	59.2 ± 6.1	0.44 ± 0.05
HT-1080	23.5 ± 3.6	0.26 ± 0.03	47.2 ± 10.4	0.53 ± 0.12
	$P < 0.01$	$P < 0.001$	$P < 0.01$	$P < 0.05$

[a] Enzyme activities were assayed as described under Experimental Procedures and are expressed per milligram of extractable cell protein and in relation to prolyl 4-hydroxylase activity. The results are given as means ± SD for eight samples of each cell type.

protein and only slightly higher, about 1.2-fold, when expressed per unit of prolyl 4-hydroxylase (Table IV).

Secretion of Collagen by HT-1080 Cells Incubated in Suspension. Cells incubated in suspension are more suitable for short pulse–chase experiments with radioactive amino acids than those cultured on plastic dishes, since isotope uptake is more rapid and several aliquots can be taken from the same incubation at various points in time. Experiments were therefore carried out to study whether the HT-1080 cells would synthesize type IV collagen in suspension in a simple amino acid free medium, as described for freshly isolated chick-embryo tendon (Dehm & Prockop, 1971, 1972) and cartilage (Dehm & Prockop, 1973) cells.

The HT-1080 cells synthesized radioactive hydroxyproline in suspension linearily for about 4–6 h (Figure 2A), there being a lag of about 100 min before its secretion became linear (Figure 2B). This lag is similar to that reported for the secretion of type IV collagen in other cell types [see Kivirikko & Myllylä (1979)]. The percentage of collagen production was similar to that in cells incubated on plastic dishes (not shown), and the extents of modification of the proline and lysine residues were identical with those given in Table II (not shown).

Modification of Proline and Lysine Residues When Studied by Pulse–Chase Experiments in Suspension. The HT-1080 cells were first preincubated in a modified Krebs medium for 15 min, then incubated with [^{14}C]proline or [^{14}C]lysine for 5 min, and finally incubated in the presence of a large excess of unlabeled proline or lysine for an additional 5–60 min. Small samples were taken after the incubation times indicated, and at each time point the extent of modification of proline or lysine residues was assayed in dialyzable peptides produced from the total labeled protein in the cells plus medium by exhaustive digestion with highly purified bacterial collagenase.

The extent of 4-hydroxylation of the proline residues (Figure 3A) had reached about 25% of its maximal value by the end of the 5-min pulse (for maximal values, see Table II). The

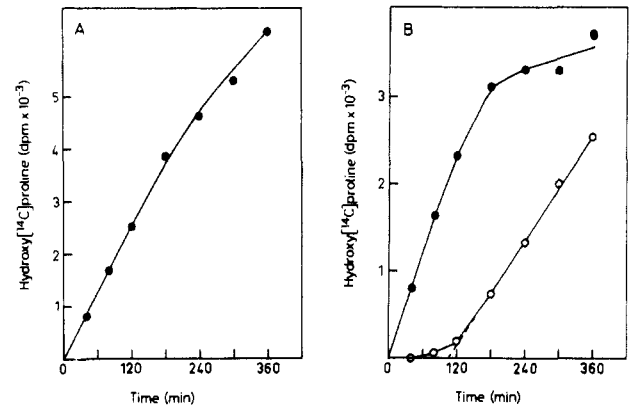

FIGURE 2: Synthesis and secretion of procollagen hydroxy[^{14}C]proline by HT-1080 cells incubated in suspension. The cells (80×10^6) were incubated in 26 mL of modified Krebs medium containing 30 μCi of [^{14}C]proline. At each time point, 2 mL of the incubation system (cells plus medium) was pipetted into test tubes containing 0.1 volume of modified Krebs medium with cycloheximide and α,α′-dipyridyl to provide a final concentration of 100 μg of cycloheximide and 1 μmol of α,α′-dipyridyl/mL. The samples were centrifuged at 1200*g* for 10 min at room temperature, and the cell pellet was washed with 1.5 mL of modified Krebs medium containing 100 μg of cycloheximide and 1 μmol of α,α′-dipyridyl/mL. The medium and cells were then dialyzed against running tap water for 3 days, and the nondiffusible hydroxy[^{14}C]proline was assayed by the procedure of Juva & Prockop (1966). (A) Total nondiffusible hydroxy[^{14}C]proline in the cells plus medium. (B) Nondiffusible hydroxy[^{14}C]proline in the cells (●) and medium (○).

reaction proceeded further during the chase period, being completed only at about 40 min. The 3-hydroxylation of proline residues proceeded at a slightly lower rate (Figure 3B), and thus the ratio of 3-hydroxyproline to total hydroxyproline increased slighlty with time.

The hydroxylation of lysine residues occurred intitially at a higher rate than that of proline residues. Lysine hydroxylation had reached about 50% of its maximal value by the end of the 5-min pulse (Figure 4A) but was completed at about the same time as the two proline hydroxylations. The galactosylation of hydroxylysine residues (Figure 4B) and glucosylation of galactosylhydroxylysine residues (Figure 4C) occurred at a slightly lower rate than lysine hydroxylation, and the ratio of galactosylhydroxylysine and glucosylgalactosylhydroxylysine to total hydroxylysine increased slightly with time.

Lack of Conversion of the Newly Synthesized Polypeptide Chains to Shorter Chains. No conversion of the newly synthesized polypeptide chains was found during continuous labeling of the HT-1080 cells in culture for 24 h (Figure 1). In additional experiments, the cells in culture were labeled by incubation with [^{14}C]proline for 6 h and then chased for up to 72 h. The mobilities of the two polypeptide chains of type IV collagen were identical at 6 and 72 h, there being no evidence for the presence of any shorter chains (not shown).

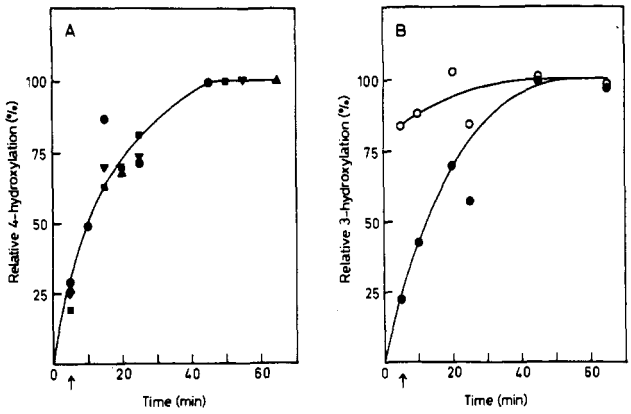

FIGURE 3: 4-Hydroxylation and 3-hydroxylation of [^{14}C]proline in HT-1080 cells when studied in a pulse–chase experiment. The cells (200 × 10^6) were suspended in 24 mL of modified Krebs medium, preincubated for 15 min, and then incubated with 200 μCi of [^{14}C]proline for 5 min. The pulse was stopped by adding 0.5 mL of modified Krebs medium containing 8.1 mg of unlabeled proline. At each time point, 4 mL of the incubation system (cells plus medium) was pipetted into tubes containing 0.4 mL of modified Krebs medium containing 1 mg of cycloheximide, 10 μmol of α,α'-dipyridyl, 10 μmol of ethylenediaminetetraacetic acid, 9 μmol of *N*-ethylmaleimide, and 2 μmol of phenylmethanesulfonyl fluoride per mL. The samples were heated for 5 min at 100 °C, dialyzed against running tap water for 3 days, digested with highly purified bacterial collagenase, and assayed for collagenase-digestible 4-hydroxy[^{14}C]proline, 3-hydroxy[^{14}C]-proline, and [^{14}C]proline. The final values of the ratios of 4-hydroxy[^{14}C]proline to total [^{14}C]proline (A), 3-hydroxy[^{14}C]proline to total [^{14}C]proline [(●) in (B)], and 3-hydroxy[^{14}C]proline to total hydroxyproline [(○) in (B)] were taken as 100% (for actual values, see Table II), and the data obtained at various time points are given in relation to these values. The terms "total proline" and "total hydroxyproline" are used as explained in the legend to Table II. Values obtained in four separate experiments are summarized in part A and indicated by different symbols (●, ■, ▲, and ▼). The arrow indicates the beginning of the chase period.

Discussion

HT-1080 cells cultured on plastic dishes or incubated in suspension were found to produce type IV collagen at quite a high rate, about one-third of the production of interstitial collagens by cultured human skin fibroblasts. The percentage of collagen production in the HT-1080 cells was much lower, however, this being due to the combined effect of a lower absolute rate of collagen production and a higher rate of synthesis of other proteins. A substantial proportion of the noncollagenous protein was retained in the cell layer of the HT-1080 cells, and hence type IV collagen formed about 3% of the protein secreted into the medium (data not shown). It seems that HT-1080 cells are highly suitable for studies on the synthesis of type IV collagens.

Comparison of the intracellular enzyme activities and the extents of modification in the collagens synthesized by these two cell types is somewhat complicated by the differences in the rates of collagen production. It has recently been demonstrated that a decrease in the rate of collagen synthesis can in itself increase the extent to which the newly synthesized pro α chains are modified (Myllylä et al., 1981). Expression of the data in terms of ratios of other enzyme activities to that of prolyl 4-hydroxylase in part corrects this difference, since changes in the latter roughly parallel changes in the rate of collagen synthesis in many cases [see Kivirikko & Myllylä (1980)]. An approximate correlation of this kind was found here between prolyl 4-hydroxylase activity and the rate of 4-hydroxyproline formation.

The high ratios of lysyl hydroxylase and hydroxylysyl galactosyltransferase activity to prolyl 4-hydroxylase activity and the moderately high corresponding ratio regarding galactosylhydroxylysyl glucosyltransferase activity in the HT-1080 cells when compared to that in the skin fibroblasts may largely explain the differences in the extents of the respective collagen modifications. It is notable, however, that the difference in galactosyltransferase activity was not as great as that in the extent of galactosylation of hydroxylysine residues. No difference in prolyl 3-hydroxylase activity was found between the two cell types, however, even though the extent of 3-hydroxylation of the proline residues was about 6-fold in type IV collagens. This finding is surprising, since a very high ratio has previously been found in rat kidney cortex when compared to rat skin (Tryggvason et al., 1979).

All the enzyme activities were measured with substrates consisting primarily of type I proto- or procollagens. If some

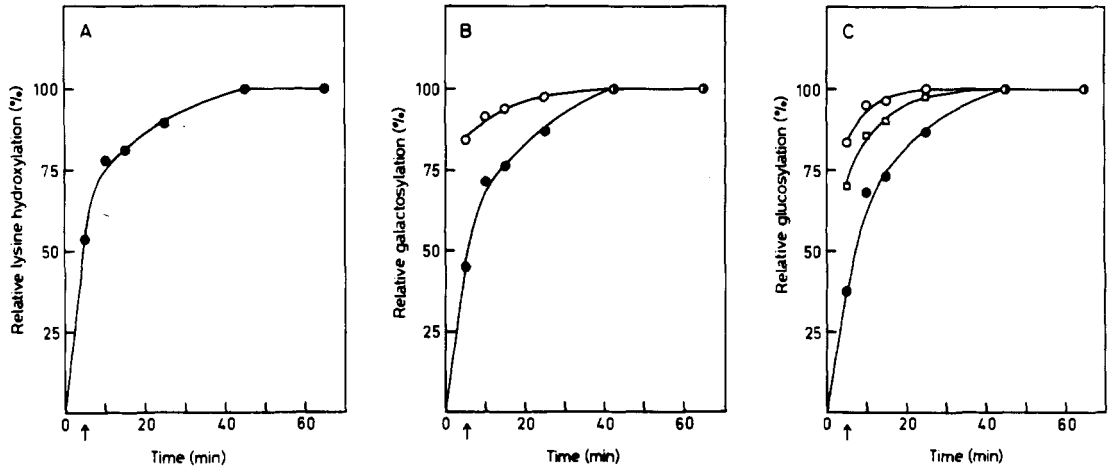

FIGURE 4: Hydroxylation of [^{14}C]lysine, galactosylation of hydroxy[^{14}C]lysine, and glucosylation of galactosylhydroxy[^{14}C]lysine in HT-1080 cells when studied in a pulse–chase experiment. The cells (200 × 10^6) were suspended in 24 mL of modified Krebs medium, preincubated for 15 min, and then incubated with 200 μCi of [^{14}C]lysine for 5 min. The pulse was stopped by adding 0.5 mL of modified Krebs medium containing 8.1 mg of unlabeled lysine. A sample was taken at each time point and treated as described in the legend to Figure 3, except that collagenase-digestible [^{14}C]lysine, hydroxy[^{14}C]lysine, galactosylhydroxy[^{14}C]lysine, and glucosylgalactosylhydroxy[^{14}C]lysine were measured. The final values of the ratios of hydroxy[^{14}C]lysine to total [^{14}C]lysine (A), galactosylhydroxy[^{14}C]lysine to total [^{14}C]lysine [(●) in (B)], galactosylhydroxy[^{14}C]lysine to total hydroxy[^{14}C]lysine [(○) in (B)], glucosylgalactosylhydroxy[^{14}C]lysine to total [^{14}C]lysine [(●) in (C)], glucosylgalactosylhydroxy[^{14}C]lysine to total hydroxy[^{14}C]lysine [(□) in (C)], and glucosylgalactosylhydroxy[^{14}C]lysine to total galactosylhydroxy[^{14}C]lysine [(○) in (C)] were taken as 100% (for actual values, see Table II), and the data obtained at various time points are given in relation to these values. The terms "total lysine", "total hydroxylysine", and "total galactosylhydroxylysine" are used as explained in the legend of Table II. The arrow indicates the beginning of the chase period.

of these enzymes have collagen type specific isozymes, then the differences between the two cell types might alter when measured with substrates of type IV structure. Nevertheless, such a situation does not seem to exist in cases of lysyl hydroxylase and galactosylhydroxylysyl glucosyltransferase at least. Studies on the immunological properties of both these enzymes (Myllylä, 1981; Turpeenniemi-Hujanen, 1981) and on the catalytic properties of lysyl hydroxylase (Turpeenniemi-Hujanen et al., 1981; U. Puistola, unpublished experiments) from various tissues and cells argue against the existence of collagen type specific isozymes. The data on lysyl hydroxylase (U. Puistola, unpublished experiments) in fact indicate that the differences in this enzyme activity between the two cell types do not alter when measured with type IV protocollagen as the substrate. It thus seems likely that the differences in these two enzyme activities, and perhaps in all the enzyme activities, between the two cell types are due to regulation of the amounts of a single type of the corresponding enzyme activity rather than the presence of collagen type specific isozymes.

The time required for triple-helix formation varies greatly, being shortest, about 10–15 min, in tendon cells synthesizing type I collagen, intermediate in cartilage cells synthesizing type II, and longest, about 1 h or more, in various cells synthesizing type IV collagens [see Kivirikko & Myllylä (1979)]. Secretion of all these collagens has been found to take place soon after helix formation [see Prockop et al. (1976)]. None of the five intracellular enzymes acts on triple-helical substrates in vitro, and it has been speculated, therefore, that one factor contributing to the differences in the extent of modification between various collagen types concerns differences in the time required for helix formation [see Kivirikko & Myllylä (1979, 1980)]. In support of this suggestion, it has been demonstrated that when triple-helix formation is experimentally accelerated in cells synthesizing interstitial collagens, the modifications in the newly synthesized protein are reduced in extent, while the opposite changes occur if triple-helix formation is prevented (Uitto & Prockop, 1974; Oikarinen et al., 1976, 1977; Uitto et al., 1978; Majamaa, 1981).

The secretion of type IV collagen by the HT-1080 cells required about 100 min, suggesting a rate of triple-helix formation similar to that reported for other cells synthesizing this collagen type. The finding that the intracellular enzyme reactions proceeded for up to about 40 min likewise suggests that helix formation did not take place before this point in time at least. The hydroxylation of lysine residues proceeded very rapidly initially, reaching 50% of the maximal value during the 5-min pulse and about 80% during an additional 10 min of chase time. Accordingly, the slow rate of triple-helix formation could not have been a major factor in the high extent of lysine hydroxylation in the type IV collagen synthesized by the HT-1080 cells. Similar conclusions can be made concerning the role of the helix formation rate in the extent of the glycosylation of the hydroxylysine residues and the 3-hydroxylation of proline residues. It is significant, however, that none of the modifications was entirely completed within 15 min of the beginning of the experiment, indicating that the slow rate of triple-helix formation did contribute to the high extent of all the modifications.

An additional factor affecting the extent of the modifications is the number of potential substrate sites in the various pro α chains. Lysyl hydroxylase, for example, acts only on lysine residues in the Y positions of the repeating -X-Y-Gly- triplets [see Kivirikko & Myllylä (1980)]. As about 80% of all the lysine residues in the collagenase sensitive parts of the pro α (IV) chains were hydroxylated, a substantially higher proportion of the lysine residues in type IV collagens must have been in the Y positions than is found in type I [for amino acid sequences of type I collagen, see Fietzek & Kühn (1976)].

It is not known whether differences in the concentrations of the cofactors, cosubstrates, and inhibitors of the five enzymes also contributed to the differences in the extents of the modifications between the two cell types studied. It is likewise not known whether the enzyme activities extracted from the cells and measured in vitro correspond exactly to those within the cisternae of the rough endoplasmic reticulum, where the enzymes might exist as multienzyme complexes and where the substrate consists in part of growing polypeptide chains. It should be noted in particular that the high extent of proline 3-hydroxylation in type IV collagen cannot be explained at all in terms of high enzyme activity and probably not entirely by a slow rate of triple-helix formation.

Previous studies on the processing of the newly synthesized polypeptide chains of type IV collagen to shorter chains have given no evidence for such a conversion in vitro (Minor et al., 1976; Heathcote et al., 1978, 1980; Crouch & Bornstein, 1979; Kefalides et al., 1979; Alitalo et al., 1980b; Tryggvason et al., 1980). The present data confirm and extend such findings, in that no conversion of the pro α(IV) chains was observed even during a chase period of 72 h in culture. It is not known, however, whether a similar situation exists in vivo, as processing of the pro α(IV) chains to shorter ones may take place at least in the EHS sarcoma of mice (K. Tryggvason, T. Pihlajaniemi, L. A. Liotta, G. R. Martin, and K. I. Kivirikko, unpublished experiments).

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References

Alitalo, K., Kurkinen, M., Vaheri, A., Krieg, T., & Timpl, R. (1980a) *Cell (Cambridge, Mass.) 19*, 1053–1062.

Alitalo, K., Vaheri, A., Krieg, T., & Timpl, R. (1980b) *Eur. J. Biochem. 109*, 247–255.

Bonner, W. M., & Laskey, A. B. (1974) *Eur. J. Biochem. 46*, 83–88.

Bornstein, P., & Sage, H. (1980) *Annu. Rev. Biochem. 49*, 957–1003.

Breul, S. D., Bradley, K. H., Hance, A. J., Schafer, J. P., Berg, R. A., & Crystal, R. G. (1980) *J. Biol. Chem. 255*, 5250–5260.

Crouch, E., & Bornstein, P. (1979) *J. Biol. Chem. 254*, 4197–4204.

Crouch, E., Sage, H., & Bornstein, P. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 745–749.

Dehm, P., & Prockop, D. J. (1971) *Biochim. Biophys. Acta 240*, 358–369.

Dehm, P., & Prockop, D. J. (1972) *Biochim. Biophys. Acta 264*, *375*–382.

Dehm, P., & Prockop, D. J. (1973) *Eur. J. Biochem. 35*, 159–166.

Eyre, D. R. (1980) *Science (Washington, D.C.) 207*, 1315–1322.

Fessler, J. H., & Fessler, L. I. (1978) *Annu. Rev. Biochem. 47*, 129–162.

Fietzek, P. P., & Kühn, K. (1976) *Int. Rev. Connect. Tissue Res. 7*, 1–60.

Heathcote, J. G., Sear, C. H. J., & Grant, M. E. (1978) *Biochem. J. 176*, 283–294.

Heathcote, J. G., Bailey, A. J., & Grant, M. E. (1980) *Biochem. J. 190*, 229–237.
Juva, K., & Prockop, D. J. (1966) *Anal. Biochem. 15*, 77–83.
Kefalides, N. A., Alper, R., & Clark, C. C. (1979) *Int. Rev. Cytol. 61*, 167–228.
Kivirikko, K. I., & Prockop, D. J. (1972) *Biochim. Biophys. Acta 258*, 366–379.
Kivirikko, K. I., & Myllylä, R. (1979) *Int. Rev. Connect. Tissue Res. 8*, 23–72.
Kivirikko, K. I., & Myllylä, R. (1980) in *The Enzymology of Post-Translational Modifications of Proteins* (Freedman, R. B., & Hawkins, H. C., Eds.) pp 53–104, Academic Press, London.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Lembach, K. J., Branson, R. E., Hewgley, P. P., & Cunningham, L. W. (1977) *Eur. J. Biochem. 72*, 379–383.
Majamaa, K. (1981) *Biochem. J. 196*, 203–206.
Minor, R. R., Clark, C. C., Strause, E. L., Koszalka, T. R., Brent, R. L., & Kefalides, N. A. (1976) *J. Biol. Chem. 251*, 1789–1794.
Myllylä, R. (1981) *Biochim. Biophys. Acta 658*, 299–307.
Myllylä, R., Risteli, L., & Kivirikko, K. I. (1975a) *Eur. J. Biochem. 52*, 401–410.
Myllylä, R., Risteli, L., & Kivirikko, K. I. (1975b) *Eur. J. Biochem. 58*, 517–521.
Myllylä, R., Risteli, L., & Kivirikko, K. I. (1976) *Eur. J. Biochem. 61*, 59–67.
Myllylä, R., Alitalo, K., Vaheri, A., & Kivirikko, K. I. (1981) *Biochem. J. 196*, 683–692.
Oikarinen, A., Anttinen, H., & Kivirikko, K. I. (1976) *Biochem. J. 156*, 545–551.
Oikarinen, A., Anttinen, H., & Kivirikko, K. I. (1977) *Biochem. J. 166*, 357–362.
Prockop, D. J., Berg, R. A., Kivirikko, K. I., & Uitto, J. (1976) in *Biochemistry of Collagen* (Ramachandran, G. N., & Reddi, A. H., Eds.) pp 163–273, Plenum Press, New York.
Prockop, D. J., Kivirikko, K. I., Tuderman, L., & Guzman, N. A. (1979a) *N. Engl. J. Med. 301*, 13–23.
Prockop, D. J., Kivirikko, K. I., Tuderman, L., & Guzman, N. A. (1979b) *N. Engl. J. Med. 301*, 77–85.
Risteli, J., & Kivirikko, K. I. (1976) *Biochem. J. 158*, 361–367.
Risteli, J., Tryggvason, K., & Kivirikko, K. I. (1977) *Eur. J. Biochem. 73*, 485–492.
Risteli, J., Tryggvason, K., & Kivirikko, K. I. (1978) *Anal. Biochem. 84*, 423–431.
Schwartz, C. E., Hellerqvist, C. G., & Cunningham, L. W. (1979) *Biochem. Biophys. Res. Commun. 90*, 240–246.
Tryggvason, K., Majamaa, K., & Kivirikko, K. I. (1979) *Biochem. J. 178*, 127–131.
Tryggvason, K., Gehron Robey, P., & Martin, G. R. (1980) *Biochemistry 19*, 1284–1289.
Tuderman, L., Kuutti, E.-R., & Kivirikko, K. I. (1975) *Eur. J. Biochem. 52*, 9–16.
Turpeenniemi-Hujanen, T. M. (1981) *Biochem. J. 195*, 669–676.
Turpeenniemi-Hujanen, T. M., Puistola, U., Kivirikko, K. I. (1981) *Collagen Relat. Res.: Clin. Exp. 1*, 355–366.
Uitto, J., & Prockop, D. J. (1974) *Biochim. Biophys. Acta 336*, 234–251.
Uitto, V.-J., Uitto, J., Kao, W. W.-Y., & Prockop, D. J. (1978) *Arch. Biochem. Biophys. 185*, 214–221.

Reversed Unfolding–Refolding Process of Cobra Neurotoxin†

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ABSTRACT: Circular dichroism and nuclear magnetic resonance spectroscopies have been used to study the unfolding process of cobrotoxin upon addition of fluoro alcohols and/or sodium dodecyl sulfate to its aqueous solution. In each final unfolded state, the protein had its disulfide bonds intact. The unfolding process has been found to be reversible in the case of fluoro alcohol/water mixtures, while no such reversibility was found in the case of sodium dodecyl sulfate. However, when hexafluoro-2-propanol is added to the sodium dodecyl sulfate unfolded protein, refolding is induced. The mechanism of unfolding is discussed in terms of the different interactions which govern the protein conformation in solution.

Neurotoxins constitute a group of homologous proteins found in snake venoms. They block transmission of the nerve impulses by binding with high affinity to the acetylcholine receptor of the motor end plate on the postsynaptic membrane (Lee, 1972; Tu, 1973; Yang, 1974). During the last few years, a large number of studies including optical spectroscopy (Chicheportiche et al., 1972; Harada et al., 1976; Chen et al., 1977; Menez et al., 1976; Visser & Louw, 1978), nuclear magnetic resonance (Arseniev et al., 1976; Bystrov et al., 1978; Lauterwein et al., 1977; Fung et al., 1979; Inagaki et al., 1978; Endo et al., 1979), and X-ray crystallography (Low et al., 1976; Tsernoglou & Petsko, 1976, 1977) as well as theoretical predictions of the molecular conformations (Ryden et al., 1973; Eterovic & Ferchmin, 1977) and the use of chemically modified toxins (Seto et al., 1970; Karlsson & Sundelin, 1976; Yang, 1976, 1974) have been made with the aim of understanding the relations between the spatial structures of these proteins and their biological functions.

Cobrotoxin from Taiwan cobra (*Naja naja atra*) venom is a postsynaptic neurotoxin. It consists of a single polypeptide chain of 62 amino acid residues and contains 4 disulfide bridges which divide the molecule into 4 loops. In a recent NMR[1]

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