REMOVAL OF AMINO-TERMINAL AND CARBOXY-TERMINAL EXTENSION PEPTIDES FROM PROCOLLAGEN DURING SYNTHESIS OF CHICK EMBRYO TENDON COLLAGEN

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Summary: Matrix-free chick embryo tendon cells were incubated with [^{14}C]proline for 60 minutes and protein synthesis was stopped by the addition of cycloheximide. Newly synthesized collagen precursors recovered in the incubation medium were mostly intact procollagen molecules which contain both amino-terminal and carboxy-terminal extensions. If the cells were further incubated for 2 hours in the presence of cycloheximide, most of the procollagen was converted to precursor molecules which were devoid of amino-terminal extensions. Removal of the carboxy-terminal extensions from procollagen was not observed. Similar experiments with intact tendons demonstrated that procollagen synthesized by the intact tissues *in vitro* was readily converted to an intermediate form devoid of amino-terminal extensions and then to collagen. The results suggest that the removal of the amino-terminal and carboxy-terminal extensions from procollagen is catalyzed by two separate enzymic activities.

Collagen is initially synthesized as a higher molecular weight precursor, called procollagen*, which has additional non-collagenous peptide extensions at both the amino-terminal and carboxy-terminal ends of the molecule (1-3; for review see 4-7). During intracellular assembly the collagenous portions of the three polypeptide chains of procollagen fold into a triple-helical conformation (7). Procollagen molecules are then converted to collagen by limited proteol-

*The term procollagen is used to signify a precursor form of collagen which has intact extension peptides at both carboxy-terminal and amino-terminal ends of the molecule. In accordance with previously suggested nomenclature (4, and J.M. Davidson, J.G. McEneany, and P. Bornstein, personal communication) $p_C$collagen refers here to a precursor form of collagen which contains carboxy-terminal extension peptides but is devoid of amino-terminal extensions. Similarly, $p_N$collagen refers to a molecule which contains amino-terminal extensions but is devoid of carboxy-terminal extension peptides.

ysis which removes the peptide extensions. Here we have studied the cleavage of the extension peptides from procollagen by matrix-free cells isolated from chick embryo tendons (8,9); this system allows one conveniently to examine the extracellular processing of procollagen separate from the intracellular biosynthesis (7,10). The results obtained with matrix-free cells were compared with those obtained by using intact tendons from which the matrix-free cells were isolated.

Materials and Methods. Cells were prepared by enzymic digestion of leg tendons from 17-day old chick embryos as described previously (8,9), and the cells were incubated in modified Krebs medium (8) containing 20% fetal calf serum and 50 μg/ml ascorbic acid, at 37°C. At the end of each incubation period 1/10 volume of modified Krebs medium containing 0.5 mg/ml α-toluenesulfonyl fluoride, 0.1 M N-ethylmaleimide and 0.2 M ethylenediamine tetraacetic acid was added to the incubation. The sample was cooled to 0°C and the medium was separated from the cells by centrifugation at 600 x g for 3 min. ^{14}C-Proline-labeled collagen precursor molecules were precipitated with ammonium sulfate (30% of saturation) at 4°C for 60 min, and the precipitate was dissolved in 5 ml of buffer consisting of 2 M urea in 25 mM Tris-HCl, pH 7.5, at 4°C. The sample was dialysed against 500 ml of the same buffer for 2 hr, changing the dialysis buffer three times. The sample was then chromatographed on a 2.5 x 10.0 cm column of microgranular (preswollen) DEAE† cellulose (DE52; Whatman Biochemicals, Ltd.)(11). The sample was eluted with a linear gradient prepared with 300 ml of starting buffer containing 2 M urea in 25 mM Tris-HCl, pH 7.5, and 300 ml of limit buffer consisting of 0.3 M NaCl and 2 M urea in 25 mM Tris-HCl, pH 7.5, at 8°C.

^{14}C-Radioactivity was assayed by using a liquid scintillation counter (Beckman LS 3155 P), and [^{14}C]hydroxyproline was assayed by a specific radiochemical method (12).

Results. Previous studies with matrix-free tendon cells from 17-day old chick embryos have demonstrated that essentially all ^{14}C-procollagen recovered in the medium, when the cells are incubated with [^{14}C]proline for 1 to 2 hr, consists of intact procollagen molecules which contain extension peptides both at the carboxy- and amino-terminal ends of the molecule (13). To study the removal of extension peptides from procollagen, the cells were pre-incubated for 15 min and then pulse-labeled for 60 min with [^{14}C]proline. Further protein synthesis was stopped by the addition of cycloheximide, and the incubation was continued for an additional 15 min in order to allow completed intracellular

†Abbreviation: DEAE, diethylaminoethyl.

molecules to be secreted into the medium (14). Examination of the medium ^{14}C-protein on DEAE cellulose chromatography demonstrated that about 95% of the radioactive protein eluted in fractions 46-50 or in the position which has been previously (2,3,13) shown to correspond to intact procollagen molecules with extension peptides at both ends of the molecules (Fig. 1A). Only a small amount of ^{14}C-protein eluted in the position where $p_c$collagen, or molecules containing carboxy-terminal extensions but devoid of amino-terminal extensions, elute. No radioactivity appeared in the positions where collagen or $p_n$collagen, i.e. molecules which have lost the carboxy-terminal extensions, would elute. If the incubation of cells following the addition of cycloheximide was extended to 135

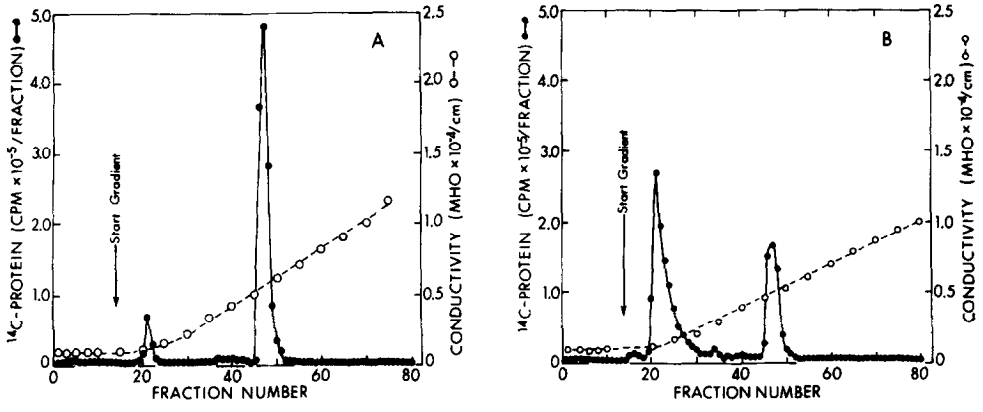

Figure 1. DEAE cellulose chromatography of medium ^{14}C-protein synthesized by matrix-free tendon cells. Cells, 3.2 x 10^8, were incubated with 30 μC of [^{14}C]proline for 60 min (see Methods), and 100 μg/ml cycloheximide was then added to the incubation medium. Incubation in the presence of cycloheximide was continued either for 15 min or for 135 min at 37°C. At the end of each incubation a mixture of protease inhibitors was added, and the ^{14}C-protein in the medium was isolated and prepared for DEAE cellulose chromatography as described in the text. The chromatography was performed with a flow rate of 120 ml/hr; fractions of 8 ml were collected and 0.4 ml of each fraction was used for assay of radioactivity. Recovery of ^{14}C-protein in 16 different chromatograms was 71-96%. The radioactivity eluting in fractions 45-49 has previously (13) been shown to represent procollagen with extension peptides at both ends of the molecule, and radioactivity eluting in fractions 21-29 represents $p_c$collagen. In this chromatographic system, ^{14}C-collagen elutes in fractions 4-10 (see Fig. 3), and $p_n$collagen elutes in fractions 51-55 (Uitto, J., Lichtenstein, J.R., and Bauer, E.A., manuscript in preparation.)
A: ^{14}C-Protein in the medium after 15 min incubation with cycloheximide.
B: ^{14}C-Protein in the medium after 135 min incubation with cycloheximide.

min, about 65 percent of the intact ^{14}C-procollagen was now converted to $p_c$collagen. However, little if any radioactivity appeared in the positions where molecules devoid of carboxy-terminal extensions elute (Fig. 1B). Analysis of the ^{14}C-protein demonstrated that the cycloheximide effectively stopped the incorporation of radioactive proline into protein, since the amount of ^{14}C-protein in the incubate (cells plus medium) at the end of 15 min and 135 min chase periods was 4.63 and 4.52 x 10^6 cpm, respectively. Also, the amount of radioactive procollagen or collagen in the medium was unchanged during the chase period, since the values for [^{14}C]hydroxyproline in the medium at the end of 15 min and 135 min chase periods were 1.41 and 1.36 x 10^6 dpm, respectively.

In further studies, a similar pulse-chase experiment was performed by pre-incubating cells for 240 min in the medium prior to the addition of [^{14}C]proline; such an extended pre-incubation would allow regeneration of membrane proteins, and also, enzymes secreted by the cells might accumulate in the medium. The analysis of ^{14}C-protein in the medium after 60 min incubation with radioactive proline and further 15 min incubation with cycloheximide demonstrated that about 55 percent of radioactive protein appeared in the position where $p_c$collagen elutes (Fig. 2A). If the incubation with cycloheximide was extended to 135 min, 92% of the ^{14}C-protein was recovered as $p_c$collagen (Fig. 2B). Little if any radioactivity was recovered on DEAE cellulose chromatography as collagen or $p_n$collagen. Analysis of the ^{14}C-protein demonstrated again that no [^{14}C]hydroxyproline containing protein was lost from the medium during the incubation with cycloheximide.

Further studies demonstrated that addition of a mixture of 50 μg/ml α-toluenesulfonyl fluoride, 10 mM N-ethylmaleimide and 20 mM ethylenediamine tetraacetic acid into the medium at the end of pulse-labeling period prevented the removal of the amino-terminal extensions from newly-synthesized procollagen during the subsequent 135 min incubation with cycloheximide. On the other hand, addition of 10 mM Ca^{2+} to the incubation or omission of fetal calf serum from

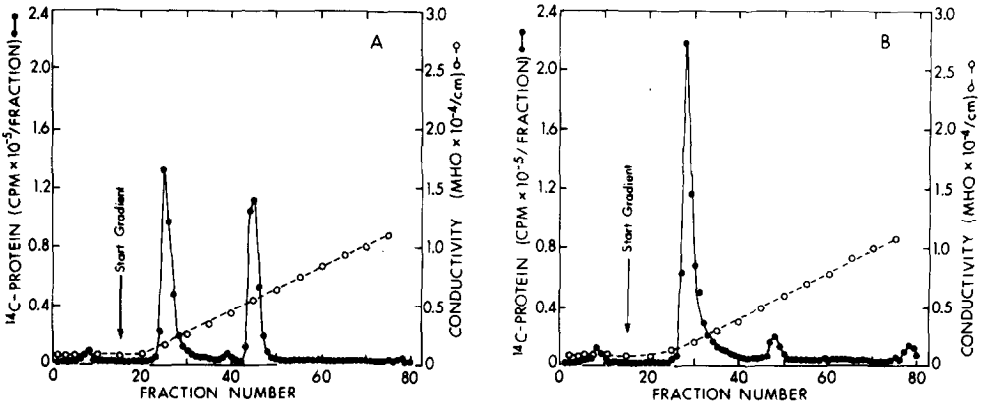

Figure 2. DEAE cellulose chromatography of medium ^{14}C-protein synthesized by tendon cells which had been pre-incubated in the medium for 240 min prior to the addition of [^{14}C]proline. The experimental conditions are otherwise the same as in Fig. 1.
A: ^{14}C-Protein in the medium after 15 min incubation with cycloheximide.
B: ^{14}C-Protein in the medium after 135 min incubation with cycloheximide.

the incubation medium did not affect the removal of the amino-terminal extensions in experiments similar to that in Fig. 1.

Control experiments demonstrated that procollagen synthesized by intact tendons from which the matrix-free cells were initially prepared was readily converted to collagen. In these experiments isolated tendons were pulse-labeled for 60 min with [^{14}C]proline in the presence of 20 μg/ml of β-aminopropionitrile and further protein synthesis was stopped by adding cycloheximide. Fifteen min later ^{14}C-protein in the incubate was extracted and chromatographed on DEAE cellulose column. The results demonstrated that most of the ^{14}C-protein was recovered as $p_c$collagen and a large fraction of the ^{14}C-protein eluted at the front of the chromatogram (Fig. 3A). Assay of [^{14}C]hydroxyproline and examination of ^{14}C-protein by polyacrylamide gel electrophoresis in SDS demonstrated that most of the ^{14}C-protein in fractions 4-8 was ^{14}C-collagen. If the incubation in the presence of cycloheximide was extended to 135 min, most of the ^{14}C-protein recovered in the chromatogram was ^{14}C-collagen (Fig. 3B).

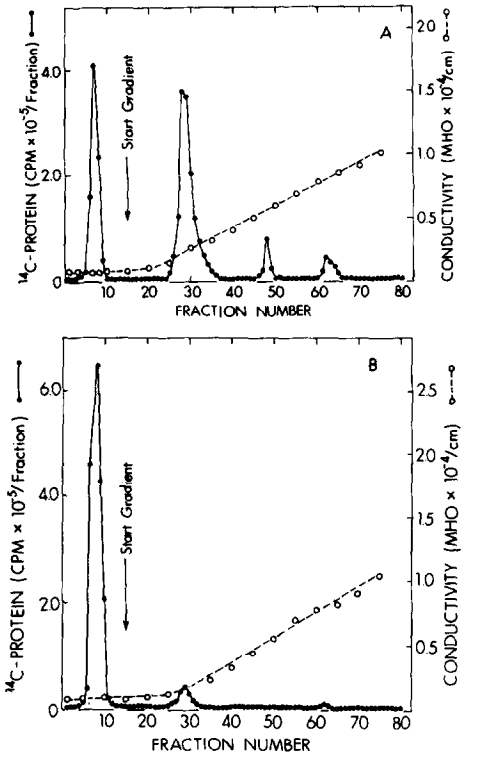

Figure 3. DEAE cellulose chromatography of ^{14}C-protein synthesized by leg tendons of 17-day old chick embryos. Tendons from 12 embryos were incubated in 10 ml of modified Krebs medium containing 20% fetal calf serum, 20 µg/ml β-aminopropionitrile, 50 µg/ml ascorbic acid and 30 µC [^{14}C]proline (22). After 60 min incubation, 100 µg/ml of cycloheximide was added and the incubation was continued at 37°C for either 15 min or 135 min. At the end of each incubation, a mixture of protease inhibitors (see text) was added, and the samples were homogenized in a Teflon and glass homogenizer at 4°C. The samples were centrifuged at 18,000 x g for 30 min at 4°C. The recovery of ^{14}C-protein in the soluble form was 82-86%. The ^{14}C-protein in supernatant fluid was isolated by ammonium sulfate precipitation and prepared for DEAE cellulose chromatography as described in Materials and Methods and in Fig. 1.
A: ^{14}C-Protein isolated after 15 min incubation with cycloheximide.
B: ^{14}C-Protein isolated after 135 min incubation with cycloheximide.

Discussion. This study demonstrates that there is enzymatic activity which cleaves the amino-terminal extensions from procollagen in the medium from matrix-free cells in suspension. However, the matrix-free tendon cell system demonstrated little if any enzymic activity which could remove the carboxy-terminal extensions,

while intact tendons from which the matrix-free cells were initially isolated readily converted the newly-synthesized procollagen to collagen. This observation suggests that the intact tissue contains peptidases which catalyze the removal of both amino- and carboxy-terminal extension peptides and that the carboxy-terminal peptidase is removed or inactivated during the isolation of the matrix-free cells.

Previously, an endopeptidase which cleaves the amino-terminal extensions from procollagen has been purified from calf connective tissues (15,16). A similar peptidase has also been demonstrated in rat calvaria (17) and in the medium of cultured human fibroblasts (18,19). Endopeptidases which convert procollagen to collagen have been isolated from mouse 3T3 fibroblast culture medium (20). This medium appears, therefore, to contain enzymatic activity which catalyzes the removal of the large carboxy-terminal extension fragment from procollagen, as well as enzyme activity which cleaves the amino-terminal extensions (20).

Further support for the existence of separate amino- and carboxy-terminal peptidases comes from the isolation of discreet precursor intermediates with only the carboxy- or amino-terminal extensions. In this study, as in several other prior studies (2,3,13), a precursor form of collagen which contains carboxy-terminal extensions but is devoid of amino-terminal extension peptides has been isolated. Failure to detect significant amounts of intermediate forms of procollagen which have lost only the carboxy-terminal extensions during the incubation of intact tendons, where procollagen was efficiently converted to collagen, suggests that the removal of the amino-terminal extensions may precede the cleavage of the carboxy-terminal extensions from procollagen *in vivo*. The cleavage of the amino-terminal extensions is not, however, a rigorous prerequisite for the removal of the carboxy-terminal extensions, since in an animal disease of fragile skin, dermatosparaxis, $p_n$collagen accumulates in tissues (6,21).

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