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Characterization of Procollagen Synthesized by Matrix-Free Cells Isolated from Chick Embryo Tendons†

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ABSTRACT: The genetic type and molecular structure of the precursor forms of collagen synthesized by matrix-free tendon cells isolated from 17-day old chick embryos were examined by chromatographic and electrophoretic techniques. The [^{14}C]proline-labeled collagenous proteins secreted by the cells resolved on diethylaminoethylcellulose into two peaks, A and B. Both peaks contained type I collagenous proteins since on chromatography on carboxymethylcellulose, after limited pepsin proteolysis, both peaks contained α1 and α2 chains of collagen in a 2:1 ratio, and cyanogen bromide peptide maps of the ^{14}C-labeled protein in both peaks were similar to cyanogen bromide peptide maps derived from authentic type I collagen. Enzymatic digestion with purified mammalian collagenase demonstrated that the collagen precursor in peak B contained noncollagenous peptide extensions at both the amino- and carboxy-terminal ends of the molecule, while peak A had only carboxy-terminal extension peptides. Although both the amino- and carboxy-terminal extensions incorporated radioactive cystine, only the carboxy-terminal extensions contained interchain disulfide bonds. The carboxy-terminal extensions were also shown to incorporate radioactive tryptophan. Since most of the precursor forms of collagen recovered in the incubation medium chromatographed in peak B, it is concluded that matrix-free tendon cells secrete only type I procollagen with extension peptides at both the amino- and carboxy-terminal ends of the molecule.

The biosynthesis of collagen involves elaboration of a precursor molecule, called procollagen (for recent reviews on procollagen, see Bornstein, 1974; Miller and Matukas, 1974; Gross, 1974; Martin et al., 1975; Veis and Brownell, 1975; Uitto and Lichtenstein, 1976a; Prockop et al., 1976). The polypeptide chains of procollagen, pro-α chains, are larger than collagen α chains, because they contain additional noncollagenous peptide extensions. In studies utilizing cultured fibroblasts (Tanzer et al., 1974) and chick calvaria (Byers et al., 1975; Fessler et al., 1975), the largest form of the collagen precursors has been shown to contain extension peptides at both the amino- and carboxy-terminal ends of the molecule. In addition, collagen precursors with only amino-terminal extension peptides (Lenaers et al., 1971) and with only carboxy-terminal extension peptides (Byers et al., 1975; Fessler et al., 1975) have been isolated.

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Matrix-free cells isolated from chick embryo tendons have been extensively used to study the biosynthesis of collagen since almost exclusively collagenous proteins are synthesized by these cells (Dehm and Prockop, 1971, 1972; Prockop et al., 1976). These collagenous proteins are known to be larger than collagen, and in a recent study we have demonstrated that the medium of matrix-free tendon cells contains at least two different forms of collagen precursor molecules (Uitto and Lichtenstein, 1976b). However, the characterization of the genetic type and the molecular structure of these precursor molecules has been incomplete.

In this report we demonstrate that most of the collagen precursors synthesized by matrix-free tendon cells contain peptide extensions at both the amino- and carboxy-terminal ends, but also a precursor form with extension peptides at only the carboxy-terminal ends of the molecule can be isolated from the incubation medium. We further establish on the basis of α-chain composition and cyanogen bromide peptide mapping, that only type I collagen chains are synthesized by the matrix-free tendon cells.

Materials and Methods

Materials. Unless otherwise indicated, all the materials were purchased from the same suppliers as indicated previously (Dehm and Prockop, 1971, 1972; Schofield et al., 1974; Uitto and Prockop, 1974a).

Isolation and Incubation of Matrix-Free Tendon Cells. Cells from leg tendons of 17-day old chick embryos were prepared by digesting the tissues with bacterial collagenase and trypsin in modified essential medium as indicated previously (Dehm and Prockop, 1971, 1972). The cells in the digest were filtered through a lens paper and then recovered by centrifugation at 600*g* for 6 min at room temperature. The cells were resuspended in modified Krebs medium containing 10% fetal calf serum and washed three times by centrifugations. For incubation, the cells were suspended in modified Krebs medium containing 20% fetal calf serum and 50 μg/ml ascorbic acid. After a 15-min preincubation, radioactive amino acids were added to the medium and the incubation was continued for 2 h at 37 °C. Incubation was stopped by adding cycloheximide (100 μg/ml) and α,α'-dipyridyl (1 mM). The incubation mixture was immediately cooled to 0 °C and the following protease inhibitors were added: *N*-ethylmaleimide (final concentration 10 mM), α-toluenesulfonyl fluoride (0.3 mM), and EDTA[1] (20 mM). The medium was then separated from the cells by centrifugation at 1200*g* for 3 min.

To prepare the radioactive proteins from the medium for different chromatographic procedures, the medium proteins were precipitated with 176 mg of ammonium sulfate per ml (30% of saturation) at 4 °C for 60 min. The precipitate was then recovered by centrifugation at 18 000*g* for 30 min at 4 °C.

Chromatographic Procedures. For DEAE-cellulose chromatography, the ammonium sulfate precipitate of the radioactive proteins was dissolved in 5 ml of starting buffer consisting of 2 M urea in 0.025 M Tris-HCl, pH 7.5, at 4 °C. The sample was dialyzed against 500 ml of the same buffer for 2 h, changing the dialysis buffer three times, and then chromatographed on a 2.5 × 10 cm column of microgranular (preswollen) DEAE-cellulose (DE52; Whatman Biochemicals, Ltd.) (Smith et al., 1972; Lichtenstein et al., 1975; Uitto and Lichtenstein, 1976b). The sample was eluted with a linear gradient prepared with 300 ml of starting buffer containing 2 M urea in 0.025 M Tris-HCl, pH 7.5, and 300 ml of limit buffer consisting of 0.3 M NaCl and 2 M urea in 0.025 M Tris-HCl, pH 7.5, at 8 °C. Chromatography was performed at a flow rate of 150 ml/h; 8-ml fractions were collected and 0.4 ml of each fraction was used for assay of radioactive protein. The radioactive peaks in the chromatogram were pooled (see Results) and protease inhibitors in concentrations indicated above were added. The samples were then dialyzed against 0.4 M NaCl in 0.1 M Tris-HCl, pH 7.5, at 4 °C, and the radioactive proteins concentrated by precipitations with 176 mg of ammonium sulfate per ml, as indicated above.

To prepare the radioactive proteins either from unfractionated medium or from peaks isolated by DEAE-cellulose chromatography for gel filtration in sodium dodecyl sulfate, the ammonium sulfate precipitates were dispersed in 2 ml of 2% sodium dodecyl sulfate, and 0.05 M iodoacetamide in 0.1 M sodium phosphate buffer, pH 7.2. The samples were heated immediately in a boiling water bath for 3 min and then incubated at 37 °C for an additional 120 min, and subsequently dialyzed against 0.1% sodium dodecyl sulfate in 0.1 M sodium phosphate buffer overnight. In some experiments the disulfide bonds in the protein were reduced by adding 2% 2-mercaptoethanol and heating for 10 min at 37 °C just prior to gel filtration. Gel filtration was carried out at room temperature on a 1.5 × 90 cm column of 6% agarose (Bio-Gel A-5m, 200–400 mesh, Bio-Rad Laboratories) equilibrated and eluted with 0.1% sodium dodecyl sulfate in 0.1 M sodium phosphate buffer, pH 7.2, as described previously (Jimenez et al., 1971; Uitto and Prockop, 1974b,c).

For chromatography on CMC, the ^{14}C-labeled protein after limited digestion with pepsin (see below) was precipitated with 176 mg of ammonium sulfate per ml and the precipitate was then dissolved in 5 ml of starting buffer which consisted of 0.04 M sodium acetate buffer, pH 4.8 (Piez et al., 1963). Ten milligrams of chick skin collagen, prepared by pepsin digestion at 4 °C, was mixed with the sample. The sample was dialyzed against the starting buffer at 4 °C and then heated for 15 min at 55 °C just prior to being applied to the column. Chromatography was carried out on a 2.5 × 10 cm column of microgranular (preswollen) CMC resin (CM52; Whatman Biochemicals, Ltd.). The column was eluted with a linear gradient prepared with 300 ml of 0.04 M sodium acetate, pH 4.8, and 300 ml of limit buffer containing 0.1 M NaCl in 0.04 M sodium acetate buffer, pH 4.8, at 45 °C.

To chromatograph ^{14}C-labeled peptides obtained by cyanogen bromide cleavage (see below) on CMC, the lyophilized samples were dissolved in 5 ml of starting buffer consisting of 0.02 M sodium formate, pH 3.8. Chromatography was performed on a 0.9 × 2.0 cm column of CMC as described elsewhere (Lichtenstein et al., 1975). The column was eluted at 45 °C with a linear gradient prepared with 500 ml of starting buffer consisting of 0.02 M sodium formate, pH 3.8, and 500 ml of limit buffer containing 0.15 M NaCl in 0.02 M sodium formate, pH 3.8.

Polyacrylamide Slab Gel Electrophoresis in Sodium Dodecyl Sulfate. For electrophoresis on polyacrylamide slab gels, the ammonium sulfate precipitates of radioactive proteins were dissolved in 0.2 ml of 2% sodium dodecyl sulfate and 0.05 M iodoacetamide in 0.125 M Tris-HCl, pH 7.4. The samples were heated for 3 min at 100 °C, incubated at 37 °C for additional 2 h, and then dialyzed against buffer consisting of 2% sodium dodecyl sulfate, 10% glycerol, and 0.01% bromophenol blue in 0.125 M Tris-HCl, pH 6.8. In some experiments, reduction

[1] Abbreviations used: DEAE, diethylaminoethyl; CMC, carboxymethylcellulose; Me_2SO, dimethyl sulfoxide; Na_2EDTA, disodium ethylenediaminetetraacetate.

was carried out by adding 2% 2-mercaptoethanol and heating for 10 min at 37 °C just prior to electrophoretic run. The samples were electrophoresed on 10 × 14 cm slab gels using a system described elsewhere (King and Laemmli, 1971; Studier, 1973). After electrophoresis, the gels were soaked in 200 ml of Me_2SO for 30 min, and the procedure was repeated four times using fresh Me_2SO. The gels were then submerged in 4 volumes of 20% (w/w) 2,5-diphenyloxazole in Me_2SO (22.2% w/w) for 3 h, rinsed in distilled water for 1 h, and then dried under vacuum. The dried gels were exposed on x-ray films (RP Royal X-Omat; Eastman Kodak) at −70 °C usually for 24 h (Bonner and Laskey, 1974). The radioautographs were scanned using a double-beam densitometer (Joyce-Loeb, England).

Enzymic and Chemical Treatments of Proteins. For digestion with bacterial collagenase, the ammonium sulfate precipitates of the radioactive proteins were dissolved in 1 ml of 0.15 M NaCl and 0.01 M $CaCl_2$ in 0.05 M Tris-HCl buffer, pH 7.6, at 4 °C, and then dialyzed against the same buffer. Purified bacterial collagenase (Advanced Biofactors, New York, N.Y.), 50 μg/ml, and *N*-ethylmaleimide (2.5 mM) were added, and the samples were incubated for 15 h at 4 °C. The bacterial collagenase used in this study was shown to be specific for collagenous proteins, in that in control experiments no radioactive tryptophan was released from radioactive protein synthesized by human fibroblasts with [^{3}H]tryptophan (Lichtenstein et al., 1976). The incubation was stopped by adding $1/10$ volume of 0.25 M EDTA, and the samples were extensively dialyzed against 0.15 M NaCl in 0.05 M Tris-HCl buffer, pH 7.6, at 4 °C. The release of radioactive peptides by dialysis was taken as a measure of collagenous proteins in the sample.

To prepare radioactive peaks from DEAE-cellulose chromatography for digestion with a partially purified preparation of human skin collagenase, the ammonium sulfate precipitates of the proteins were dissolved in 1 ml of 0.15 M NaCl and 0.01 M $CaCl_2$ in 0.05 M Tris-HCl buffer, pH 7.5. Human skin collagenase containing 36 μg of protein was added to the sample, and the mixture was incubated at 22 °C for 20 h. The incubation was stopped by the addition of $1/10$ of a volume of 0.25 M EDTA, and the contents of the incubate were treated with sodium dodecyl sulfate and iodoacetamide in a final concentration of 2% and 0.05 M, respectively, as described above.

Human skin collagenase was prepared from serum-free culture medium of explants of human skin (Eisen et al., 1968) and partially purified by 50% ammonium sulfate precipitation followed by gel filtration on a 2.5 × 100 cm column of Sephadex G-150 equilibrated and eluted with 0.05 M Tris-HCl, pH 7.5, containing 0.15 M NaCl and 0.005 M $CaCl_2$ at 4 °C (Bauer et al., 1970). Just prior to incubation with substrate, *N*-ethylmaleimide and α-toluenesulfonyl fluoride were added to the human skin collagenase in a final concentration of 0.05 M and 0.3 mM, respectively.

To prepare samples for digestion with pepsin, ammonium sulfate precipitates of radioactive proteins were resuspended in 2 ml of 0.1 N acetic acid and then dialyzed against 0.1 N acetic acid. Pepsin (100 μg/ml) was added and the sample was incubated at 4 °C for 15 h. The pepsin was then inactivated by dialyzing the samples against 0.4 M NaCl in 0.1 M Tris-HCl buffer, pH 7.5 at 4 °C. The samples were either treated with sodium dodecyl sulfate for electrophoresis on slab gels or prepared for chromatography on CMC, as described above.

To prepare samples for treatment with cyanogen bromide, radioactive peaks obtained from DEAE-cellulose chromatography were dialyzed against distilled water, lyophilized, and then dissolved in 2 ml of 70% formic acid. The samples were flushed with N_2 for 20 min at room temperature, 200 mg of cyanogen bromide was added, and the samples were incubated at 30 °C for 3 h (Epstein et al., 1971). After incubation, the samples were diluted with 10 ml of distilled water and most of the cyanogen bromide was removed by aspiration under a water pump for 1 h. The samples were then lyophilized, redissolved in 5 ml of distilled water, and relyophilized. This procedure was repeated three times in order to completely remove cyanogen bromide from the sample. The cyanogen bromide peptides were either treated with 2% sodium dodecyl sulfate and directly electrophoresed on slab gel without dialysis, or the samples were dissolved on 0.02 M sodium formate buffer, pH 3.8, for chromatography on CMC, as described above.

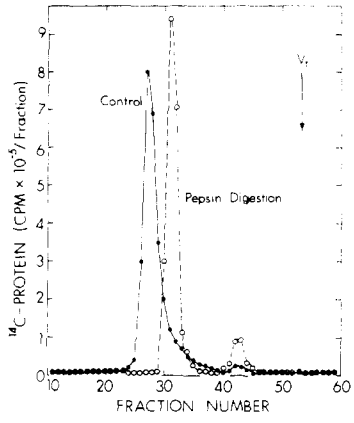

FIGURE 1: Gel filtration in sodium dodecyl sulfate of ^{14}C-labeled protein synthesized and secreted by matrix-free tendon cells. Cells (3×10^8) were incubated in 30 ml of modified Krebs medium containing 10 μCi of [^{14}C]proline, 20% fetal calf serum, and 50 μg/ml ascorbic acid. After 2 h of incubation, half of the medium ^{14}C-labeled protein was precipitated with ammonium sulfate and treated with 2% sodium dodecyl sulfate and 0.05 M iodoacetamide in 0.1 M phosphate, pH 7.2, for gel filtration, as described in Materials and Methods. The other half of the ^{14}C-labeled protein was precipitated and then digested by pepsin before treatment with sodium dodecyl sulfate. The chromatography was performed on a 6% agarose column eluted with 0.1% sodium dodecyl sulfate in 0.1 M phosphate buffer, pH 7.2, as described in Materials and Methods; 3 ml fractions were collected and 0.4 ml was used for assay of ^{14}C. The void volume of the column (V_0) was in fractions 15–16. Collagen α chains from embryonic chick skin eluted in fraction 31, and β chains eluted in fractions 22–23. The total volume of the column (V_t) is indicated by an arrow. Control: Sample prepared for chromatography without digestion with pepsin. Pepsin Digestion: Sample subjected to limited pepsin digestion before chromatography.

Results

Isolation of Collagen Precursor Molecules Synthesized and Secreted by Matrix-Free Tendon Cells. Freshly isolated tendon cells from chick embryos were incubated with [^{14}C]-proline for 2 h, and at the end of the incubation period a mixture of protease inhibitors was added to the incubation medium in order to prevent any degradation of the proteins during subsequent processing of the material. Gel filtration on agarose in sodium dodecyl sulfate demonstrated that essentially all nondialyzable ^{14}C-labeled protein in the medium eluted near the void volume of the column (not shown); if the protein was reduced with 2-mercaptoethanol prior to chromatography, the ^{14}C-labeled protein eluted as a sharp peak in a position between the α and β chains of collagen (Figure 1). The protein, therefore, consisted of polypeptide chains linked by disulfide bonds. After limited proteolytic digestion with pepsin, about 90% of the ^{14}C-labeled protein was recovered as polypeptides which

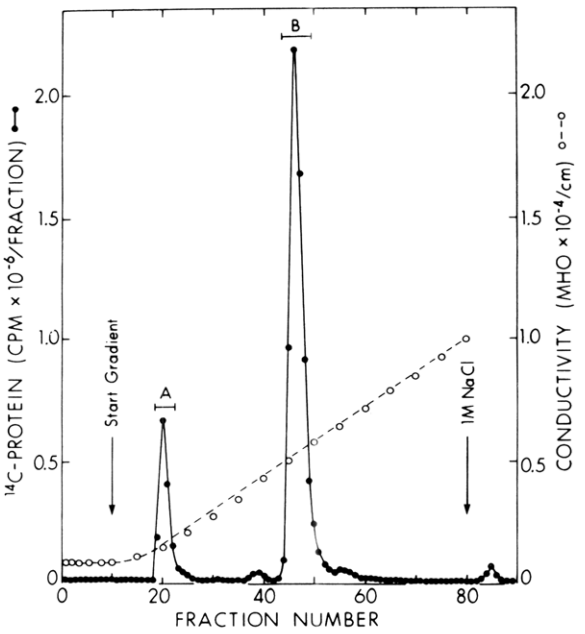

FIGURE 2: DEAE-cellulose chromatography of medium ^{14}C-labeled protein synthesized by matrix-free tendon cells. Cells (3.2×10^8) were incubated with 20 μCi of [^{14}C]proline for 120 min, and the medium ^{14}C-labeled protein was then isolated and chromatographed on DEAE-cellulose as described in Materials and Methods. The protein was eluted with 600 ml of a linear gradient from 0 to 0.3 M NaCl in 2 M urea and 0.025 M Tris-HCl, pH 7.5. The column was finally eluted with 1 M NaCl in 0.025 M Tris-HCl, pH 7.5. Regions A and B, indicated by the bars in the chromatogram, were pooled and subjected to further analysis, as described in the text.

TABLE I: Digestion of Radioactive Protein Recovered in Peaks A and B with Purified Bacterial Collagenase.[a]

Sample from Peak	Nondialyzable ^{14}C-Labeled Protein: Before Digestion (cpm $\times 10^{-4}$)	Nondialyzable ^{14}C-Labeled Protein: After Digestion (cpm $\times 10^{-4}$)	^{14}C-Labeled Peptides Released (%)
A	1.85	0.22	88.1
A	1.62	0.17	89.5
B	7.46	1.07	85.7
B	8.01	1.13	85.9

[a] ^{14}C-labeled protein in peaks A and B isolated by DEAE-cellulose chromatography (see Figure 2) was prepared for digestion with bacterial collagenase, as described in Materials and Methods. After digestion the samples were dialyzed extensively and the release of ^{14}C-labeled peptides was monitored by assaying the nondialyzable ^{14}C.

chromatographed in the same position as α chains of collagen (Figure 1); the elution position of these polypeptides was the same whether or not the protein was reduced with 2-mercaptoethanol.

When the medium ^{14}C-labeled protein was chromatographed on DEAE-cellulose under nondenaturing conditions, the ^{14}C-labeled protein resolved into two peaks, A and B (Figure 2). The ratio of the radioactivities eluting in peaks A and B was relatively constant, and peak B accounted for 73–86% of the total ^{14}C-labeled protein in the chromatograph in five different experiments. The ^{14}C-labeled protein in both peaks A and B was judged to be collagenous protein by several criteria. First, the relative proportion of [^{14}C]hydroxyproline in total ^{14}C incorporated into the protein was found to be about 42–46% (Juva and Prockop, 1966). This value agrees with

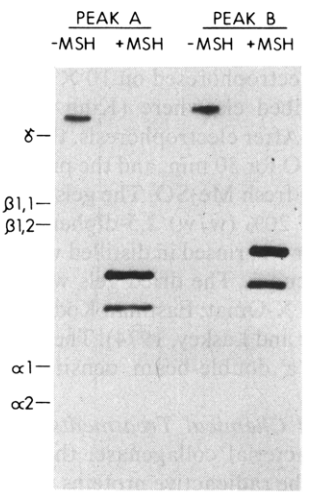

FIGURE 3: Slab gel electrophoresis in sodium dodecyl sulfate of ^{14}C-labeled protein in peaks A and B. The radioactive protein from peaks A and B in DEAE-cellulose chromatography (see Figure 2) was concentrated and treated with sodium dodecyl sulfate, as described in Materials and Methods. The samples were electrophoresed on a slab gel composed of a 2.5 cm stacking gel of 4.5% polyacrylamide, and of a separating gel of 6% polyacrylamide. The gels were then exposed to x-ray film as described in Materials and Methods. Samples were electrophoresed either without reduction (− MSH) or with reduction with 2-mercaptoethanol (+ MSH). The migration positions of γ, β, and α chains of type I collagen from chick skin are indicated.

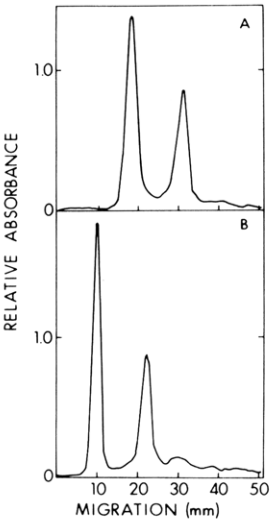

FIGURE 4: Densitometric scanning of the radioautographs of ^{14}C-labeled peptides in Figure 3. The two ^{14}C-labeled polypeptide chains observed in samples A and B after electrophoresis with 2-mercaptoethanol were quantitated by scanning as described in Materials and Methods. (Upper Frame) ^{14}C-labeled polypeptides in peak A; (lower frame) ^{14}C-labeled polypeptides in peak B.

known proportions of radioactive hydroxyproline and proline in both procollagen and collagen (Uitto and Prockop, 1974d). Secondly, incubation with highly purified bacterial collagenase in the presence of 2.5 mM *N*-ethylmaleimide converted about 90% of the ^{14}C-labeled protein into dialyzable peptides (Table I). Thirdly, after limited proteolysis with pepsin, essentially all ^{14}C-labeled protein in peaks A and B was recovered as ^{14}C-labeled peptides which eluted on sodium dodecyl sulfate agarose in the same position as α chains of collagen (see Figure 1).

Examination of the radioactive protein by gel electrophoresis in sodium dodecyl sulfate demonstrated that the ^{14}C-labeled

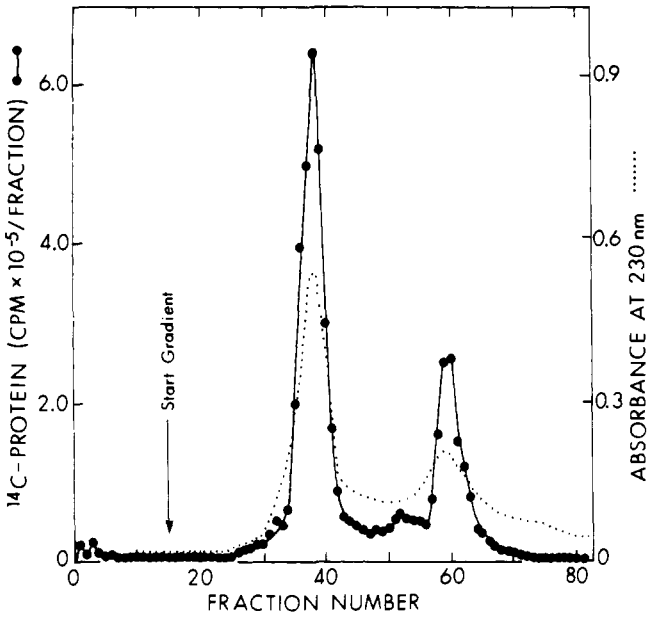

FIGURE 5: Chromatography on CMC of ^{14}C-labeled protein in peak B after limited proteolytic digestion with pepsin. ^{14}C-labeled protein recovered in peak B in DEAE-cellulose chromatography (see Figure 2) was isolated and subjected to pepsin digestion as described in Materials and Methods. Carrier type I collagen prepared by pepsin digestion and fractionated salt precipitation from 19-day old chick embryo skin was added, and the sample was chromatographed on CMC as described in the text. The absorbance of the carrier peptides was monitored by a Foci-FLEX grating monochromator (Optical Co., Inc., New York, N.Y.) at 230 nm and using a Gilson recorder RP.

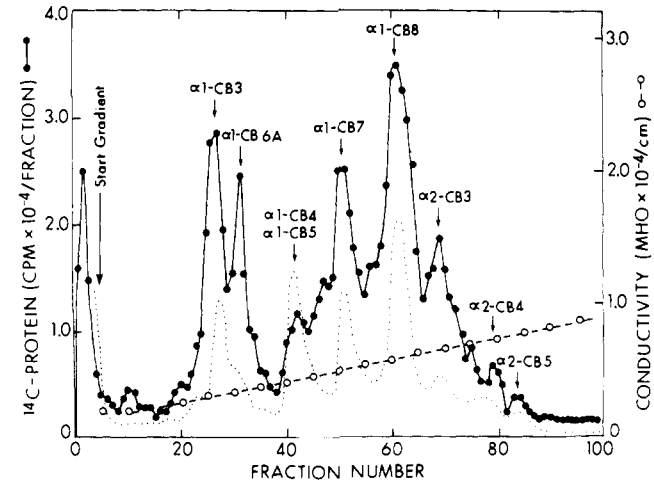

FIGURE 6: Chromatography on CMC of ^{14}C-labeled peptides obtained by cyanogen bromide cleavage of the ^{14}C-labeled protein in peak B. ^{14}C-labeled protein in peak B (see Figure 2) was isolated and subjected to cleavage by cyanogen bromide in 70% formic acid as described in the text. Sample was lyophilized and dissolved in 0.02 M sodium formate, pH 3.8, and then chromatographed on CMC with 1000 ml of a linear gradient from 0 to 0.15 M NaCl in 0.02 M sodium formate, pH 3.8, at 45 °C. The sample was chromatographed with carrier cyanogen bromide peptides derived from type I collagen isolated from rat skin. The absorbance of the carrier peptides was monitored at 230 nm as in Figure 5, and the absorbance of the carrier peptides is indicated by a dotted line (. . . .). Tentative identification of the ^{14}C-labeled peptides derived from peak B by cyanogen bromide cleavage on the basis of their elution position as compared with published chromatograms (Butler et al., 1967; Miller et al., 1969) is indicated in the figure.

protein in both peaks A and B migrated as as single band with a mobility less than γ chains of collagen (Figure 3). After reduction with 2-mercaptoethanol prior to electrophoresis, the ^{14}C-labeled protein in both A and B was composed of two kinds of peptide chains which occurred in an approximate 2:1 ratio (Figures 3 and 4). The bands derived from peak B migrated intermediate between α and β chains of collagen; the chains in peak A were smaller in size than those in peak B (Figure 3). These observations indicate that peak B consists of the largest form of the newly synthesized collagen precursor recovered from the incubation medium. Peak B, therefore, represents intact procollagen synthesized and secreted by tendon cells.

Demonstration that Procollagen Synthesized by Matrix-Free Tendon Cells is a Precursor of Type I Collagen. In order to study the chain composition of the collagenous part of the procollagen synthesized by tendon cells, procollagen in peak B from DEAE-cellulose chromatography was subjected to limited digestion with pepsin and then chromatographed on CMC. The ^{14}C-labeled collagen polypeptides derived from peak B resolved into two peaks which chromatographed in the same position as α1 and α2 chains of the carrier type I collagen (Figure 5). The ratio of radioactivity eluting in α1 and α2 positions was 2.2:1. Identical results were obtained when peak A was subjected to limited proteolysis by pepsin and then chromatographed on CMC (not shown).

To study the genetic type of procollagen synthesized by tendon cells, the ^{14}C-labeled protein in peak B was subjected to cleavage with cyanogen bromide, and the peptides were then chromatographed on CMC column with carrier peptides prepared from type I collagen. The pattern of radioactivity of ^{14}C-labeled peptides derived from peak B was similar to the cyanogen bromide peptide pattern derived from type I collagen (Figure 6). Tentative identification of the ^{14}C-labeled peptides, based on published chromatograms (Miller et al., 1969) or comparison with carrier peptides, revealed that α1-CB 6B, the carboxy-terminal fragment derived by cyanogen bromide cleavage of type I chick collagen, was absent. The lack of this peptide could be explained, however, by the presence of a carboxy-terminal extension (see below) which is not removed from collagen by cyanogen bromide cleavage. On the basis of these experiments, it was concluded that the procollagen recovered in peak B from DEAE-cellulose chromatography is a precursor of type I collagen.

Peak A from DEAE-cellulose chromatography was also subjected to cleavage by cyanogen bromide. Because of the relatively low amount of radioactivity in this peak (see Figure 2), it was not possible to obtain a reliable analysis of the cyanogen bromide peptide pattern using CMC chromatography. Therefore, the cyanogen bromide peptide patterns derived from peaks A and B were compared employing a technique which detects relatively small amounts of radioactivity in the peptides. For this purpose the cyanogen bromide peptides were electrophoresed on polyacrylamide slab gels in sodium dodecyl sulfate, and the migration positions of the peptides were detected by radioautography. The cyanogen bromide peptides were qualitatively similar from both sources (Figure 7). Densitometric scanning of the radioautographs demonstrated that the relative proportions of the different cyanogen bromide peptides derived from peaks A and B were similar (Figure 8). Thus, both peaks A and B consist of precursor forms of type I collagen.

Demonstration of Propeptide Extensions in Procollagen Synthesized by Tendon Cells. Since slab gel electrophoresis demonstrated that the two precursor chains obtained from peak A were smaller in size than those obtained from peak B, it was of interest to cleave peaks A and B with mammalian collagenase in order to determine if there were differences in the location of extension peptides. Incubation of peak A with

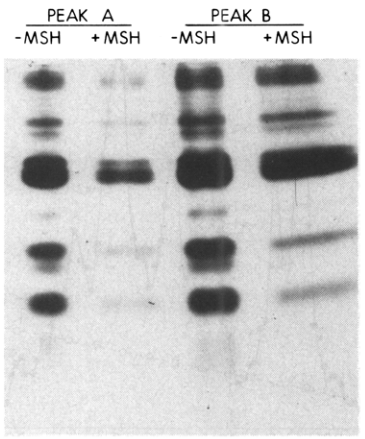

FIGURE 7: Slab gel electrophoresis of ^{14}C-labeled peptides obtained by cyanogen bromide cleavage of peaks A and B. The ^{14}C-labeled protein in peaks A and B on DEAE-cellulose was concentrated and prepared for digestion with cyanogen bromide as described in Materials and Methods. Samples were then electrophoresed on an 8% polyacrylamide gel and the ^{14}C-labeled peptides were visualized by radioautographic techniques as described in Materials and Methods. The samples were electrophoresed either without reduction (− MSH) or with reduction with 2-mercaptoethanol (+ MSH). Larger amounts of radioactivity were applied to the gel in samples electrophoresed without reduction in order to visualize peptides containing relatively small amounts of radioactivity.

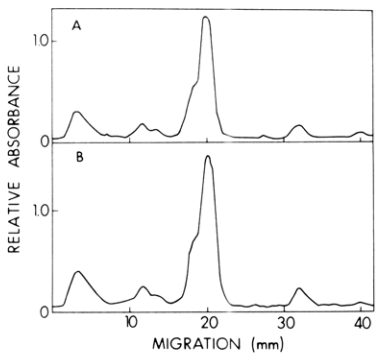

FIGURE 8: Densitometric scanning of the radioautographs of ^{14}C-labeled peptides in Figure 7. The ^{14}C-labeled peptides obtained by cyanogen bromide cleavage of peak A or peak B and electrophoresed with 2-mercaptoethanol (Figure 7) were quantitated by scanning as described in Materials and Methods. (Upper Frame) ^{14}C-labeled peptides derived from peak A; (lower frame) ^{14}C-labeled peptides derived from peak B.

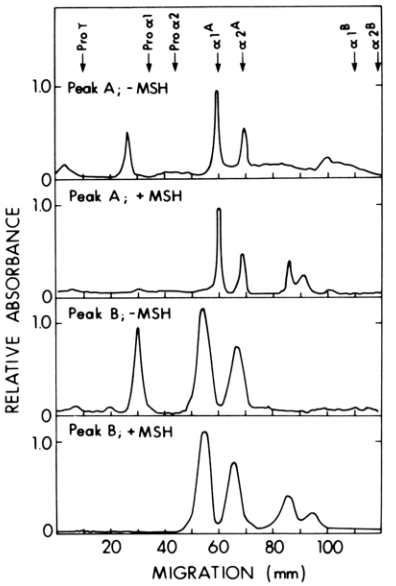

FIGURE 9: Cleavage of ^{14}C-labeled protein in peaks A and B with partially purified human skin collagenase. ^{14}C-labeled protein in peaks A and B was prepared for digestion with partially purified human skin collagenase, and the cleavage products were then electrophoresed on a slab gel of 8% polyacrylamide. The samples were electrophoresed either without reduction (− MSH) or with reduction with 2-mercaptoethanol (+ MSH). The gels were then exposed to x-ray films and the ^{14}C-labeled peptide pattern was quantitated by scanning the radioautographs with a densitometer as described in Materials and Methods. The elution positions of pro-γ, pro-α1, and pro-α2 chains from peak B, and also of α^{A} and α^{B} peptides derived by collagenase cleavage of type I guinea pig collagen are indicated in the chromatograms.

human skin collagenase released two peptides which migrated in the slab gel in the same positions as $\alpha1^{A}$ and $\alpha2^{A}$ fragments derived from the amino terminus of type I collagen (Figure 9). The migration position of these peptides in slab gel electrophoresis was the same with and without reduction with 2-mercaptoethanol. The collagenase cleavage of peak A also released a fragment which migrated about 25 mm from the origin (Figure 9). After reduction with 2-mercaptoethanol, this fragment was shown to consist of two kinds of peptide chains with a mobility less than the $\alpha1^{B}$ and $\alpha2^{B}$ fragments released from the carboxy terminus of type I collagen by mammalian collagenase (Figure 9). Limited digestion of the peptides with pepsin following the collagenase digestion but prior to denaturation produced two peptides which migrated in the same position as $\alpha1^{B}$ and $\alpha2^{B}$ peptides of type I collagen (not shown). Therefore, peak A represents a precursor form of collagen which contains peptide extensions at the carboxy-terminal end; these extension peptides also contain the interchain disulfide bonds which link together pro-α chains of procollagen. The molecules do not, however, have a detectable amino-terminal extension.

Treatment of the ^{14}C-labeled protein from peak B with mammalian collagenase released a similar disulfide-linked fragment (Figure 9); this fragment was also converted by pepsin digestion into two peptides migrating in the same positions as $\alpha1^{B}$ and $\alpha2^{B}$ peptides of type I collagen (not shown). In addition, collagenase digestion released two peptides which were larger than $\alpha1^{A}$ and $\alpha2^{A}$ peptides (Figure 9). The migration positions of these peptides were unaffected by reduction, but they were converted to $\alpha1^{A}$ and $\alpha2^{A}$ peptides by limited pepsin digestion following the collagenase cleavage. Peak B, therefore, consists of procollagen molecules which have peptide extensions at both amino-terminal and carboxy-terminal ends of the molecule, the carboxy-terminal extensions being disulfide linked.

Incorporation of Radioactive Cystine and Tryptophan into Extension Peptides. In order to partially characterize the amino acid composition of extension peptides of procollagen, the cells were incubated with radioactive cystine and tryptophan, two amino acids which are not found in the collagenous portion of type I collagen (Piez et al., 1963). The results demonstrated that both peaks A and B in DEAE-cellulose chromatogram were labeled with radioactive cystine and tryptophan (Figure 10). The observed radioactivities incorporated into peak A were 0.41×10^5 and 1.20×10^5 cpm for [^{35}S]cystine and [^{3}H]tryptophan, respectively, and the corresponding radioactivities observed in peak B were 1.60×10^6 and 1.72×10^6 cpm. The ratios of radioactive cystine to radioactive tryptophan in peaks A and B were 0.34 and 0.93, respectively. Thus, precursor forms of collagen in both peaks A and B contained cystine and tryptophan, but peak B was relatively enriched with respect to cystine. In further studies,

the radioactive protein in peak B was subjected to cleavage by mammalian collagenase, as described above. The cleavage products of the collagenase were then examined on slab gel electrophoresis in sodium dodecyl sulfate employing radioautographic detection technique. [^{35}S]Cystine could be detected in both extension peptides of procollagen, whereas [^{3}H]tryptophan was detected only at the carboxy-terminal end of the molecule (not shown). Incubation of the cleavage products with pepsin at a temperature at which the α^{A} and α^{B} fragments of collagen produced by mammalian collagenase retained their helical conformation completely removed the radioactivity from the cleavage products. The results indicate that the nonhelical carboxy-terminal extension contains both cystine and tryptophan, whereas the amino-terminal nonhelical extensions contain only cystine.

Discussion

Matrix-free cells prepared by controlled enzymic digestion of several tissues from 17-day old chick embryos are particularly useful for studies on the biosynthesis of collagen, since essentially all radioactive protein synthesized when the cells are incubated with radioactive proline is collagenous (for discussion on matrix-free cell systems, see Prockop et al., 1976). Previous studies employing matrix-free cells have demonstrated that cells prepared from aortae and associated large blood vessels of 17-day old chick embryos synthesize and secrete procollagen which is predominantly of genetic type I (Uitto et al., 1976b), while cells obtained from sternal cartilages synthesize procollagen of type II (Dehm and Prockop, 1973; Uitto et al., 1976c). The most extensively used matrix-free cell system employs cells which are isolated from leg tendons of 17-day old chick embryos (Dehm and Prockop, 1971, 1972). In this study we have demonstrated that essentially all collagenous protein synthesized and secreted by these cells is of genetic type I.

The precursors of type I collagen isolated from the incubation medium of tendon cells were shown to exist in two different forms. Most of the [^{14}C]procollagen consisted of molecules which contained peptide extensions at the carboxy-terminal and amino-terminal ends. Previous studies using the same tendon cell system have demonstrated that the precursor polypeptides recovered in the medium have the same apparent molecular size as the intracellular chains which have been completed and released from the ribosomes (Uitto and Prockop, 1974c,d). It appears that the newly synthesized protein isolated in peak B in DEAE-cellulose chromatography represents intact procollagen. Demonstration of extension peptides at both ends of the procollagen molecules synthesized and secreted by chick embryo tendon cells confirms previous observations that procollagen synthesized by chick calvaria or cultured fibroblasts is composed of molecules which have peptide extensions at both the carboxy-terminal and amino-terminal ends (Tanzer et al., 1974; Park et al., 1975; Byers et al., 1975; Fessler et al., 1975). Although radioactive cystine was incorporated into both extensions, only the carboxy-terminal extension contained interchain disulfide bonds. Another amino acid which is not present in type I collagen, tryptophan, was also incorporated into carboxy-terminal extension peptides but not to the amino-terminal extensions. The results confirm previous observations that the amino acid composition of the extension peptides of procollagen is different from that of the collagen portion of the molecule (Bornstein, 1974; Martin et al., 1975) and that the interchain disulfides which link the three pro-α chains of procollagen are present at the carboxy-terminal end of the molecule (Byers et al., 1975; Fessler et al., 1975).

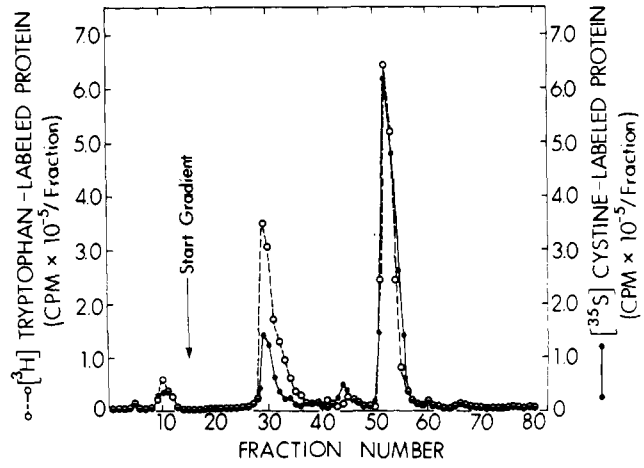

FIGURE 10: Incorporation of radioactive tryptophan and cystine into the two precursor forms of collagen recovered in the medium of tendon cells. Cells (2.3 × 10^{8}) were incubated with 300 μCi of [^{3}H]tryptophan or with 100 μCi of [^{35}S]cystine for 2 h in the modified Krebs medium containing 20% fetal calf serum and 50 μg/ml ascorbic acid. The radioactive protein in the medium was then isolated and chromatographed on DEAE-cellulose column as indicated in the text and Figure 2.

A small portion of the precursors of type I collagen were shown to contain the carboxy-terminal extensions but were devoid of amino-terminal extension peptides. These molecules represent a partially modified form of procollagen, and their existence has also been reported in other systems synthesizing procollagen and collagen (Byers et al., 1975; Fessler et al., 1975; Lichtenstein et al., 1975). Our previous studies with intact tendons from 17-day old chick embryos have demonstrated that the initially synthesized procollagen containing extension peptides at both ends of the molecule is readily converted to the intermediate form devoid of amino-terminal extensions and then to collagen (Uitto et al., 1976a; Uitto and Lichtenstein, 1976b). The matrix-free cell system employed here, however, was shown to lack enzymic activity which cleaves the carboxy-terminal extensions, but a time-dependent removal of the amino-terminal extensions occurred in the medium (Uitto and Lichtenstein, 1976a,b). The results, therefore, suggest that the intermediate form of collagen precursor detected in peak A in DEAE-cellulose chromatography is produced by the removal of the amino-terminal extensions by a secreted enzyme.

Several previous studies have demonstrated that many tissues contain type III collagen in association with type I collagen (Chung and Miller, 1974; Chung et al., 1974; Epstein, 1974; Trelstad, 1974; Byers et al., 1974). Also, cultured human skin fibroblasts (Lichtenstein et al., 1975) or cloned fibroblasts from dermatosparactic calves (Church et al., 1974) synthesize both type I and type III collagens. Although skin from 8-week old chicks contains type III collagen (Herrmann and von der Mark, 1975), the synthesis of type III collagen by embryonic chick skin appears to occur primarily prior to the 13th day of embryonic development (Vinson and Seyer, 1974). Here we failed to detect any significant amounts of newly synthesized type III collagen or procollagen in the incubation medium. The present study, therefore, provides conclusive evidence that fibroblasts can synthesize a single genetic type of collagen. Since the existence of type III collagen in chick embryo tendons has not been reported, it is possible that tendon cells do not synthesize type III procollagen, and the fibroblasts in tendon would, therefore, be different from skin fibroblasts. Alternatively, skin and tendon fibroblasts represent the same cell type,

but there are tissue specific factors which control the genetic types of collagen synthesized by the fibroblasts.

Note Added in Proof

Following the submission of this manuscript, a paper from another laboratory was published providing further evidence to the conclusion made here that tendon procollagen contains both amino- and carboxy-terminal extensions and that the interchain disulfide bonds are located in the carboxy-terminal region (Olsen et al., 1976).

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