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Cell-Free Synthesis of Procollagen: L-929 Fibroblasts as a Cellular Model for Dermatosparaxis

(procollagen peptidase/polysomes/protein synthesis)

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ABSTRACT **A cell-free system that actively synthesizes collagen was prepared from L-929 fibroblasts. Chromatographic and electrophoretic techniques were used to demonstrate that the only collagenous products are pro α_1 and pro α_2 chains. The collagen synthesized by the cell-free system was also compared to the collagen extracted from the cells. The cellular collagen was composed of aggregates of pro-α chains, while no α chains were found. Procollagen peptidase activity could not be detected in the cells, and the activity present in the medium was low, comparable to that in dermatosparaxic cell cultures. These properties indicate that L-929 cells may be a model system for dermatosparaxis.**

Most collagen molecules contain three polypeptide chains of two different kinds, α_1 and α_2, present in a 2:1 ratio. Recent studies from several laboratories have indicated that precursor forms of these chains (pro α_1 and pro α_2) are the initial synthetic products and that these forms contain an additional peptide at their NH_2-termini (1–8). Procollagen, which contains the precursor chains in a triple helical arrangement, is a more soluble form of collagen and has been proposed as the structure transported from the intracellular to extracellular space (3, 9). Before its extracellular deposition, the extra NH_2-terminal peptides are cleaved by a specific enzyme, procollagen peptidase, to form the collagen triple helix containing α_1 and α_2 chains (10).

Studies by Gribble *et al.* (11) have shown that L-929 fibroblasts grown in culture actively synthesize collagen. The [^{14}C]proline incorporated into collagen paralleled cell growth, and the collagen that was synthesized was partially secreted into the culture medium. In the present report the collagen extractable from L-929 cells is characterized, and a cell-free system prepared from L-929 fibroblasts and active in the synthesis of precursor chains is described. No α chains could be detected in the extracted collagen, and the pro α_1 and pro α_2 chains extracted from the cells were present in an aggregated form stabilized by disulfide bonds. This aggregated form of pro α chains could be converted to pro α_1 and pro α_2 by reduction and carboxymethylation. Pro α_1 and pro α_2 chains were also the only collagenous proteins synthesized by L-929 polysomes that were active in the synthesis of collagen. Maximal synthesis of these precursor chains by the polysomes required a high-speed supernatant fraction and a ribosomal salt wash. Unlike the pro α chains extracted from the cells, however, the pro α_1 and pro α_2 chains synthesized by the polysomes were not in an aggregated form.

The observation that collagen extracted from L-929 cells contains only pro α chains is consistent with the finding that only low amounts of procollagen peptidase are present in the medium. Since similarly low amounts are found in cultures of dermatosparaxic cells, L-929 cells may serve as a model system for this genetic disease of cattle (3).

Abbreviations: CM, carboxymethyl; RSB, reticulocyte-swelling buffer.

MATERIALS AND METHODS

Uniformly labeled [^{3}H]proline (4.7 Ci/mmol) and [^{14}C]-proline (285 Ci/mol) were obtained from New England Nuclear Corp., Boston, Mass. Bacterial collagenase, free of nonspecific proteases, was a product of Advance Biofactures, Lynbrook, N.Y. L-929 fibroblasts were obtained as monolayers from Microbiological Associates, Bethesda, Md. Dermatosparaxic collagen was extracted from the skin of dermatosparaxic calves and was a gift from Dr. Charles Lapière, University of Liège, Belgium.

Analysis of Collagen of L-929 Cells. 25 μCi of [^{14}C]proline was added to confluent L-929 fibroblasts. After an additional incubation for 3 hr, the cells were harvested by scraping and washed three times with Puck's saline A; the collagen was extracted with hot trichloroacetic acid (12). The hot acid-extracted material was extensively dialyzed against 20 mM sodium acetate buffer (pH 4.8) containing 1 M urea. One aliquot of the dialyzed material was analyzed for collagen by carboxymethyl (CM)–cellulose chromatography as described below. The pH of the other aliquot was adjusted to 7.0, and 2-mercaptoethanol was added to a concentration of 0.1 M. After addition of Triton X-100 to a concentration of 0.1%, the material was heat denatured at 60° for 30 min. The sample was brought to room temperature, and iodoacetamide was added to a concentration of 0.2 M. After incubation for 60 min at 37°, 10 mg of carrier collagen was added. The sample was dialyzed overnight against 20 mM sodium acetate (pH 4.8) buffer containing 1 M urea. The control and the reduced, carboxymethylated sample were heat denatured at 60° for 30 min; the clear material was loaded on a 1.5 × 10 cm CM–cellulose column. The column was developed with a gradient containing 150 ml of 20 mM sodium acetate buffer (pH 4.8) containing 1 M urea in the mixing chamber and 150 ml of 20 mM sodium acetate–urea buffer (pH 4.8) containing 0.1 M NaCl in the reservoir. The column was developed at 42° (13), and fractions of 3 ml were collected.

Fractions were assayed for radioactivity (14) and for absorbance at 226 nm.

Preparation of Polysomes. L-929 fibroblasts were grown in suspension in 10-liter carboys containing minimal essential medium (F-13) supplemented with 10% fetal-calf serum. Cells were grown to a density of 4×10^5 per ml. At this time, crushed frozen Earle's basal medium was added to rapidly chill the culture. Cells were harvested by centrifugation at $2000 \times g$ for 5 min. The pelleted cells were washed three times with Earle's balanced salt solution and resuspended in a reticulocyte-swelling buffer (RSB), 10 mM Tris·HCl (pH 7.4)–10 mM NaCl–3 mM $MgCl_2$, at a final concentration of 2×10^8 cells per ml. After addition of NP-40 to a final concentration of 0.5%, the cells were lysed by gentle homogenization in a Dounce homogenizer. $MgCl_2$ and KCl were added to a final concentration of 10 mM and 0.25 M, respectively, and the homogenate was centrifuged at $30,000 \times g$ for 10 min. The postmitochondrial supernatant (20 ml) was layered on a 10–30% glycerol gradient containing 10 mM Tris·HCl (pH 7.4)–10 mM $MgCl_2$–0.24 M KCl–1 mM dithiothreitol. After centrifugation at 27,000 rpm for 150 min in an SW-27 rotor, 50% glycerol was pumped through the bottom of the tube. Fractions (1 ml) were collected from the top. Fractions 1–12 (light), 13–26 (medium), and 27–37 (heavy) were pooled. The polysomal pellet was pooled with the heavy fraction. The various polysomal fractions were centrifuged at $265,000 \times g$. The polysomal pellets were suspended in a buffer containing 50 mM Tris·HCl (pH 7.4)–10 mM KCl–5 mM $MgCl_2$–1 mM dithiothreitol and assayed for their ability to synthesize procollagen.

Preparation of L-929 Ribosomal Salt Wash and the S-30 (Supernatant) Fraction. About 4×10^9 cells were suspended in 50 ml of RSB and allowed to swell at 4° for 60 min. The cells were homogenized in a Dounce homogenizer and centrifuged at $4000 \times g$ to remove nuclei. The supernatant was adjusted to 20 mM Tris·HCl (pH 7.4)–10 mM $MgCl_2$–0.24 M KCl–1 mM dithiothreitol and centrifuged at $30,000 \times g$ for 15 min. An aliquot of the supernatant fraction (S-30) was saved and used as a source of soluble components required for protein synthesis. To the remainder, potassium fluoride was added to a concentration of 15 mM and incubated at 37° for 10 min. After centrifugation at $265,000 \times g$ for 2 hr, the ribosomal pellet was washed with 5 ml of buffer containing 10 mM Tris·HCl (pH 7.4)–0.5 M KCl–1 mM $MgCl_2$–1 mM dithiothreitol. Ribosomes were removed by centrifugation at $265,000 \times g$ for 1 hr, and the supernatant fraction was dialyzed for 12 hr against buffer containing 10 mM Tris·HCl (pH 7.4)–100 mM KCl–0.1 mM EDTA. The dialyzed ribosomal salt wash was used as a source of chain-initiation factors.

TABLE 1. *Analysis of the various polysome fractions for activity in collagen synthesis*

Polysome fraction	pmol [^{3}H]proline incorporated into protein	
	−Collagenase	+Collagenase
Light	2.3	2.3
Medium	3.2	3.0
Heavy	6.0	4.6

The amount of radioactivity solubilized after collagenase treatment is used as a measure of collagen synthesis.

TABLE 2. *Requirements for protein and collagen synthesis by the heavy polysome fraction*

	pmol [^{3}H]proline incorporated into	
	Total protein	Collagen
Complete	5.4	1.6
− S-30	0.8	0.25
− Ribosomal salt wash	2.1	0.4
− tRNA	4.7	1.1

The amount of radioactivity retained by the filters after incubations in the absence of polysomes (0.5 pmol) was used as a blank. Other details of the assay are described in the text.

Assays for Procollagen Synthesis. The reaction mixture in a total volume of 0.05 ml contained 20 mM Tris·HCl (pH 7.4), 5 mM $MgCl_2$, 100 mM KCl, 2 mM ATP, 0.4 mM GTP, 3 mM phosphoenolpyruvate, 6 μg of pyruvate kinase, 1 mM dithiothreitol, 0.4–0.8 A_{260} units of polysomes, 0.8 mM each of a mixture of 19 amino acids (minus proline), 10 μCi of [^{3}H]proline (2 nmol), 130 μg of protein from the S-30 fraction, 120 μg of the ribosomal salt wash, and 50 μg of tRNA (yeast and rabbit liver). After incubation at 37° for 30 min, 12.5 units of purified collagenase were added to one set of samples. The control and the collagenase-treated samples were incubated for an additional 30 min at 37°. To all samples, 0.1 ml of 1 M Tris·HCl (pH 10.0) was added to deacylate [^{3}H]prolyl-tRNA; after incubation for 10 min at 37°, cold 5% trichloroacetic acid was added. The amount of labeled proline incorporated into the synthesized protein and into collagen or procollagen was assayed by filtering samples through a nitrocellulose filter (0.45 μm) (Millipore Corp., Bedford, Mass.) (7). The filter was washed at least three times with cold 5% trichloroacetic acid, dried, and then assayed for radioactivity with an Omnifluor liquid scintillation counting fluid. In this assay, a decrease in radioactivity attached to the filter after collagenase treatment was a preliminary measure of procollagen or collagen synthesis.

Analysis of the In Vitro Polysomal Product. The incubation mixture containing heavy polysomes was scaled up 3-fold (0.15 ml). After addition of 0.3 ml of 1 M Tris·HCl (pH 10.0), the mixture was incubated for 10 min at 37°. 5 ml of 20 mM sodium acetate buffer (pH 4.8) containing 1 M urea and 10 mg of carrier collagen was added, and the mixture was dialyzed against this buffer. The dialyzed material was freed of polysomes and ribosomes by centrifugation at $265,000 \times g$ for 1 hr. After the mixture was denatured at 60° for 30 min, the clear material was analyzed by CM-cellulose chromatography as described above.

Acrylamide gel electrophoresis was done by the method of Sakai and Gross (15). Proline and hydroxyproline content of the collagenous protein was analyzed after acid hydrolysis in a Beckman Amino Acid Analyzer. A purified preparation of procollagen peptidase was prepared by the method of Lapière *et al.* (10).

RESULTS

Analysis of the Collagen Synthesized In Vitro by L-929 Polysomes. Of the three polysome fractions prepared from

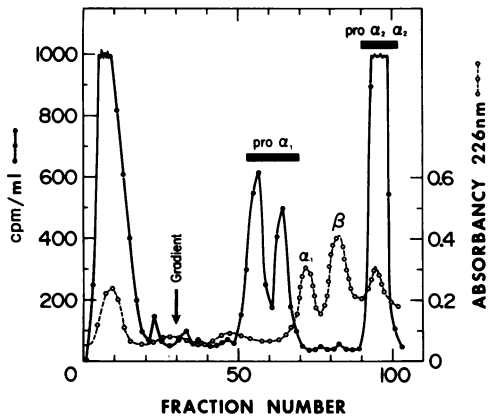

FIG. 1. CM-cellulose chromatography of the product synthesized by the heavy polysome fraction. The dialyzed reaction mixture containing 6.1×10^4 cpm of proline incorporated into protein was applied on the column. Before the gradient was applied, 2.5×10^4 cpm was eluted. After the gradient was started, 7.5×10^3 cpm was eluted in the area marked pro α_1 and 1.8×10^4 cpm eluted in the pro α_2/α_2 region. As seen in Fig. 2, 18% of this material represents pro α_2.

L-929 fibroblasts, only those polysomes that had sedimented in the heavy region of the glycerol gradient were active in collagen synthesis (Table 1). With the collagenase assay as a measure of collagen synthesis, 24% of the protein synthesized by these polysomes represented collagenous material.

The requirements for collagen synthesis by the heavy polysome fraction were investigated. Maximal synthesis was dependent upon the addition of both the S-30 fraction and the

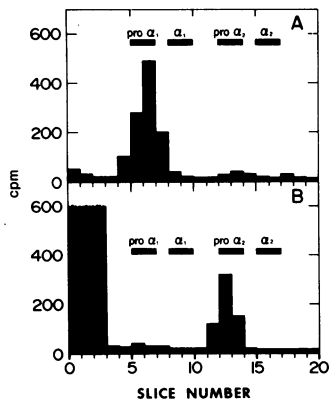

FIG. 2. Acrylamide gel electrophoresis of the column peaks labeled pro α_1 and pro α_2/α_2. The column peaks (Fig. 1) were concentrated by ultrafiltration (Amicon UM-10 filters). To 0.05 ml of the concentrated material was added 50 μg of dermatosparaxic collagen. After heat denaturation at 60° for 30 min, the samples were layered on gels. *Panel A* shows that CM-cellulose column peaks labeled pro α_1 (2400 cpm) coelectrophoresed with carrier pro α_1 of dermatosparaxic collagen. *Panel B* shows the analysis of CM-cellulose (Fig. 1) peak labeled pro α_2/α_2 (5700 cpm applied). Electrophoresis was performed for 4 hr, and the gels were stained with Amido-Schwarz. The bands corresponding to dermatosparaxic collagen are shown in the top of the figure. The gels were sliced manually and digested with NCS tissue solubilizer (19). Recovery of radioactivity after electrophoresis was about 40%. About 18% of the counts applied in *panel B* migrated with pro α_2. No radioactivity was detected in either α_1 or α_2 regions of the gel.

TABLE 3. *CM-cellulose analysis of pro α_1 before and after digestion with procollagen peptidase*

	cpm eluting as	
	pro α_1	α_1
Before digestion	2480	0
After digestion with procollagen peptidase	140	2508

5900 cpm of proline-labeled pro α_1 (Fig. 3*A*) was divided into two equal aliquots. One aliquot was treated with procollagen peptidase and the other aliquot served as control. After addition of carrier collagen, both samples were reanalyzed by CM-cellulose chromatography as described in the text.

ribosomal salt wash (Table 2). Since a requirement of a ribosomal salt wash (which presumably contains chain initiation factors) can be used as an indication of chain initiation (16), it appears that these polysomes were able to complete nascent chains and initiate synthesis of new polypeptide chains.

The collagenous protein synthesized by these polysomes was first analyzed by CM-cellulose chromatography. The column was loaded with 6.1×10^4 cpm of the proline-labeled protein synthesized by the polysome fraction. As seen in Fig. 1, a large peak of radioactivity was not retained on the column and two peaks of radioactivity eluted before carrier α_1‡ in the pro α_1 area§. No radioactive material was present in the α_1 region, but a large peak of radioactivity eluted in the pro α_2/α_2 area of the column. To further characterize the column peak labeled pro α_1 (Fig. 1), this material was pooled and dialyzed against 0.5 M acetic acid for 12 hr. The dialyzed protein was concentrated to dryness by lyophilization and incubated with purified prolyl hydroxylase as described by Rhoads *et al.* (17). After dialysis against 0.5 M acetic acid, the lyophilized protein was hydrolyzed with 6 N HCl for 18 hr and assayed for hydroxyproline and proline. The hydroxyproline/proline ratio of this material was 0.63.

Additional characterization of column peaks labeled pro α_1 and pro α_2/α_2 (*black bars*, Fig. 1) was obtained as follows. The peaks were separately pooled and concentrated. After addition of carrier dermatosparaxic collagen as a marker, the concentrated material was analyzed by acrylamide gel electrophoresis by the method of Sakai and Gross (15). The radioactivity (7.5×10^3 cpm) contained in the CM-cellulose peaks labeled pro α_1 coelectrophoresed as one band with pro α_1 of dermatosparaxic collagen (Fig. 2*A*), whereas the radioactivity contained in the CM-cellulose peak labeled pro α_2/α_2 gave two bands on electrophoresis (Fig. 2*B*). A major portion of the pro α_2/α_2 area radioactivity was retained on top of the gel, but 18% (3.2×10^3 cpm) of the radioactive material coelectrophoresed with carrier pro α_2. No radioactivity was detected in the α_2 region of the gel. These results

‡ Previous data have recorded two peaks of pro α_1 in dermatosparaxic procollagen samples (3). At present there is no explanation for this finding; however, both peaks have the same amino-acid composition and behavior on analytical disc gel electrophoresis (3).

§ The pro α_1 and pro α_2/α_2 areas have been defined by chromatography of dermatosparaxic procollagen standards (3).

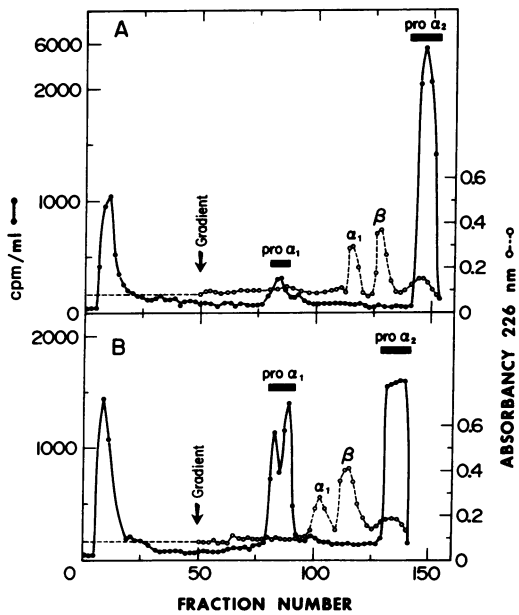

FIG. 3. CM-cellulose chromatography of the collagen extracted from L-929 fibroblasts. *Panel A* presents analysis before reduction and carboxymethylation. *Panel B* shows the chromatographic analysis after reduction and carboxymethylation of the extracted collagen. Recovery of radioactivity from these columns was about 70%.

indicate that pro α_1 and pro α_2 were the only collagenous proteins synthesized by the polysomes.

Analysis of Cellular Collagen. The labeled collagenous protein extracted from cells grown in the presence of [^{14}C]-proline was analyzed by chromatography on CM-cellulose. The column was loaded with 9.8×10^4 cpm. As seen in Fig. 3*A*, 15% of the radioactive material was not retained on the column, only 5% of the radioactivity was present as pro α_1, and at least 50% of the radioactive material was eluted coincident with or just after the α_2 standard. No radioactivity was detectable in the α_1 region of the column. The radioactive material eluting in the pro α_1 region of the column (Fig. 3*A*) was dialyzed against 0.5 M acetic acid and lyophilized. The lyophilized material was dissolved in 10 mM Tris·HCl buffer (pH 7.4) and one half of the material was digested with procollagen peptidase for 4 hr at 20° (10). The digested and the control samples were again analyzed by CM-cellulose chromatography. As seen in Table 3, digestion of pro α_1 with procollagen peptidase results in its conversion to α_1.

Since both pro α_1 and pro α_2 chains of procollagen contain half-cystine residues (3, 4, 8, 9), and since these half-cystine residues might cause aggregation and altered chromatographic properties of pro α chains by disulfide bond formation (8, 9), the collagen extracted from the cells was reduced, carboxymethylated, and reanalyzed by CM-cellulose chromatography. As seen in Fig. 3*B*, 15% of the radioactive material eluted in the pro α_1 region rather than 5%, and less than 35% of the material eluted in the pro α_2/α_2 area[¶]. The hydroxyproline/proline ratio of the material eluting in the pro α_1 region was 0.31 and that eluting in the pro α_2/α_2 region was 0.34. Similarly, low ratios for hydroxyproline/proline have been obtained by Peterkofsky (18) for collagen extracted from L-929 cells grown in the absence of ascorbate. Analogous CM-cellulose column data were obtained before and after reduction and carboxymethylation on the collagenous protein extracted from the medium in which the L-929 cells were labeled with proline. These results would suggest that precursor forms synthesized by the L-929 polysome system *in vitro* were nonaggregated pro α chains, whereas the precursor forms synthesized *in vivo* and extracted from L-929 cells or extruded into the medium existed as aggregated chains stabilized by disulfide bonds.

[¶] It should be noted that the amount of radioactivity eluting in the pro α_2 is much higher than that in pro α_1. No explanation is offered at the present time for this finding.

DISCUSSION

The present report describes a cell-free system derived from L-929 fibroblasts, which is active in the synthesis of procollagen. The synthesis is dependent on the addition of a homologous S-30 fraction and on a ribosomal salt wash, the latter dependence suggesting that reinitiation occurred. In a chick embryo polysomal system, which also has been shown to synthesize procollagen (7), 10–15% of the total synthetic product was collagenous compared to the 24% found in the present system derived from L-929 cells.

Dermatosparaxis is a genetic disease of cattle characterized by an extreme fragility of the skin and associated with an inability to form normal collagen fibers (20–23). Studies by Lapière *et al.* have shown that the collagen extracted from the skin of these calves contains high amounts of pro α_1 and pro α_2 chains (3) and that this abnormality is due to the absence of procollagen peptidase, an enzyme that removes the amino-terminal peptides from the pro α chains to yield α chains (10). The L-929 fibroblasts would appear to be similar to dermatosparaxic tissues. As in dermatosparaxis, the L-929 cells do not form normal fibers (Dr. S. Udenfriend, personal communica-

TABLE 4. *Procollagen peptidase activity in the cells and medium of L-929 fibroblast cultures*

Cell culture	Procollagen peptidase activity	
	Medium	Cells
	Relative enzyme activity	
3T3	100	Nondetectable
3T6	123	Nondetectable
L-929	14*	Nondetectable

Procollagen peptidase activity was measured and quantitated as described (10). 5-ml Aliquots of the medium were concentrated 10-fold, and the concentrates were tested for activity at multiple enzyme concentrations. Cells from four 250-ml flasks were harvested by scraping, sonicated, and assayed at several protein concentrations. The amount of activity in 3T3 medium served as the 100% value to compare activities in the different fibroblast systems. The medium was in contact with confluent cells for 4 days. Activity was corrected for the volume of medium, the number of cells in the flask at the time of harvesting, or the protein concentration of the media. Independent of the correction method used, the relative activity values were not significantly altered.

* Tissue cultures of dermatosparaxic cells have been shown to have similarly low levels of peptidase activity (personal communication, Dr. Charles Lapière).

tion) and have in their media and cells large amounts of pro α chains. Preliminary studies also indicate a very low amount of procollagen peptidase in the media of these cells as opposed to the media from other fibroblast systems (Table 4). This analogy to dermatosparaxic animals and the high percentage of collagenous protein in their *in vitro* polysomal product would seem to confirm previous suggestions by Gribble *et al.* that L-929 cells might provide an excellent system for studies of collagen biosynthesis, biosynthetic control, and genetic abnormalities such as dermatosparaxis (11).

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