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Production of Procollagen by Human Fibroblasts in Culture

(collagen synthesis/connective tissue/chromatography/disulfide linkage)

BARBARA D. SMITH, PETER H. BYERS, AND GEORGE R. MARTIN

National Institutes of Health, National Institute of Dental Research, Bethesda, Maryland 20014

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ABSTRACT **Three hydroxyproline-containing proteins secreted into the medium by human fibroblasts in culture were isolated and characterized. A minor fraction was identical to the collagen monomer. The major fraction was a form of procollagen, which contained, in addition to pro α and α chains, a component estimated to have a molecular weight of 250,000. This component was a dimer of pro α chains joined by disulfide bonds. The third fraction, much lower in hydroxyproline and hydroxylysine content, was of still greater size. Pro α chains were released upon denaturation and reduction, indicating that this fraction may contain pro α chains linked by disulfide bonds to noncollagenous material.**

While studying collagen synthesized by human skin fibroblasts in culture, Layman *et al.* (1) found that these cells secreted an unusual collagen into the medium that was more soluble than normal collagen and that, upon denaturation, yielded components larger than α chains. Incubation with pepsin caused a limited digestion that converted this protein into a collagen-like molecule. They suggested that this medium protein was a precursor form of collagen possibly involved in transport. Since then, precursors of collagen have been observed in several preparations, including organ culture (2, 3), cultures of cells freed from tendon with enzymes (4), and in tissues of calves with dermatosparaxis (5). The precursors of the α chains, designated pro α1 and pro α2, are about 5–15% larger than α chains, although larger precursors have also been reported (6, 7). We have now been able to isolate the proteins secreted into the medium by fibroblasts and to establish their relation to the other precursors of collagen.

MATERIALS AND METHODS

Fibroblasts were obtained from the bank of normal human cells maintained by the American Type Culture Collection, Rockville, Md., and were cultured as described (1). To prepare labeled medium protein, confluent cultures were rinsed with Dulbecco–Vogt medium (7*a*) lacking glutamine and serum, but supplemented with 50 μg/ml of β-amino propionitrile·HCl and 50 μg/ml of ascorbic acid. The cells were then incubated in 10 ml of the same medium to which was added 20 μCi per flask of [*U*-^{14}C]proline, [*U*-^{14}C]lysine, and [*U*-^{14}C]glycine. 24 hr later the medium was removed and dialyzed against 1 M NaCl–50 mM Tris·HCl–20 mM EDTA (pH 7.4) at 4°. For separation of collagen from procollagen and other proteins in the medium, samples were dialyzed against 10 mM Tris·HCl (pH 7.4)–2 M urea and chromatographed on DEAE–cellulose at 10° as described by Müller *et al.* (8), with a linear salt gradient from 0 to 0.3 M NaCl over a total volume of 600 ml. Collagen and procollagen chains in denatured samples were fractionated by carboxymethyl (CM)–cellulose chromatography as described by Piez *et al.* (9), except that the pH 4.8 acetate buffer used as the starting buffer was 0.05 N in Na^+. Molecular sieve chromatography of denatured proteins was performed with 8% agarose in 1 M $CaCl_2$–50 mM Tris·HCl at pH 7.5 as described by Piez (10). Some samples isolated by DEAE–cellulose chromatography were studied in the denatured state after treatment with 8 M urea and 10 mM 2-mercaptoethanol in 50 mM Tris·HCl (pH 7.4) to reduce disulfide bonds. Total radioactivity was determined by liquid scintillation counting. [^{14}C]Proline, [^{14}C]hydroxyproline, [^{14}C]lysine, and [^{14}C]-hydroxylysine in the various chains and proteins were measured after hydrolysis and separation on an automatic amino-acid analyzer by continuously monitoring the column effluent (11).

RESULTS

Initial attempts to characterize the hydroxyproline-containing proteins in the medium by size revealed a complex mixture. Further study was hampered by low recoveries from ion-exchange columns and by a lack of adequate techniques for separating collagen from its precursor. After modification of the CM–cellulose chromatography by lowering the ionic strength of the sodium acetate buffer to 0.05, some pro α1 could be demonstrated, but the recovery of hydroxyproline-containing proteins remained low. These problems were resolved when it was found that native collagen samples, prepared from rat or chick, could be separated from native procollagen, as well as from denatured collagen, by DEAE–cellulose chromatography (8). Therefore, we initially fractionated the proteins in the medium by DEAE–cellulose chromatography. Three radioactive peaks, designated 1, 2, and 3, were eluted by the salt gradient (Fig. 1). Each contained hydroxyproline and hydroxylysine (Table 1). Peak 2 accounted for 70–75% of the peptide-bound hydroxyproline in the medium, while the remaining peaks each contributed about 10–15%.

Several experiments indicated that the material in peak 1 was native collagen. First, it eluted from the DEAE–cellulose column at the same salt concentration as native collagen from other sources (between 10 and 50 mM NaCl). Second, α1 and α2 chains were obtained in good yield and in a 2:1 ratio when the material was denatured and chromatographed on CM–cellulose (not shown). Third, the denatured protein yielded a single radioactive peak on molecular sieve chromatography that exactly coincided with authentic α chains from acid-extracted rat-skin collagen (not shown). Finally, the measured ratio of hydroxyproline to proline ranged from 0.8 to 0.97 in several experiments.

TABLE 1. *^{14}C-Labeled amino acids in medium proteins*

Fraction*	Hydroxyproline (cpm)	Proline (cpm)	Hydroxylysine (cpm)	Lysine (cpm)	Hydroxyproline / proline	Hydroxylysine / lysine
Peak 1	5,200	6,600	1,800	9,500	0.80	0.19
Peak 2	38,000	53,000	14,000	54,000	0.71	0.25
Peak 3	9,000	55,000	2,800	59,000	0.16	0.05

* See Fig. 1.

With similar techniques, the material in peak 2 was identified as procollagen. It eluted from the DEAE–cellulose column in the position observed for procollagen from other species (100–150 mM NaCl). The ratio of hydroxyproline to proline was slightly lower than that in collagen, ranging from 0.71 to 0.8. When the radioactive material in peak 2 was chromatographed with lathyritic rat-skin collagen carrier on a CM–cellulose column after denaturation, radioactivity was found in pro α1, pro α2 (including α2), and α1 (Fig. 2, *top*). However only 50–60% of the radioactivity applied was recovered in these peaks, and the ratio of α1 plus pro α1 to α2 plus pro α2 was less than 2:1. Two distinct molecular weight species were observed when the same material was applied to a molecular sieve column (Fig. 3, *top*): a 250,000 molecular weight fraction and a broad peak that chromatographed in part with, and in part ahead of, authentic α chains. Since cysteine has been found in procollagen (5, 10–12), it seemed possible that the high molecular weight peak consisted of dimers of pro α chains linked by disulfide bonds. To test this hypothesis, we reduced aliquots of peak 2 material with 2-mercaptoethanol in 8 M urea. The 250,000 molecular weight fraction previously observed largely disappeared and was replaced by additional material of about 120,000 molecular weight (Fig. 3, *bottom*). After reduction, the radioactivity in reduced peak 2 samples was recovered in good yield (80%) (Fig. 2, *bottom*) by CM–cellulose chromatography. The increased recovery after reduction was due entirely to an increase in the pro α1 peak, yielding a 2:1 ratio of α1 plus pro α1 to α2 plus pro α2. From these data we concluded that peak 2 contained procollagen in which about half of the pro α1 chains were linked as dimers by disulfide bonds.

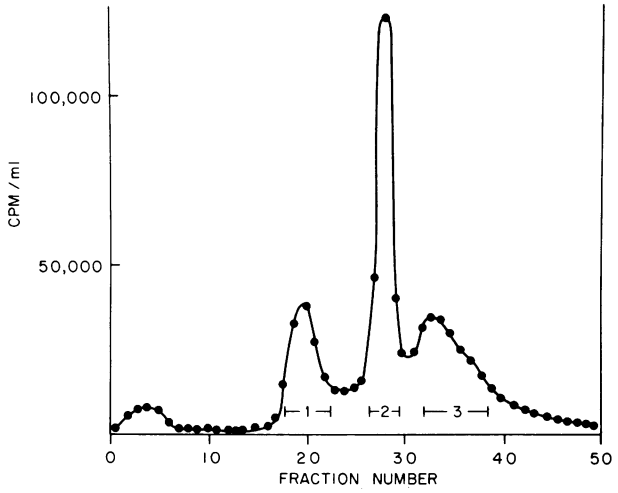

FIG. 1. Separation of medium proteins by DEAE–cellulose chromatography.

The material in peak 3 had a still-lower ratio of hydroxyproline to proline (0.16–0.28). The denatured protein yielded a single peak on molecular sieve chromatography that emerged in the exclusion volume of the column and still had a low ratio of hydroxyproline to proline (0.09–0.15). When material from peak 3 was denatured with 8 M urea in the presence of 2-mercaptoethanol, we observed an additional radioactive peak identical in its migration on molecular sieve columns to pro α chains (not shown). Denaturation with 8 M urea alone did not change the migration of peak 3. We concluded that peak 3 contained noncollagenous material linked to pro α chains, possibly by disulfide bonds.

DISCUSSION

The data presented here indicate that most of the peptide-bound hydroxyproline found in the medium is a form of procollagen. Small amounts of hydroxyproline are found in collagen, as well as in a larger molecular weight fraction. The procollagen fraction, when denatured, contains pro α chains, α chains, and a component that appears to be a disulfide-linked dimer of pro α1. In this respect, it differs from the pro-

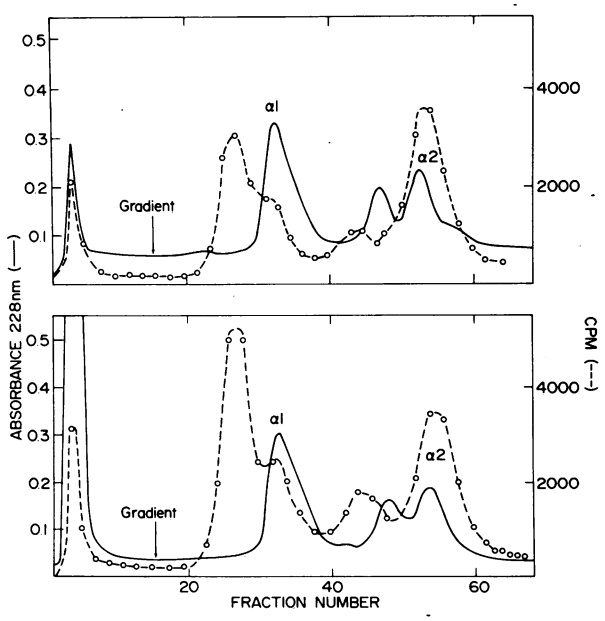

FIG. 2. CM–cellulose chromatography of denatured procollagen from medium (peak 2 from DEAE–cellulose chromatography) (O– – –O); plus carrier lathyritic rat-skin collagen (*solid line*). (*Top*) Before reduction with 2-mercaptoethanol; (*Bottom*) after reduction with 2-mercaptoethanol.

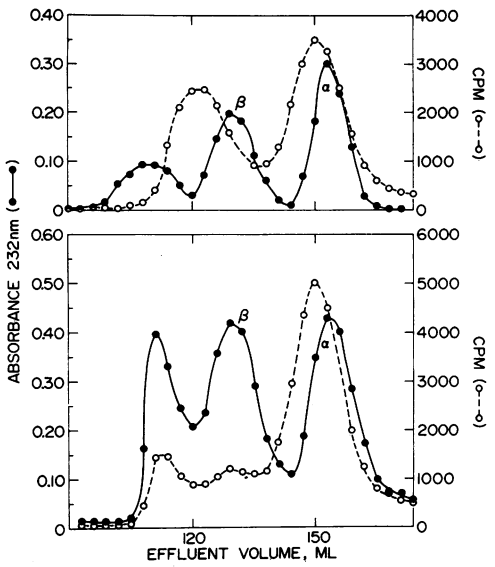

FIG. 3. Molecular sieve chromatography of denatured procollagen from medium (peak 2 from DEAE-cellulose chromatography) (O- - -O); plus denatured acid-extracted rat-skin collagen carrier (●——●). (*Top*) Before reduction with 2-mercaptoethanol; (*Bottom*) after reduction with 2-mercaptoethanol.

collagen reported in some other systems (2, 3), which can be converted to pro α chains solely by denaturation. The preferential incorporation of pro α1 chains into these dimers may be explained by the higher content of cysteine in pro α1 than in pro α2 (5, 12, 13).

A wide range of molecular weights has been reported for collagen precursors. While an exact size cannot yet be given to pro α chains due to anomalies in the behavior of collagen in comparison to other proteins, many estimates are in the range of 120,000, so that procollagen would be about 360,000. While collagen was well resolved from procollagen on DEAE-cellulose, peak 2 contained both proα and α chains. We suggest that hybrid molecules composed in part of pro α and in part of α chains cochromatograph with procollagen. These hybrid molecules may arise as a result of incomplete conversion of procollagen to collagen or by cleavage with nonspecific proteases. It is also possible that the conversion of procollagen to collagen does not occur as a single step. Proteins that may represent intermediates in the conversion of pro α1 to α1 chains have been observed in tissues of dermatosparaxic cattle (5) and in pulse-labeled calvaria extracts (14).

The possibility that precursors of collagen larger than pro α chains might arise from disulfide linkage of precursor chains was suggested by Dehm *et al.* (15) and Fessler *et al.* (16). Church *et al.* (7) have also reported a high molecular weight, hydroxyproline-containing fraction and have suggested that this may be an early precursor much larger than pro α chains, possibly a single chain containing sequences corresponding to both pro α1 and pro α2. Since kinetic studies (14) indicate that α1 and α2 are synthesized at the same time, and *in vitro* studies of collagen synthesis (17) indicate that messenger RNA for the α chains is monocistronic, such a model is unlikely. Our studies indicate that the high molecular weight species consists of pro α chains (perhaps in the form of procollagen) linked to noncollagenous material through disulfide linkages generating a complex of considerable size. Such a complex might occur as a normal step during biosynthesis of collagen, as a structural component of cells, or adventitiously.

It is difficult to determine whether the disulfide bonds observed in procollagen play a role in biosynthesis or whether they are artifacts. In our experience (unpublished observations), disulfide-linked dimers are more common in fibroblast cultures than in procollagen obtained by pulse-labeling calvaria. Still, even a transient linkage between pro α chains could play an important role in stabilizing the interactions that cause the correct assemblage of pro α chains into procollagen molecules. Formation and perhaps cleavage of these disulfide bonds may occur within the cell as a step essential for normal processing of the molecule.

1. Layman, D. L., McGoodwin, E. B. & Martin, G. R. (1971) *Proc. Nat. Acad. Sci. USA* **68,** 454–458.
2. Müller, P. K., McGoodwin, E. & Martin, G. R. (1971) *Biochem. Biophys. Res. Commun.* **44,** 110–117.
3. Bellamy, G. & Bornstein, P. (1971) *Proc. Nat. Acad. Sci. USA* **68,** 1138–1142.
4. Jimenez, S. A., Dehm, P. & Prockop, D. J. (1971) *FEBS Lett.* **17,** 245–248.
5. Lenaers, A., Ansay, M., Nusgens, B. V. & Lapière, C. M. (1971) *Eur. J. Biochem.* **23,** 533–543.
6. Ramaley, P. B. & Rosenbloom, J. (1971) *FEBS Lett.* **15,** 59–64.
7. Church, R. L., Pfeiffer, S. E. & Tanzer, M. L. (1971) *Proc. Nat. Acad. Sci. USA* **68,** 2638–2642.

7*a*. Smith, J. D., Freeman, G., Vogt, M. & Dulbecco, R. (1960) *Virology* **12,** 185–195.

8. Müller, P. K., McGoodwin, E. B. & Martin, G. R. (1972) in *Biology of the Fibroblast,* ed. Kulonen, E., in press.
9. Piez, K. A., Eigner, E. A. & Lewis, M. S. (1963) *Biochemistry* **2,** 58–66.
10. Piez, K. A. (1968) *Anal. Biochem.* **26,** 305–312.
11. Piez, K. A. (1962) *Anal. Biochem.* **4,** 444–458.
12. Bornstein, P., von der Mark, K., Wyke, A. W., Ehrlich, H. P. & Monson, J. M. (1972) *J. Biol. Chem.* **247,** 2808–2813.
13. Tsai, R. L. & Green, H. (1972) *Nature New Biol.* **237,** 171–173.
14. Vuust, J. & Piez, K. A. (1970) *J. Biol. Chem.* **245,** 6201–6207.
15. Dehm, P., Jimenez, S. A., Olsen, B. R. & Prockop, D. J. (1972) *Proc. Nat. Acad. Sci. USA* **69,** 60–64.
16. Burgeson, R. E., Wyke, A. W. & Fessler, J. H. (1972) *Biochem. Biophys. Res. Commun.* **48,** 892–897.
17. Lazarides, E. & Lukens, L. N. (1971) *Nature New Biol.* **232,** 37–40.