CLEAVAGE OF HUMAN FIBROBLAST TYPE I PROCOLLAGEN BY MAMMALIAN COLLAGENASE: DEMONSTRATION OF AMINO-AND CARBOXY-TERMINAL EXTENSION PEPTIDES*+

Jack R. Lichtenstein, Eugene A. Bauer, and Jouni Uitto

Division of Dermatology, Department of Medicine
Washington University School of Medicine, St. Louis, Missouri 63110

Received October 4,1976

SUMMARY. Human skin fibroblasts in monolayer culture synthesize and secrete precursor forms of collagen into the culture medium. The type I collagen precursor, the major precursor in the culture medium, was isolated on DEAE cellulose chromatography and subjected to mammalian collagenase cleavage. The amino terminal cleavage fragments had a higher molecular weight than $\alpha 1^A$ and $\alpha 2^A$, but did not contain interchain disulfide bonds. The carboxy-terminal cleavage fragments formed high molecular weight aggregates which contained interchain disulfide bonds. These results indicate that human type I procollagen contains noncollagenous amino and carboxy-terminal extension peptides and that all of the interchain disulfide bonds are on the carboxy-terminal portion of the molecule.

Collagenous proteins are synthesized and secreted by cultured fibroblasts as higher molecular weight precursors (1-3). These precursors have been isolated as several distinct biochemical species. Recent studies have demonstrated that procollagen has noncollagenous extensions on both the amino and carboxy ends of the molecule (4-7). In addition, precursor intermediates have been isolated with extension peptides only on the amino-terminal portion [p_n collagen (8)] or only on the carboxy end [p_c collagen (5-7)].

---

*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; p_c collagen refers here to a precursor form of collagen which contains carboxy-terminal extension peptides but is devoid of amino-terminal extensions; and p_n collagen refers to a molecule which contains amino-terminal extensions but is devoid of carboxy-terminal extension peptides. $p\alpha^A$ refers to the amino-terminal fragment from mammalian collagenase cleavage with an amino-terminal extension peptide and $p\alpha^B$ refers to the carboxy-terminal fragment from mammalian collagenase cleavage with a carboxy-terminal extension peptide.

+ABBREVIATIONS. EDTA, ethylene diamine tetraacetic acid; NEM, N-ethyl-maleimide; PMSF, phenylmethylsulfonyl fluoride; DEAE, diethylaminoethyl; SDS, sodium dodecyl sulfate; DVMEM, Dulbecco-Vogt minimal essential medium.

Cultured human skin fibroblasts secrete both type I and type III collagenous precursors into the culture medium (9-11). Since this system has been used to identify defects in collagen metabolism in human diseases (see 3), it is important to characterize precisely these collagenous precursors. In this study both radiolabeled and quantitative amounts of type I procollagen were cleaved with mammalian collagenase to demonstrate that human fibroblast type I procollagen has extensions on both the amino and carboxy portions of the molecule and that all of the interchain disulfide bonds are in the carboxy-terminal portion.

MATERIALS AND METHODS. Human type I procollagen was prepared in both quantitative and radioisotopic amounts as previously described (11). Normal human skin fibroblasts in culture (Robel, CRL 1187) were purchased from American Type Culture Collection. Cells were grown to confluence in DVMEM (Microbiological Associates) (11), containing 10% fetal calf serum. For the preparation of quantitative amounts of procollagen, each roller bottle was incubated with 50 ml of DVMEM supplemented with 100 μg/ml of ascorbic acid for 24 hrs, 25 mM EDTA, 5 mM NEM and 0.3 mM PMSF were added, and the medium was precipitated with 20% saturation of ammonium sulfate overnight at 4°C. The precipitate was collected by centrifugation, washed with 20% methanol and 5% trichloroacetic acid, and then solubilized in 0.15 M NaCl, 50 mM Tris, pH 7.4, containing the above protease inhibitors. The solubilized precipitate was dialyzed against 2 M urea in 25 mM Tris, pH 7.4. This material was then applied to a 1.5 cm x 4.5 cm DEAE cellulose column in the above buffer and eluted from the column over a 600 ml gradient of 0 to 0.2 M NaCl, at 8°C. To prepare radiolabeled procollagen, confluent monolayer cultures were incubated with DVMEM supplemented with 100 μg/ml of ascorbic acid and 100 μCi of [^{3}H]proline for 20 hrs. The radiolabeled medium procollagen was then prepared as above. The type I procollagen from DEAE cellulose was dialyzed exhaustively against 0.1% acetic acid, lyophilized, and resolubilized in 0.15 M NaCl, 50 mM Tris, pH 7.4. Mammalian collagenase was prepared from human skin explants and partially purified as previously described (12). Collagenase digestion was done in 0.15 M NaCl, 5 mM $CaCl_2$, 50 mM Tris, pH 7.4, at 27°C. Collagenase digestion of the human type I procollagen was monitored by viscometry using a guinea pig collagen standard. Limited chymotrypsin cleavage was performed at 4°C with a 1:50 w/w ratio of enzyme to substrate.

SDS polyacrylamide gel electrophoresis was performed as previously described using 5% gels (13). Reduction of samples was performed with 0.1 M β-mercaptoethanol at 60°C for 30 min. In order to determine the migration of radiolabeled proteins on the SDS acrylamide gel electrophoresis, the radiolabeled proteins were electrophoresed with collagen standards, the gels were stained with 0.1 % Coomassie Blue in 7% acetic acid, destained in 7% acetic acid and 4% methanol and the positions of the marker collagen chains noted. The gels were then sliced with a Bio-rad gel slicer into 2 mm sections, the sections digested with 30% hydrogen peroxide at 37° for 4 hrs and the solubilized gel was mixed with 10 ml Aquasol scintillation fluid (New England Nuclear), and counted in a liquid scintillation counter. Collagen standards were prepared as previously described (11).

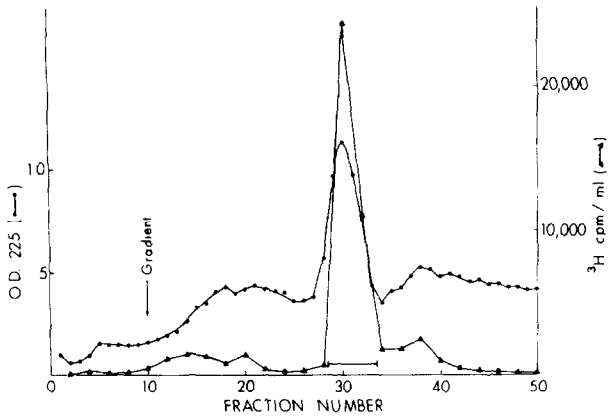

FIG. 1 - DEAE cellulose chromatography of the medium procollagen secreted by human fibroblasts. The fractions indicated by the bar contained type I procollagen (9,11).

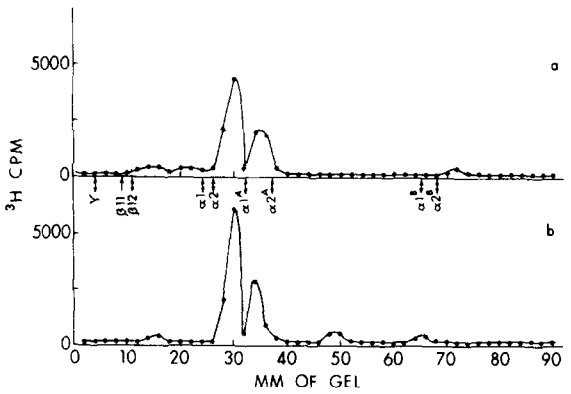

FIG. 2 - 5% SDS acrylamide gel electrophoresis of the supernatant of the mammalian collagenase digest of [^{14}C] type I procollagen before (a) and after (b) reduction with mercaptoethanol.

RESULTS. As previously noted (11), procollagen eluted essentially as a single large peak from DEAE cellulose chromatography whether present in quantitative or tracer amounts (Fig. 1). The small peak eluting from DEAE cellulose chromatography in fractions 35 to 40 has previously been shown to contain the precursor of type III collagen. The small peaks eluting early from DEAE cel-

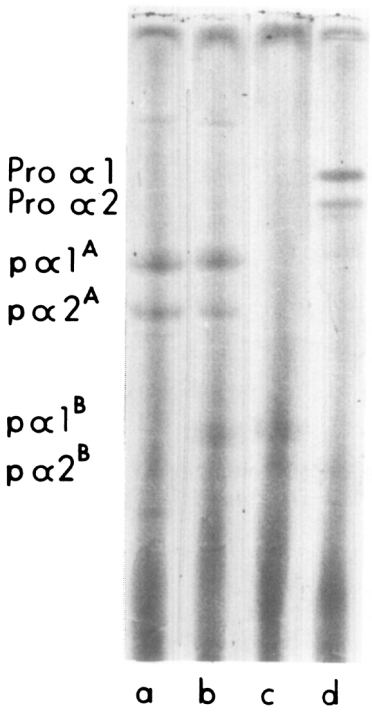

FIG. 3 - SDS acrylamide gel electrophoresis of the type I procollagen and mammalian collagenase digests of type I procollagen.

a) mammalian collagenase digest supernate of human type I procollagen
b) the same as (a) reduced with mercaptoethanol
c) 10 M urea, 0.1% SDS solubilized precipitate of mammalian collagenase digest of human type I procollagen, reduced with mercaptoethanol
d) human type I procollagen reduced with mercaptoethanol

lulose chromatography have previously been shown to contain intermediate collagen precursors (6,7,11). On SDS gel electrophoresis the type I procollagen migrated as a single band of higher molecular weight. After reduction with β-mercaptoethanol, two bands were present whose migration distance indicated a size larger than collagen α-chains (not shown). These bands have previously been shown to be pro-α1 and pro-α2 chains (11).

During the collagenase digestions of type I procollagen, a heavy precipitate formed spontaneously. On SDS acrylamide gel electrophoresis the supernate of the collagenase digest resolved into two bands. One band migrated

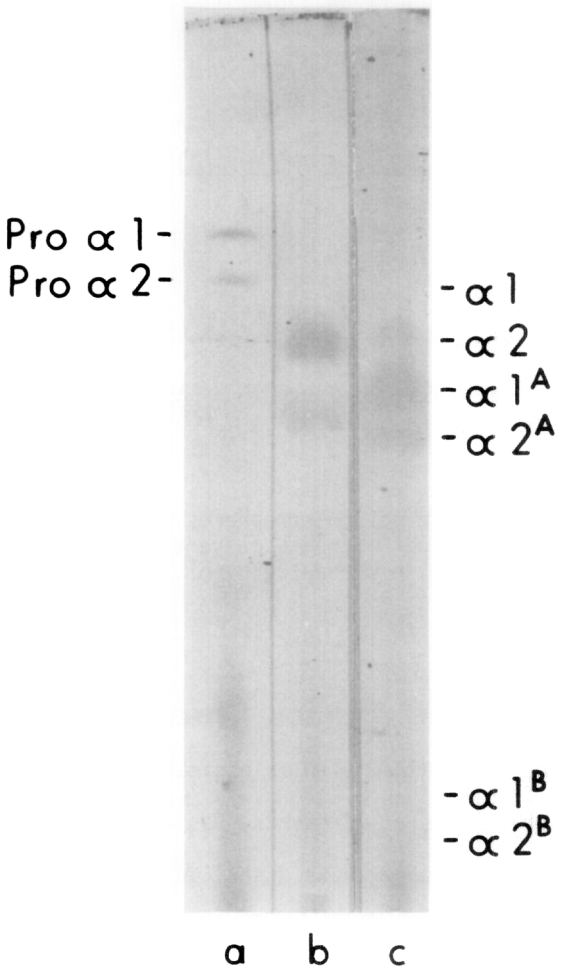

FIG. 4 - 5% SDS acrylamide gel electrophoresis of the chymotrypsin cleavage of the mammalian collagenase fragments from type II procollagen.

a) human type I procollagen, reduced with mercaptoethanol
b) mammalian collagenase digest supernate of human type I procollagen
c) limited chymotrypsin cleavage of the mammalian collagenase digest supernate of human type I procollagen

between the α2 chain and the $\alpha 1^A$ band, and the other migrated between the $\alpha 1^A$ and $\alpha 2^A$ bands. The migration of these two bands was not altered after reduction with mercaptoethanol (Fig. 2 and 3). Limited chymotrypsin cleavage of the mammalian collagenase digest resulted in an altered mobility of the bands on SDS electrophoresis, with the two major bands now having the same

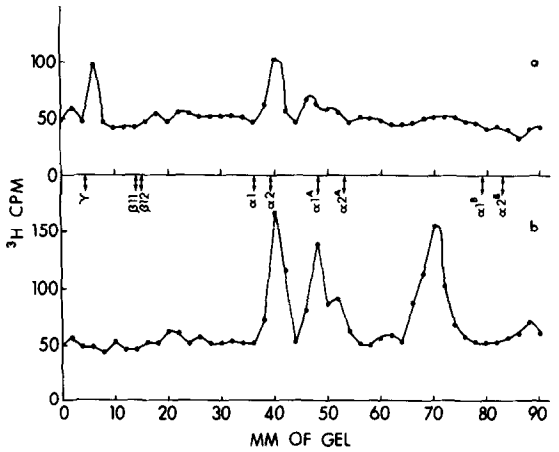

FIG. 5 - 5% SDS acrylamide gel electrophoresis of the precipitate from the mammalian collagenase digest of radioactive type I procollagen before (a) and after (b) reduction with mercaptoethanol.

mobility as $\alpha 1^A$ and $\alpha 2^A$ (Fig. 4). Thus, primarily the amino terminal portion of the molecule, or $p\alpha^A$ chains, with an estimated molecular weight of 85,000 using collagen α or β chains as standards, was visualized on SDS gel electrophoresis after mammalian collagenase digestion of type I procollagen.

The precipitates formed during collagenase incubation were partially solubilized in 10 M urea, 0.1% SDS, 0.1 M PO_4, pH 7.4, at 100°C for 1 hr. When the [^{14}C] precipitate was examined on SDS gel electrophoresis, a high molecular weight band which migrated near the γ chains was seen in addition to the $p\alpha 1^A$ and $p\alpha 2^A$ bands on SDS gel electrophoresis (Fig. 5). After reduction with mercaptoethanol, in addition to the $p\alpha 1^A$ and $p\alpha 2^A$ bands, a new radioactive peak which migrated between the α^A and α^B bands was seen. This band has been tentatively identified as the carboxy-terminal portion of the molecule or $p\alpha^B$. The estimated molecular weight for this $p\alpha^B$ was 40,000. Similarly the precipitate formed from the collagenase digestion of unlabeled procollagen contained primarily $p\alpha^B$ (Fig. 3).

DISCUSSION. In this study we have utilized mammalian collagenase digestion

to demonstrate that the type I procollagen from human skin fibroblasts has extensions on both the amino and carboxy-terminal portions of the molecule. The amino terminal extension peptides contain no interchain disulfide bonds, since there was no alteration in the migration of $p\alpha1^A$ and $p\alpha2^A$ on SDS acrylamide gel electrophoresis following reduction.

The fragments containing the carboxy-terminal extensions precipitated during mammalian collagenase cleavage and formed high molecular weight aggregates. These aggregates could be partially solubilized in urea-SDS buffer. The appearance of a single band of an approximate molecular weight of 40,000 following reduction indicates that the aggregated $p\alpha^B$ chains had intermolecular disulfide bonds.

In both chick calvaria (5,6) and matrix-free chick tendon cells (7), both procollagen and collagenous precursor intermediates with only a carboxy-terminal extension, p_c collagen, have been isolated. In the latter system, the amino terminal extension is rapidly cleaved from procollagen, although the carboxy-terminal extension remains intact (14). However, in the cultured human fibroblast system, the major portion of the collagenous protein contains both amino and the carboxy-terminal extensions, even after a twenty hour incubation. These results confirm previous studies which suggest that fibroblasts synthesize little enzyme capable of converting procollagen to other precursor intermediates or collagen (15,16).

ACKNOWLEDGEMENTS

This work was supported in part by U.S. Public Health Service, N.I.H. grants I R01 AM 18515 01, T01 AM 05611 and AM 12129, and by grant 1357 from the National Foundation. J.R.L. is an investigator for the Howard Hughes Medical Institute. E.A.B. is the recipient of Research Career Development Award 5K04 AM 00077 from the N.I.H.

REFERENCES

1. Bornstein, P. (1974), Annu. Rev. Biochem. 43, 567-603.
2. Martin, G.R., Byers, P.H., and Piez, K.A. (1975), Adv. Enzymol. 42, 161-191.
3. Uitto, J., and Lichtenstein, J.R. (1976), J. Invest. Dermatol. 66, 59-79.
4. Tanzer, M.L., Church, R.L., Yaeger, J.A., Wampler, D.E., and Park, E.D. (1974), Proc. Natl. Acad. Sci. U.S.A. 71, 3009-3013.
5. Byers, P.H., Click, E.M., Harper, E., and Bornstein, P. (1975), Proc. Natl. Acad. Sci. U.S.A. 72, 3009-3013.
6. Fessler, L.I., Morris, N.P., and Fessler, J.H. (1975), Proc. Natl. Acad. Sci. U.S.A. 72, 4905-4909.
7. Uitto, J., Lichtenstein, J.R., and Bauer, E.A. (1976), Biochemistry (in press).
8. Lenaers, A., Ansay, M., Nusgens, B.V., Lapiere, C.M. (1971), Eur. J. Biochem. 23, 533-543.
9. Smith, B.D., Byers, P.H., and Martin, G.R. (1972), Proc. Natl. Acad. Sci. U.S.A. 69, 3260-3262.
10. Goldberg, B., Epstein, E.H., Jr., and Sherr, C.J. (1972), Proc. Natl. Acad. Sci. U.S.A., 69, 3655-3659.
11. Lichtenstein, J.R., Byers, P.H., Smith, B.D., and Martin, G.R. (1975), Biochemistry 14, 1589-1594.
12. Bauer, E.A., Eisen, A.Z., and Jeffrey, J.J. (1970), Biochim. Biophys. Acta 206, 152-159.
13. Weber, K., and Osborn, M. (1969), J. Biol. Chem. 244, 4406-4412.
14. Uitto, J., and Lichtenstein, J.R. (1976), Biochem. Biophys. Res. Comm. 71, 60-67.
15. Goldberg, B., Taubman, M.B., and Radin, B. (1975), Cell 4, 45-50.
16. Taubman, M.B., and Goldberg, B. (1976), Arch. Biochem. Biophys. 173, 490-494.