The Biosynthesis of Basement Membrane Collagen in Embryonic Chick Lens

III. INTRACELLULAR FORMATION OF THE TRIPLE HELIX AND THE FORMATION OF AGGREGATES THROUGH DISULFIDE BONDS*

(Received for publication, June 11, 1973)

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SUMMARY

The basement membrane [14C]collagen secreted into the medium by matrix-free lens cells was found to be largely resistant to pepsin at 15°C, suggesting that it was in a triple helical conformation. In contrast, [14C]collagen in the lens cells themselves was largely digested by pepsin at 15°C, suggesting it was in a random coil form. Gel filtration in sodium dodecyl sulfate showed that the 14C-polypeptides of the collagen in the medium were recovered in aggregates greater than 140,000 daltons when reduction with mercaptoethanol was omitted prior to chromatography. With the cell fraction, essentially all of the [14C]collagen polypeptides were found to elute with molecular weights of 140,000, whether or not reduction with mercaptoethanol was carried out. The observations are consistent with the possibility that the formation of disulfide bonds among the polypeptide chains is of considerable importance in promoting the formation of the triple helix.

Basement membranes are specialized forms of connective tissue which are widely distributed in animal tissues and are comprised of a collagen-like protein and at least two non-collagenous glycoproteins (for review, see Ref. 1). Studies on the structural organization of these extracellular matrices indicate that the macromolecules interact through both noncovalent and covalent bonds of which disulfide bridges may be the major cross-link, since reduction and alkylation of the membranes has been shown to solubilize the basement membrane components (1-6).

The collagen-like protein, which is the major component of all basement membranes (6), has been shown to differ from vertebrate fibrillar collagens in several respects. Noteworthy differences are the presence of significant amounts of 3-hydroxyproline and much higher quantities of 4-hydroxyproline and hydroxylysine, the presence of 4 to 10 residues of half-cystine/1000 amino acid residues, and up to 12% carbohydrate which is found primarily as glucosylgalactosyl disaccharides linked to the hydroxyl groups of the hydroxylysyl residues in the basement membrane collagen (6).

We have recently attempted to study the biosynthesis of basement membrane collagen in embryonic chick lenses in vitro and in matrix-free cells from the lenses (7, 8). Incubation of the cells with [14C]proline for varying periods of time indicated that the secretion of the [14C]collagen by the matrix-free cells did not begin until about 1 hour after incorporation of [14C] into protein became linear. The results indicated that in the lens cells there is a greater lag between the incorporation of [14C]-proline into protein and the secretion of the [14C]collagen than is observed with matrix-free tendon cells under the same conditions (9, 10). Further studies showed that the first collagen synthesized by both the intact lenses and isolated cells consisted of polypeptide chains which have molecular weights of approximately 140,000 and which were considerably larger than the polypeptide chains from the precursor form of collagen synthesized by interstitial connective tissues (11-15).

In the present study we have used proteolytic digestion to test the conformation of the collagen synthesized by lens cells and we have examined the formation of aggregates through disulfide bonds.

EXPERIMENTAL PROCEDURE

Materials—[3H]Glycine-labeled procollagen was prepared by incubating 50 x 10⁶ cells from embryonic chick tendons with...
100 µCi of [3H]glycine for 4 hours at 37° in 10 ml of modified Krebs medium (9). The medium was removed by centrifugation at 1200 × g for 10 min, dialyzed against 0.1 M acetic acid, and stored frozen at 15°.

All other materials were obtained from the sources described previously (7, 8).

**Incubation of Lenses and Cells with [14C]Proline**—Lenses were removed from 19- or 20-day-old chick embryos and incubated either as the intact tissue or matrix-free cells isolated from the lenses by the methods described previously (7, 8). Whole lenses were preincubated in 3 ml of modified Krebs medium (9) for 60 min at 37° prior to incubation with [14C]proline which was added in a total of 2 ml of modified Krebs medium. At the end of the incubation period 0.4 ml of medium containing 540 µg of cycloheximide and 5.4 µmoles of α,α'-dipyridyl was added to stop any further incorporation of [14C]proline and hydroxylation of non-hydroxylated [14C]collagen. Lenses plus medium were then cooled to 0°, homogenized, and the homogenate was either adjusted to 1% sodium dodecyl sulfate and 5% 2-mercaptoethanol in preparation for direct sodium dodecyl sulfate-agarose gel filtration or dialyzed against 0.1 M acetic acid alone. Matrix-free lens cells isolated by enzymatic dissociation of the embryonic lenses were incubated with [3H]proline in 10 ml of modified Krebs medium containing 10% fetal calf serum (unless otherwise indicated) in siliconized 25-ml Erlenmeyer flasks. At the end of the incubation period, cycloheximide and α,α'-dipyridyl were added as described above. The samples were centrifuged at 1200 × g for 10 min at room temperature to separate medium from cells (7).

**Pepsin Digestion of Basement Membrane Collagen**—The susceptibility to proteolytic attack of the polypeptides of basement membrane collagen synthesized by the matrix-free lens cell system and by intact lenses in vitro was investigated. The medium from lens cells which had been incubated for 4 hours with [14C]proline was dialyzed at 3° for 24 hours against two changes of 1 liter of 0.1 M acetic acid. Approximately 2 mg of carrier lathyritic collagen from chick skin was added as the internal marker for standard proteins and collagen polypeptides remained the same as previously reported (8). A few of the chromatograms were obtained with a second column which was prepared from a different batch of collagen. The Vα : Vt values for large polypeptides were less in the second column, but a standard curve of Vα : Vt versus log of molecular weight of well-characterized polypeptides was linear and the performance of the column was similar to that of several different columns previously used by other investigators in the same laboratory (12).

The sizes of the intracellular and extracellular collagen polypeptides were determined by gel filtration without prior reduction with 2-mercaptoethanol. Lens cells and medium were separated and the collagen polypeptides in the medium were obtained as described above. The lens cells and the pellet of medium polypeptides were solubilized by heating at 100° for 1 min in 1% sodium dodecyl sulfate and 0.1 M sodium phosphate buffer (pH 7.4). Incubation was continued at 37° for 2 hours in either the absence or presence of 2-mercaptoethanol followed by dialysis against 0.1 M sodium dodecyl sulfate in 0.1 M sodium phosphate buffer as described above. When treatment with 2-mercaptoethanol was included in the procedure, the [3H]glycine-labeled tendon procollagen was used as internal marker and was added prior to sodium dodecyl sulfate-mercaptoethanol treatment. The procollagen did not elute as a single peak when the mercaptoethanol treatment was omitted (16), about 3.5 mg of lathyritic chick skin collagen were added as the internal marker for samples which were not reduced.

**Assay Procedures**—The % in the fractions from the agarose chromatograms was determined by taking 0.1 ml together with 0.2 ml of distilled water in the solvent system for liquid scintillation counting described previously (17). 4-Hydroxy[14C]proline in the fractions was assayed by hydrolyzing the remainder of the fractions in 6 N HCl at 120° for 18 hours and then using a specific radiochemical assay procedure (17). The localization of the α chains of lathyritic chick skin collagen in the chromatogram was determined by taking an aliquot of the acid hydroly-
RESULTS

Pepsin Digestion of Basement Membrane Collagen Synthesized by Matrix-free Cells—The medium from matrix-free cells which had been incubated with [14C]proline for 4 hours was dialyzed against 0.1 M acetic acid for 24 hours and precipitated with 5% NaCl. In samples not treated with pepsin, essentially all the 4-hydroxy[14C]proline eluted from the sodium dodecyl sulfate-agarose column ahead of embryonic chick tendon [3H]procollagen in a peak with an apparent molecular weight of about 140,000 (Fig. 1A). After treatment with pepsin, about 70% of the 4-hydroxy[14C]proline eluted in a peak with an apparent molecular weight of about 120,000 (Fig. 1B). In the experiment shown the peak eluted in about the same position as an internal standard of [H]labeled pro-α chains of procollagen from chick embryo tendon cells and the apparent molecular weight was 115,000 to 120,000. In later experiments in which the gel filtration was carried out in a second agarose column, the peak eluted slightly ahead of the same internal standard and the apparent molecular weight was 125,000 to 130,000.

The amount of the collagen 4-hydroxy[14C]proline digested by pepsin was greater in the cell fraction. Before treatment with pepsin the elution pattern of the 4-hydroxy[14C]proline from the cells was essentially the same as the medium (Fig. 2A). After treatment with pepsin only 30% of the initial 4-hydroxy[14C]proline was recovered in a peak with an apparent molecular weight of about 120,000 and 70% was recovered as smaller peptides (Fig. 2B). Similar results (not shown) were obtained with the [14C]collagen in both the medium and the cells when the pepsin concentration was reduced from 200 to 100 µg per ml and the conditions for the digestion were 6 hours at 15° followed by 18 hours at 3° instead of 18 hours at 15°.

Pepsin Resistance of Collagen Synthesized by Intact Lenses—Intact lenses from chick embryos were incubated with [14C]proline in modified Krebs medium, the tissues were homogenized in acetic acid, and the homogenates were treated with pepsin as described above. In lenses which were incubated with [14C]proline for 15 min, most of the 4 hydroxy[14C]proline eluted in a peak with an apparent molecular weight of about 140,000 (Fig. 3A). After the same samples were treated with pepsin, about 20% of the 4-hydroxy[14C]proline was recovered in a peak with an apparent molecular weight of about 120,000 and about 80%...
was recovered in smaller peptides. Intact lenses were also labeled by incubating them for 60 min with [14C]proline and the label was chased by adding 100 μg per ml of [12C]proline and continuing the incubation for an additional 180 min. In lenses which were pulse-labeled and chased in this manner, most of the [14C]collagen was resistant to proteolysis (Fig. 3B).

Control Experiments on Degradation of Tendon [3H]Procollagen—It was previously shown that if lens cells were incubated in Krebs medium containing fetal calf serum, native [14C]collagen added to the incubation system was not degraded to dialyzable [14C]-peptides (8). The results suggested therefore that there was little proteolysis of triple helical collagen secreted into the medium. This observation was confirmed here by experiments in which the size of the collagen polypeptides were examined before and after incubation with the cells. When native [3H]-procollagen from tendon cells was incubated with the lens cells, no change in the molecular weight of the [3H]-polypeptides was observed and there was little evidence of degradation to smaller molecular species. Also, virtually all of the [3H] counts were recovered in the medium and less than 0.6% of the [3H] remained associated with the cell pellet. When the [3H]-procollagen was denatured and then incubated with lens cells in medium containing 10% fetal calf serum, considerable degradation of the polypeptides occurred (Fig. 4).

Extracellular Aggregation of Collagen—To test for the presence of disulfide bonds either among collagen polypeptides or between collagen and other macromolecules, gel filtration on sodium dodecyl sulfate-agarose was carried out with and without reduction of the [14C]-protein with mercaptoethanol. Omission of mercaptoethanol during the sodium dodecyl sulfate treatment produced a marked effect on the elution pattern of the [14C]-collagen secreted into the medium, since most of the 4-hydroxy-[14C]proline eluted in the void volume of the column (Fig. 5A). The same results were obtained whether or not the medium in which the cells were incubated contained serum. In contrast to the results obtained with the medium [14C]collagen, omission of mercaptoethanol had no effect on the elution pattern of the...
4-hydroxy[14C]proline from the cell fraction (compare Fig. 5B with Fig. 2A).

Further experiments were designed to determine under what conditions the [14C]collagen polypeptides in the cells would form aggregates similar to the aggregates of the [14C]collagen secreted into the medium. For these experiments lens cells were incubated with [14C]proline for 4 hours so that they contained [14C]collagen. The cells were lysed by repeated freezing and thawing under conditions previously shown to make the intracellular [14C]collagen accessible to proteolytic enzymes (7). The lysate was diluted with Krebs medium containing 10% fetal calf serum and then incubated in air at 37°C for 30 min. After this treatment, the [14C]collagen polypeptides eluted from the sodium dodecyl sulfate-agarose column in an aggregated form (Fig. 5C).

**DISCUSSION**

Extensive previous work has shown that if proteolytic digestion of collagen is carried out with moderate amounts of enzyme and below the denaturation temperature of collagen, only the nonhelical extensions of the molecule are removed by pepsin (19, 20) or by other hydrolytic enzymes such as trypsin, chymotrypsin (21), or cathepsins (22, 23). Studies presented here on the pepsin digestion of basement membrane collagen synthesized by lens cells suggest therefore that a major fraction of the intracellular, newly synthesized [14C]collagen is in a random coil form and that the collagen becomes triple helical prior to secretion from cells.

The [14C]collagen secreted into the medium by matrix-free lens cells was found to be largely resistant to pepsin digestion at 15°C, and the 4-hydroxy[14C]proline-labeled precursor polypeptides decreased in size after pepsin treatment, indicating that a small portion or portions of the chains were susceptible to proteolysis and were cleaved to basement membrane collagen α chains in a manner analogous to the time-dependent cleavage shown to occur in intact lenses in vitro (8). Since the addition of denatured [3H]procollagen to the matrix-free lens cells resulted in the rapid degradation of the random coil [3H]-polypeptides, it was apparent that the basement membrane collagen molecules became triple helical before they were secreted; otherwise they too would have been degraded in the lens cell system.

Pepsin digestion of the lens cells themselves suggested that most of the intracellular [14C]collagen was in a random coil form. After the matrix-free cells were incubated with [14C]proline for several hours, 90% of the intracellular nondialyzable 4-hydroxy-[14C]proline was in polypeptide chains with molecular weights of about 140,000. Therefore, the observation that 70% of the intracellular 4-hydroxy[14C]proline was digested by pepsin at 15°C suggested that most of the intracellular collagen consisted of polypeptides which were complete in terms of size but which were still largely random coil. It might be noted that although about 30% of the intracellular [14C]collagen was resistant to pepsin at 15°C, this observation does not necessarily mean that fully 30% was triple helical during incubation of the cells at 37°C. Recent observations with [14C]procollagen from tendon cells have shown that the molecule is random coil (24) and pepsin-digestable (24-26) at 30 or 37°C, but it rapidly becomes pepsin-resistant on cooling at 15 or 20°C. Digestion with pepsin at 15°C as employed here may therefore overestimate the degree of helicity of the intracellular [14C]collagen, particularly since exposure of cell homogenates to air tends to promote disulfide bond formation (see below).

The experiments with the intact lenses showed that the observations made with the matrix-free cells were not artifacts introduced by the technique for preparing the cells. Previous studies showed (7, 8) that after lenses are incubated for 1 hour or less with [14C]proline, most of the collagen 4-hydroxy[14C]-proline was intracellular. If longer labeling times were employed, or if the label was chased for 180 min, most of the...
collagen 4-hydroxy[14C]proline was extracellular. Since the [14C]collagen was pepsin-digestable at 15° after a labeling period of 15 min and it was largely resistant after a chase period of 180 min, the results confirmed the impression that the intracellular [14C]collagen was largely random coil and the extracellular [14C]collagen was largely triple helical.

The results obtained with the lens systems are in marked contrast to observations with matrix-free cells from the tendons of chick embryos in that with the latter system 60 to 70% of the intracellular 4-hydroxy[14C]proline was found to be resistant to pepsin at 15° and under essentially the same conditions employed here (27–29). At the moment it is difficult to explain why the lens cell system should differ in this respect from cells synthesizing tendon collagen. It was previously observed that there was a longer delay in the secretion of basement membrane collagen than in the secretion of tendon collagen and it was suggested that this longer lag might be explained by the additional time required to introduce the larger number of hydroxylysyl residues and glycosylated hydroxylysyl residues found in basement membrane collagen (7). The results presented here raise the further possibility that the longer lag in secretion may in part be explained by the time required for the collagen to become triple helical.

One possible role of the NH2-terminal extensions of procollagen polypeptides might be that they provide disulfide bridges among the polypeptides and that they thereby facilitate chain association leading to triple helix formation (16, 30–32). In this respect, the effects of reduction or nonreduction of intracellular and extracellular basement membrane collagen prior to sodium dodecylsulfate gel filtration are of interest. The collagen 4C-polypeptides in the medium were recovered in large aggregates which eluted at or near the void volume of the sodium dodecyl sulfate-agarose column when reduction was omitted prior to chromatography. Similar observations have been reported with medium [14C]procollagen from tendon cells (16) and from fibroblasts in culture (30–32). With the lens cells examined here, however, essentially all of the collagen 4C-polypeptides were found to elute with molecular weights of 140,000 whether or not reduction or nonreduction of intracellular 4-hydrosy[14C]proline was extracellular. Since the intracellular [14C]collagen was pepsin-digestable at 15° after a labeling period of 60 min and it was largely resistant after a chase period of 180 min, the results confirmed the impression that the intracellular [14C]collagen was largely random coil and the extracellular [14C]collagen was largely triple helical.

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Acknowledgments—We wish to thank Miss Judy Parris, Ms. Carolyn Rizzo, and Miss Judy Nawalinski for valuable technical assistance.

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*J. Biol. Chem.* 1973, 248:7432-7437.

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