Basement Membrane Procollagen Is Not Converted to Collagen in Organ Cultures of Parietal Yolk Sac Endoderm

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Basement membrane procollagen biosynthesis was studied in organ cultures of embryonic rat parietal yolk sac endoderm by following [14C]proline incorporation into nondialyzable proteins. After reduction with 2-mercaptoethanol the [14C]-proteins synthesized were characterized by agarose gel filtration and disc electrophoresis in the presence of sodium dodecyl sulfate. The labeled procollagen was identified by its content of hydroxy[14C]proline, its sensitivity to digestion with bacterial collagenase, and its resistance to digestion with pepsin. In cultures which were continuously labeled for periods from 6 hours to 4 days, the pro-α chains consistently eluted as a single peak with an apparent molecular weight of 160,000. After pepsin digestion the resultant α chains had an apparent molecular weight between 125,000 and 140,000. This suggests that basement membrane procollagen either contains non-triple helical pepsin-resistant regions or a triple helical region which is larger than the corresponding region of interstitial procollagen.

Two experiments were performed to determine whether the chains of newly synthesized basement membrane procollagen were cleaved to a smaller molecular species. In the first, the hydroxylation and secretion of procollagen were blocked with a,a'-dipyridyl, and the resulting intracellular chains of basement membrane protocollagen were found to co-elute with fully hydroxylated and secreted pro-α chains. In the second, cultures were labeled for 1 day and chased for 3 days with unlabeled medium. Autoradiography had shown that most of the label was chased into new basement membrane. Agarose chromatography showed that after a 3-day chase the pro-α chains still eluted with an apparent molecular weight of 160,000. Thus, the data indicated that basement membrane procollagen was deposited in new basement membrane without undergoing a time-dependent extracellular conversion.

It is now well established that basement membranes consist of a collagen component in association with one or more non-collagen glycoproteins (3, 4). In recent studies we have found that organ cultures of embryonic rat parietal yolk sac tissues offer many advantages for studies of the biosynthesis of basement membrane (5-9). For example, the initial studies showed that the parietal endodermal cells on the surface of existing basement membrane could be cleanly isolated without exposure to enzymes, that it was only the endodermal cells which synthesized basement membrane, that basement membrane was the only structured extracellular matrix synthesized, and that new basement membrane was deposited on the surface of existing basement membrane (5-9). When organ cultures of PEM1 were labeled with [14C]proline, 4-hydroxy-[14C]proline constituted ~20% of the total [14C], and 3-hydroxy[14C]proline constituted ~10% of the total hydroxy-[14C]proline. In cultures labeled with [14C]lysine, ~85% of the hydroxy[14C]lysine residues were glycosylated, and ~95% of these residues were glucosylgalactosylhydroxy[14C]lysine (5, 7, 9). Autoradiography showed that in cultures of PEM labeled with [1H]proline or [1H]glucosamine, more than 10 μm of heavily labeled new basement membrane were deposited on the surface of existing membrane during the first 4 days of culture (5, 8). Because of the relatively high rate of basement membrane synthesis, cultures of PEM therefore appeared to be appropriate for further studies of both basement membrane collagen biosynthesis, and the steps involved in the deposition of this collagen in new basement membrane.

In their studies of basement membrane collagen synthesis by * This investigation was supported by United States Public Health Service Grants AM 14326, AM 14866, and HD 650. Preliminary reports of this work have been published (1, 2).
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1 The abbreviation used is: PEM, the parietal endoderm on the surface of the existing basement membrane.
lens and glomeruli, Grant et al. (10, 11) suggested that this molecule, like interstitial collagens, was synthesized as a precursor or procollagen molecule which appeared to undergo a time-dependent extracellular conversion to basement membrane collagen. The pro-α chains of basement membrane procollagen secreted by the lens had an apparent molecular weight of ~140,000 and these chains appeared to undergo an extracellular conversion to chains with a molecular weight of ~115,000 (12). In contrast, our initial studies of the collagen component of basement membrane deposited in cultures of PEM showed a higher molecular weight for the procollagen molecule, and failed to show any evidence for conversion to a lower molecular weight species (1).

The present study deals with investigations of the molecular weight and lack of conversion of basement membrane procollagen. The pro-α chains extracted from new basement membrane had an apparent molecular weight of 160,000, and the α chains resulting from pepsin digestion had an apparent molecular weight between 125,000 and 140,000. However, there was no evidence of an extracellular conversion of this basement membrane procollagen in the cultures. Unhydroxylated intracellular pro-α chains, hydroxylated pro-α chains in 6-hour to 4-day continuously labeled cultures, and pro-α chains extracted from new basement membrane after a 1-day pulse and 3-day chase all had the same apparent molecular size.

**EXPERIMENTAL PROCEDURE**

**Materials**—Pregnant Wistar rats (150 to 200 g) in their 15th day of gestation (embryo age, 14.5 days) were used in all experiments. Formula 947 mixture, L-[U-14C]proline (> 200 mCi/mmol) and L-[2,5,6-3H]proline (30 to 50 Ci/mmol) were purchased from New England Nuclear Corp. (Boston, Mass.). Pepsin (EC 3.4.1.4, twice crystallized), collagenase (Clostridium histolyticum type III, Fraction A), β-amino- propionitrile fumarate, and α,α'-dipyridyl were purchased from Sigma Chemical Co. (St. Louis, Mo.). Fetal calf serum and a modified F12X nutrient mixture (8) were purchased from Grand Island Biological Co. (Grand Island, N. Y.). Organ culture dishes (No. 3010) were purchased from Falcon Plastics (Oxnard, Calif.). Bio Gel A-5m (200 to 400 mesh) agarose beads were purchased from Bio-Rad Laboratories (Richmond, Calif.). Simm’s balanced salt solution was prepared according to the formula of Lash et al. (13).

**Organ Culture Preparations**—All cultures consisted of the single layer of cells of the parietal yolk sac endoderm on the surface of the existing parietal yolk sac basement membrane, which were isolated by dissection without the aid of enzymes (8). Six halves of PEM were grown on the surface of 1 ml of a nutrient agar substrate and fed 80 μl of liquid nutrient medium at 12-hour intervals as described previously (8, 9). Sodium ascorbate was added to a concentration of 200 μg/ml in the liquid nutrient medium, giving a concentration of 16 μg of ascorbate/ml of nutrient substrate. β-Aminopropionitrile was added to a concentration of 60 μg/ml in both the nutrient agar and liquid nutrient medium. For radioisotopic labeling, L-proline was omitted from the F12X and 5 μCi/ml of [14C]proline or 10 μCi/ml of [3H]proline was added to both the nutrient agar and liquid nutrient medium. Cultures were continuously labeled for 6 hours to 4 days, or were labeled for 1 day and then chased for 3 days on unlabeled agar. For the preparation of unhydroxylated basement membrane procollagen (pretocollagen), the tissues were preincubated 20 min at 37° in 0.5 mM serum and 20 μCi of [14C]proline. The reaction was stopped and the collagen was precipitated by raising the pH to 8.5 with 0.5 M NaOH and adding KCl to a final concentration of 15% at 4°. The precipitate was collected by centrifugation at 4° and was redissolved in 1 ml of 0.1% sodium dodecyl sulfate/0.1 mM phosphate buffer, pH 7.4, at room temperature, prior to reduction with sodium dodecyl sulfate-mercaptoethanol in preparation for gel filtration and disc electrophoresis as described below.

For collagenase digestion, cultures of PEM were homogenized at 4° in 1 ml of 0.4 M NaCl/0.1 M Tris-HCl, pH 7.4. Digestion with 100 μg/ml of collagenase in the presence of 10 mM N-ethylmaleimide as a protease inhibitor was performed as described by Dehm et al. (17). Digestion was stopped by boiling for 5 min, and the sample was dialyzed against 0.4 M NaCl/0.1 M Tris-HCl containing 10 mM N-ethylmaleimide prior to reduction and gel filtration as described below.

**Gel Filtration on Sodium Dodecyl Sulfate-agarose**—For gel filtration and disc electrophoresis, cultures of PEM were incubated for 6 hours in 0.5 ml of 0.1 M phosphate buffer, pH 7.4, containing 10 mM N-ethylmaleimide, and were placed in boiling water for 3 min. Then, 1 mg of acid soluble rat tail tendon collagen was added, and the tissue was homogenized and solubilized under reducing conditions with stirring in 1% sodium dodecyl sulfate and 5% mercaptoethanol for 3 hours at 45°. After dialysis against several changes of 0.1 M phosphate buffer containing 0.1% sodium dodecyl sulfate, the total 14C and 4-hydroxy[14C]proline were determined and duplicate aliquots containing ~25,000 cpm were removed for disc electrophoresis (see below). The remainder of the sample was chromatographed on a calibrated column of agarose A-5m (200 to 400 mesh) as described previously (10). Fractions of 2 ml were collected, and aliquots of each fraction were taken for the determination of total 14C; the remainder of appropriate fractions was hydrolyzed and aliquots were taken for the determination of labeled and unlabeled 4-hydroxyproline. The positions of β and α components from the added rat tail tendon collagen were used to calibrate the column for molecular weight determinations.

**Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis**—Aliquots of the reduced homogenate described above were dialyzed against several changes of water containing Dowex 1 resin prior to lyophilization. The lyophilized sample was incubated for 2 hours at 45° in 50 μl of 0.1% sodium phosphate, pH 6.5, containing 10 mM N-ethylmaleimide, and were placed in boiling water for 3 min. Then, 1 mg of rat tail tendon collagen was added, and the tissue was homogenized and solubilized under reducing conditions with stirring in 1% sodium dodecyl sulfate and 5% mercaptoethanol, pH 7.0 (18). Standards containing 50 μg of rat tail tendon collagen were treated in the same way. A modification of the Weber and Osborn (19) procedure was used for electrophoresis in that the polymerizing solution contained 7% acrylamide monomer and 0.25% N,N'-diallyltartardiamide. After electrophoresis against several changes of 0.1 M phosphate buffer containing 0.1% sodium dodecyl sulfate, the total 14C and 4-hydroxy[14C]proline were determined and duplicate aliquots containing ~25,000 cpm were removed for disc electrophoresis (see below). The remainder of the sample was chromatographed on a calibrated column of agarose A-5m (200 to 400 mesh) as described previously (10). Fractions of 2 ml were collected, and aliquots of each fraction were taken for the determination of total 14C; the remainder of appropriate fractions was hydrolyzed and aliquots were taken for the determination of labeled and unlabeled 4-hydroxyproline. The positions of β and α components from the added rat tail tendon collagen were used to calibrate the column for molecular weight determinations.

**RESULTS**

**Apparent Size of Chains of Collagen in New Basement Membrane**—Sodium dodecyl sulfate-agarose gel filtration and sodium dodecyl sulfate-acrylamide gel electrophoresis were used to determine the size of the polypeptide chains of the collagen component of new basement membrane which was deposited in continuously labeled 1, 2, and 4 days cultures of PEM. The results showed that all of the cultures yielded a single major peak of both 14C and 4-hydroxy[14C]proline which eluted between the α chains and β components of the rat tail tendon collagen (Fig. 1). The apparent molecular weight of this peak was ~100,000 with both gel filtration (Fig. 1A) and disc gel electrophoresis (Fig. 1B). This peak on sodium dodecyl
underwent an extracellular cleavage. In the first series of experiments, the apparent size of intracellular chains was determined whether the basement membrane procollagen was in collagen.

4-hydroxy[\textsuperscript{14}C]proline was in collagen.

agarose (Fig. 1C). These results indicated that all of the 4-hydroxy[\textsuperscript{14}C]proline in the chromatograms was in peptides which were too small to be resolved by the sodium dodecyl sulfate-agarose chromatography of cultures of PEM which were labeled for 1 day. The labeled tissues were boiled, and homogenized with 0.5 mg of acid-extracted rat tail tendon collagen prior to solubilization and reduction with sodium dodecyl sulfate-mercaptoethanol. After dialysis, an aliquot of the retentate containing ~300,000 cpm was charged to a column (1.5 × 95 cm) of agarose A 5m and eluted at 30°C with 0.1 M phosphate/0.1% sodium dodecyl sulfate, pH 7.4. The total volume (V\textsubscript{i}) was determined by the elution position of \textsuperscript{4}H\textsubscript{2}O, and the column was calibrated by the position of the \( \alpha \) chains and \( \beta \) components of tendon collagen (\( \alpha \) and \( \beta \)).

To show that the 4-hydroxy[\textsuperscript{14}C]proline in the chromatograms was indicative of the distribution of collagen chains, cultures of PEM were digested with bacterial collagenase prior to being chromatographed on sodium dodecyl sulfate-agarose as described under "Experimental Procedure." The positions of the \( \alpha \) chains and \( \beta \) components of tendon collagen in the gel standard are shown (\( \alpha \) and \( \beta \)).

Absence of Extracellular Conversion of Basement Membrane Procollagen—Two types of experiments were performed to determine whether the basement membrane procollagen underwent an extracellular cleavage. In the first series of experiments, the apparent size of intracellular chains was compared to that of chains which were secreted and deposited in new basement membrane. In order to block secretion, unhydroxylated basement membrane procollagen (procollagen) was prepared by labeling cultures of PEM with either \textsuperscript{3}H-proline or \textsuperscript{14}C-proline in the presence of 0.5 mM \( \alpha,\alpha' \)-dipyridyl. The ratio of 4-hydroxy[\textsuperscript{14}C]proline to total \textsuperscript{14}C in these cultures was less than 0.005. Autoradiography showed that at the end of the 6 hour-pulse in the presence of \( \alpha,\alpha' \)-dipyridyl nearly all of the label remained in the endodermal cells (data not shown), whereas in control cultures much of the label was deposited in new membrane (see Ref. 8). Cultures labeled with \textsuperscript{3}H-proline in the presence of \( \alpha,\alpha' \)-dipyridyl were therefore reduced with sodium dodecyl sulfate-mercaptoethanol and co-chromatographed on sodium dodecyl sulfate-agarose with control cultures labeled with \textsuperscript{14}C-proline in the absence of \( \alpha,\alpha' \)-dipyridyl. The results of these experiments showed that the \textsuperscript{3}H-procollagen chains eluted in the same position as the \textsuperscript{14}C-procollagen chains, with an apparent molecular weight of 160,000 (Fig. 2).

The second set of experiments in this series was designed to determine whether there was a delayed conversion of basement membrane procollagen to basement membrane collagen. Cultures were labeled for 1 day with \textsuperscript{14}C-proline and chased for 3 days on unlabeled agar. Autoradiography had previously shown that in such cultures the majority of the label was chased out of the endodermal cells into heavily labeled basement membrane which subsequently became buried under poorly labeled new basement membrane (8). As shown in Fig. 3, the pro-\( \alpha \) chains in these cultures eluted in the same position as those in both continuously labeled cultures (Fig. 1A), and cultures labeled in the presence of \( \alpha,\alpha' \)-dipyridyl (Fig. 2). These results indicated that the procollagen in these cultures was secreted and deposited in new basement membrane without undergoing an extracellular cleavage.

Since Grant et al. (10) had reported that there was a time-dependent conversion of pro-\( \alpha \) chains with an apparent
molecular weight of 140,000 to α chains with an apparent molecular weight of 115,000 in cultures of the embryonic chick lens, the collagen in cultures of PEM was compared to that in cultures of 19-day-old embryonic chick lenses. After reduction with sodium dodecyl sulfate-mercaptoethanol, aliquots were co-chromatographed on sodium dodecyl sulfate-agarose. As shown in Fig. 4, the major peak of 4-hydroxy[14C]proline synthesized by the lenses eluted in the same position as the 3H-labeled pro-α chains synthesized by the endodermal cells. There were some smaller molecular weight (<85,000) peptides containing 4-hydroxy[14C]proline in these chromatograms, but there was no evidence of an extracellular conversion of the pro-α chains synthesized by either lenses or PEM (Fig. 4).

**Apparent Size of Pepsin-resistant Region of Basement Membrane Procollagen**—Sodium dodecyl sulfate-agarose gel filtration was also used to determine the apparent size of the α chains resulting from pepsin digestion of basement membrane procollagen. In 1-day cultures of PEM, ~30% of the total 14C and ~70% of the 4-hydroxy[14C]proline remained in the retentate after pepsin digestion, reduction with sodium dodecyl sulfate-mercaptoethanol, and dialysis against sodium dodecyl sulfate-phosphate buffer. When this retentate was chromatographed on sodium dodecyl sulfate agarose, between 70 and 75% of both the total 14C and 4-hydroxy[14C]proline eluted in a peak with an apparent molecular weight between 125,000 and 140,000 (Fig. 5). The ratio of 4-hydroxy[14C]proline to total 14C in this entire peak ranged from 0.65 to 0.68. This suggested that the pepsin-resistant material eluting in this region was free of non-collagen proteins.

**DISCUSSION**

Numerous studies have shown that the interstitial collagens are synthesized as precursor or procollagen molecules which undergo an extracellular cleavage to form collagen which is then deposited in fibrils and fibers (21-23). It is also well established that the peptides which are cleaved from interstitial procollagens are not triple helical and hence are removed by proteolytic enzymes such as pepsin, trypsin, or chymotrypsin. On the other hand, the triple helical region of native interstitial procollagen is digested by bacterial collagenase, but is resistant to the aforementioned proteolytic enzymes. The
shown that the procollagen which is synthesized by calvaria consists of the fact that a larger proportion of the basement membrane procollagen molecules may be glycosylated and 95% of these residues are the disaccharide glucosylgalactosylhydroxylysine (4, 7, 9), one would expect that the apparent size of the pro-α chains extracted from basement membrane should be larger than that of the unhydroxylated, unglycosylated intracellular chains of procollagen. Nevertheless, the experiments with α,α′-dipyridyl failed to show a reduction in size of the pro-α chains in the absence of the sugars. This may be due to a failure of the sugars to affect the apparent size of the procollagen chains in the presence of sodium dodecyl sulfate. It is possible, however, that there is an additional peptide extension on the intracellular chains of basement membrane procollagen which has the same effect on their apparent size as the sugar residues have on the extracellular pro-α chains. If such an extension does exist it may be cleaved intracellularly. This possibility is currently under investigation.

The studies described in this report have shown that the collagenous component of the parietal yolk sac basement membrane is synthesized as a procollagen molecule which is deposited in new basement membrane without undergoing an extracellular cleavage to a smaller molecular species. In addition, these studies have shown that a major difference between basement membrane and interstitial procollagen consists of the fact that a larger proportion of the basement membrane procollagen molecule is resistant to pepsin. Finally, these studies have shown that organ cultures of PEM on a nutrient agar substrate should permit further examination of the steps involved in the synthesis, secretion, deposition, and stabilization of basement membrane procollagen.

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