

Biosynthesis and Supramolecular Assembly of Procollagen IV in Neonatal Lung

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Abstract. The rate of biosynthesis of procollagen IV, the principal collagen of basement membranes, and the concentration of specific RNAs coding for procollagen IV were measured in neonatal rat lungs. Both decreased sharply at birth and then recovered again a few days later. The supramolecular assembly of procollagen IV was followed in neonatal rat, mouse, and chick lungs, which actively elaborate endothelial and alveolar basement membranes, and in chick embryo gizzard which is rich in smooth muscle. The tetramer of four procollagen IV molecules linked covalently through their amino ends was isolated as an assembly intermediate from all these tissues. While noncovalent

association of the carboxyl ends of two procollagen IV molecules occurred readily, the subsequent establishment of covalent cross-links was substantially slower in the junctional complexes of the carboxyl ends than of the amino ends. Both disulfide bonds and other, unidentified covalent links formed. The six component carboxyl peptides of a junctional complex became progressively covalently linked into two kinds of carboxyl peptide pairs. We conclude that both amino-linked tetramers and carboxyl-linked dimers of procollagen IV molecules are intermediates in the biological assembly of the collagen networks of these basement membranes.

ALTHOUGH there are several models of basement membranes, we have little knowledge of the physiological assembly processes of these structures. The extensive basement membranes of lung undergo substantial expansion at about the time of birth. To explore the use of rat lung as a system for the study of basement membrane synthesis, we measured incorporation of radioactive amino acids into a major component, procollagen IV. This showed unexpected changes with neonatal age and therefore we investigated the corresponding levels of the RNAs that specifically code for it. Then we found a sequence of biosynthetic assembly steps of procollagen IV and confirmed this by further studies with mouse and chick embryo lung and chick embryo gizzard. We first present the problems and our results on the supramolecular assembly of procollagen IV, and then our observations on the changes of synthesis and RNA levels with age. We regard them as related facets of one problem: the formation of basement membranes in a developing organ.

A procollagen IV molecule consists of three $\text{pro}\alpha(\text{IV})$ chains, each with specialized amino and carboxyl ends. Digestion of basement membranes with bacterial collagenase sets free two junctional complexes between the ends of procollagen IV molecules. Both junctional complexes are resistant to this enzyme. One is the so-called 7S collagen, which is a complex of the amino ends of four procollagen IV molecules ($4 \times 3 = 12$ peptides). The other complex con-

sists of the carboxyl ends of two procollagen IV molecules ($2 \times 3 = 6$ peptides) (29). The carboxyl ends are also known as NCI domains and appear as knob-like structures in electron micrographs.

Various models have been proposed for the three-dimensional organization of procollagen IV in basement membranes including the "network" model of Timpl et al. (29) and the "polygonal array" of Yurchenko and Furthmayr (34). Both of these models are based on a polygonal arrangement of procollagen IV molecules within the basement membrane; the most salient difference between the models is the emphasis on end-to-end associations in the former and lateral associations in the latter. Both groups find that limited pepsin digestion of basement membrane-containing material yields tetramers of collagen IV (18, 28, 29). The proponents of the former model propose that tetramers of procollagen IV (29) which are joined through their amino termini (11) are the primary assembly intermediates (29). The proponents of the latter model suggest that a dimer of procollagen IV joined through the carboxyl ends of the molecules is the primary assembly intermediate (34). Extensive lateral association between basement membrane collagen molecules has been deduced by Kefalides et al. (16). Furthermore, tissue-specific variations of the arrangement of procollagen IV molecules are likely to occur (16).

Previously we recovered monomers and tetramers of procollagen IV molecules from the culture medium of PF-

HR9 cells (1, 11) and formed tetramers *in vitro* from monomeric procollagen IV through the action of glutathione (10). Recently, we extended this to the *in vitro* formation of octamers, dodecamers, hexadecamers, and higher forms (9; Duncan, K. G., and J. H. Fessler, manuscript in preparation). We also suggested that the tetramer represents an assembly intermediate (10). In contrast, Yurchenko and Furthmayr (34) concluded from their *in vitro* studies with covalently linked dimers, which had been obtained by reductive extraction of murine Engelbreth-Holm-Swarm (EHS)¹ tumors, that dimers constitute the principal intermediate assembly form (34). The current studies were undertaken to find what intermediates of assembly occur in tissues that actively make basement membranes, as contrasted with the previous *in vitro* investigations. Tissues have been used previously to study the biosynthesis of monomeric procollagen IV and its constituent pro α 1(IV) and pro α 2(IV) chains (13–15, 25, 31), but not the formation of higher assemblies.

We distinguish between initial noncovalent interactions between procollagen IV molecules, which can be disrupted by changes of solvent, such as addition of urea (1, 10), and subsequent stabilization of these interactions by formation of disulfide links (10) and other covalent bonds (27, 33). Exposure of procollagen IV molecules to glutathione produces several effects: it facilitates some noncovalent interactions (9; Duncan, K. G., and J. H. Fessler, manuscript in preparation), causes the formation of disulfide links (10; Duncan, K. G., and J. H. Fessler, manuscript in preparation), and exchanges mixed disulfides (10). The facilitation of noncovalent interactions is presumably due to conformational changes of procollagen IV molecules that may be associated with a rearrangement of internal disulfide links. When cells release procollagen IV molecules into culture media that contain disulfides, then these exchange to form mixed disulfides with the procollagen IV. These mixed disulfides hinder the normal formation of disulfide bridges between adjacent procollagen IV molecules in an assembly. Subsequent addition of glutathione assists in overcoming this blockage (10).

For our biosynthetic studies we first had to establish that certain radioactive electrophoretic components did represent mouse and chick procollagens IV. The measurement of the levels of the RNAs coding for rat lung procollagen IV required specific probes. The mouse cDNA probes PE123 and PE18 are specific for, respectively, mouse pro α 1(IV)² and mouse pro α 2(IV) (21). It was necessary to establish that these probes hybridized with corresponding specificities to RNAs from rat, and in particular that they did not react with RNAs for the abundant procollagen I. For comparison and reference, RNA preparations were used from mouse and rat parietal endoderm, from F9 teratocarcinoma cells which had been induced to change to parietal endoderm-like cells and from rat calvarial bones. Parietal endoderm and induced F9 cells make procollagen IV (20). Neonatal calvaria synthesize much procollagen I but no detectable amounts of procollagen IV.

Materials and Methods

Unless otherwise stated, the methods that we have described previously (1, 10, 11) were used.

1. *Abbreviation used in this paper:* EHS tumors, Engelbreth-Holm-Swarm tumors.

2. Kurkinen, M., M. R. London, B. Blumberg, D. Barlow, S. Quinones, J. Saus, and T. Pihlajaniemi, manuscript submitted for publication.

Isolation and Incubation of Lungs and Gizzards

Rat lungs, free of trachea and major blood vessels, were freshly dissected from prenatal, newborn, and up to 17-d-old Sprague-Dawley rats. The tissue was rinsed in Dulbecco's modified Eagle's medium (DME) and then cut into ~3-mm-wide pieces. Preincubation was for 30 min at 37°C in DME bubbled with 95% oxygen, 5% carbon dioxide, devoid of proline and leucine or proline, leucine, and methionine, and containing 0.1 mg/ml ascorbic acid (Sigma Chemical Co., St. Louis, MO), 0.064 mg/ml 2-amino propionitrile fumarate (Aldrich Chemical Co., Milwaukee, WI), and 0.02 M Hepes buffer at pH 7.4 (Sigma Chemical Co.). The tissues were incubated for 2 h in DME as above containing 0.1 mCi/ml [5-³H]proline (35 Ci/mM) (Amersham Corp., Arlington Heights, IL) and 0.1 mCi/ml [4', 5'-³H]leucine (79 Ci/mM) (ICN K & K Laboratories Inc., Plainview, NY) and sometimes also containing 0.1 mCi/ml [³⁵S]methionine (1,100 Ci/mM) (New England Nuclear, Boston, MA). Then the medium was replaced with identical, fresh DME and incubation was continued for 2 h. 18-d chick gizzards, 14-d chick lungs, and neonatal mouse lungs were preincubated and incubated as above. After incubation, the tissues were rinsed in cold DME and quick-frozen in liquid nitrogen. The frozen tissue was stored at -70°C.

Extraction

The frozen tissue was weighed and then homogenized at 0°C in an extraction buffer containing 4 M guanidine-HCl, 0.05 M Tris-HCl, 0.05 M EDTA, 0.1% Triton X-100, 0.15 mg/ml phenylmethylsulfonyl fluoride (PMSF) (Sigma Chemical Co.), 1.25 mg/ml *N*-ethylmaleimide (Sigma Chemical Co.), pH 7.8. The homogenates were centrifuged at 0°C at 40 krpm for 15 min in an 80Ti rotor (Beckman Instruments, Inc., Palo Alto, CA). The clarified extract was dialyzed at 4°C into Buffer A (2 M urea, 0.05 M Tris-HCl, 5 mM EDTA, 0.1% Triton X-100, 0.02 M NaCl, pH 7.8). After dialysis, the extract was centrifuged at 10 krpm in a Sorvall SS-34 rotor at 4°C to remove suspended material. Procollagen IV and collagen I were partially purified by chromatography on DEAE-cellulose (DE-52; Whatman Inc., Clifton, NJ) equilibrated in Buffer A; DEAE-cellulose does not retain either procollagen IV or collagen I under these conditions (1). Several aliquots of the pooled effluent fractions containing procollagen IV and collagen I were reduced and electrophoresed on SDS-4.5% polyacrylamide slab gels (22) and fluorographed (2, 23). The amounts of labeled procollagen IV and collagen I were determined by densitometric analysis of the fluorograms. Fluorograms were scanned in an Optronics P-1000 scanning microdensitometer interfaced to a Digital Equipment Corporation VAX 11/780 computer and the areas under the peaks were resolved and integrated (17).

The insoluble residue was re-extracted by homogenization twice with 4 M guanidine-HCl buffer and then three times with Buffer B (0.15 M NaCl, 0.05 M Tris-HCl, 0.1% Triton X-100 at pH 7.5). These latter extracts did not contain procollagen IV. An aliquot of the residual precipitate was then suspended in 4 M guanidine-HCl buffer containing 50 mM dithiothreitol (DTT) to solubilize the remaining procollagen IV at 8°C for 18 h and then the dialyzed extract was electrophoresed on an SDS-4.5% polyacrylamide gel. The main portion of the residual precipitate was suspended in Buffer B plus 7 mM CaCl₂ and digested with bacterial collagenase (CLSPA; Worthington Biochemical Corp., Freehold, NJ) at 35°C for 18 h to yield the noncollagenous amino and carboxyl peptides.

Bacterial Collagenase Digestion and Immunoprecipitation

Aliquots of fractions containing procollagen IV were dialyzed into Buffer B at 4°C. CaCl₂ was added to 7 mM and an aliquot was digested at 35°C for 3 h with purified bacterial collagenase. The control and digested samples were electrophoresed in SDS-4.5% slab gels. Immunoprecipitation with anti-mouse procollagen IV serum and Pansorbin (Calbiochem-Behring Corp., La Jolla, CA) was performed as described (1). The immunoprecipitated material was analyzed on SDS-4.5% polyacrylamide slab gels.

DEAE-cellulose effluent fractions spanning the procollagen IV peak were pooled and concentrated 10-fold by vacuum dialysis into Buffer B. The entire sample was electrophoresed on an SDS-1.5% agarose slab gel. The bands corresponding to monomers, dimers, and tetramers of procollagen IV were located by fluorographing an adjacent standard lane. The gel was fixed in 50% methanol-7% acetic acid for 30 min and then dried. The bands of interest were removed and reswelled in Buffer B containing 7 mM CaCl₂ and bacterial collagenase. The samples were digested for 3 h at 35°C and then electrophoresed without reduction on SDS-12% polyacrylamide gels and fluorographed.

Velocity Sedimentation

Aliquots of the peak DEAE-cellulose effluent column fractions were sedimented on 5–20% sucrose gradients in Buffer A for 17 h at 51 krpm in an SW60 rotor (Beckman Instruments, Inc.) at 22°C (1). Fractions were collected and aliquots were reduced and electrophoresed on SDS-4.5% polyacrylamide slab gels. Other aliquots were denatured with 0.2% SDS and electrophoresed on SDS-1.5% agarose gels (10) and fluorographed (32) to identify tetramers, trimers, dimers, and monomers of procollagen IV molecules. Aliquots of bacterial collagenase digests were centrifuged on 25–95% D₂O gradients in 0.155 M ammonium acetate, pH 7.5, for 3.5 h at 35°C and 56 krpm in an SW60Ti rotor (Beckman Instruments, Inc.). Fractions were collected and aliquots were electrophoresed on SDS-12% polyacrylamide slab gels, fluorographed, and quantitated by densitometry.

DNA Determination

Aliquots of the clarified tissue homogenates were analyzed for DNA content by the spectrofluorometric method of Brunk et al. (4). Four successive aliquots of the homogenate were added to 3 ml of buffer containing 0.1 M NaCl, 0.01 M EDTA, 0.01 M Tris-HCl, pH 7.0, and 100 ng/ml 4',6-diamidino-2-phenylindole (a gift of Dr. Eric Mundall, UCLA). The samples were excited at a wavelength of 360 nm and the emission at 450 nm was measured after addition of each aliquot, giving a linear plot of fluorescence enhancement against volume of homogenate. To the same solution, four aliquots of a standard DNA solution were then added for internal calibration of fluorescence enhancement. This proven method is insensitive to sugar residues and to both double-stranded and single-stranded RNA. It was also used to assay the RNA samples for possible DNA contamination and none was found.

RNA Preparation and Analysis

Freshly dissected calvaria and lungs from neonatal through 21-d-old rats were homogenized in 4 M guanidinium thiocyanate (Bethesda Research Laboratories, Gaithersburg, MD) buffer containing 0.01 M sodium acetate, 0.1 M 2-mercaptoethanol (Sigma Chemical Co.), 0.1% (wt/vol) *n*-lauryl sarcosinate (Sigma Chemical Co.) in a Polytron (Brinkmann Instruments Co., Westbury, NY) homogenizer at top speed for 2–30-s bursts. The RNA was purified by centrifugation through a 2-ml cushion of 6.0 M CsCl, 0.1 M EDTA, pH 5.0, in a Beckman SW40Ti rotor at 28,000 rpm for 18 h at 20°C (6). The concentration of CsCl was increased to 6.0 M as it was empirically determined that the RNA prepared in this way was more soluble in H₂O after centrifugation, presumably because there are fewer proteoglycan contaminants in the RNA under these conditions. RNA concentration was measured spectrophotometrically (24).

Aliquots containing 20 µg of total cellular RNA were denatured with formamide and formaldehyde (24) and electrophoresed in 0.8% agarose-formaldehyde slab gels along with 20 µg rat calvarial RNA, 2 µg each of total cellular RNA from mouse parietal endoderm, mouse F9 embryonal carcinoma cells, induced mouse F9 cells, and rat parietal endoderm (20). The gels were run at 90 milliamps constant current for 5 h, the RNA was electrophoretically transferred to Gene Screen Plus (New England Nuclear), and dried to fix the RNA to the membrane. The filters were prehybridized for 2 h at 68°C in 5× SSPE (1× SSPE is 0.18 M NaCl, 0.05 M NaH₂PO₄, 1 mM EDTA, pH 7.0), 5× Denhardt's solution (1× Denhardt's is 0.02% bovine serum albumin, 0.02% polyvinyl pyrrolidone, and 0.02% Ficoll) (8), 1% SDS, 100 µg/ml denatured salmon sperm DNA. Hybridization was with the 1,800-base pair (bp) mouse cDNA (PE123) which encodes most of the NCI domain from pro α 1(IV) and the entire 3' untranslated region² or the 1,100-bp mouse cDNA (PE18) which encodes a portion of the triple-helical domain for pro α 2(IV) (21). The probes were nick-translated (26) to a specific activity of $>3 \times 10^8$ cpm/µg of input DNA [α -³²P]-dCTP at $>3,000$ Ci/mM (New England Nuclear). The hybridization was carried out at 68°C in the above buffer in a volume of 10 µl/cm².

For slot-blot hybridization, 2.5-, 5.0-, and 10.0-µg aliquots of rat lung total RNA were made in 10× SSC (1× SSC is 0.15 M NaCl, 0.015 M Na citrate, pH 7.0) after denaturation of the RNA in 2.2 M formaldehyde, 10× SSC at 65°C followed by quick cooling on ice. The samples were applied to nitrocellulose membranes (BA-85; Schleicher & Schuell, Inc., Keene, NH) held in a Schleicher & Schuell Minifold II. The RNA was fixed to the filter by baking and prehybridized and hybridized as above except that the concentration of SDS was 0.1%. Washing was at 68°C in both cases in 0.1× SSC, 0.5% SDS. Autoradiography was performed using DuPont Lightning Plus intensifying screens (DuPont Co., Wilmington, DE), and preflashed

Kodak XAR-5 x-ray film (23) at -70°C. A typical exposure time was 18 h. Autoradiographs were analyzed by densitometry as above.

Total TCA-precipitable counts were estimated as described (19).

Results

Identification of Procollagens IV and Their Associated Forms in Lung and Gizzard

The following indicates that procollagens IV from these different tissues and species are similar molecules, with similar associated forms, which include tetramers that are partly covalently linked. All procollagens were sensitive to highly purified bacterial collagenase (Fig. 1). The electrophoretic mobilities in SDS-4.5% polyacrylamide gels and SDS-1.5% agarose gels were compared with those of standard mouse procollagen IV prepared as described (1). Rat procollagen IV showed similar mobilities in SDS-4.5% polyacrylamide gels to mouse procollagen IV: the pro α 1(IV) band co-migrated with that of mouse pro α 1(IV), and rat pro α 2(IV) migrated slightly slower than mouse pro α 2(IV) (Fig. 1). Rat procollagen IV was immunoprecipitated by an antiserum prepared against mouse procollagen IV derived from PF-HR9 cells (1) (Fig. 1). Mouse pulmonary procollagen IV was indistinguishable from the materials made by murine PF-HR9 cells, as tested by immunoprecipitation, gel electrophoresis before and after reduction, and gel electrophoresis of noncollagenous peptides after digestion with bacterial collagenase (data not shown). Chick pulmonary and gizzard procollagens were also closely similar, but were not precipitated by the above antiserum. On SDS-1.5% agarose gels the electrophoretic mobilities of the procollagen IV monomers, consisting of three pro α chains, and dimers, trimers, and tetramers were indistinguishable from those of the correspondingly associated forms of mouse procollagen IV (Fig. 2).

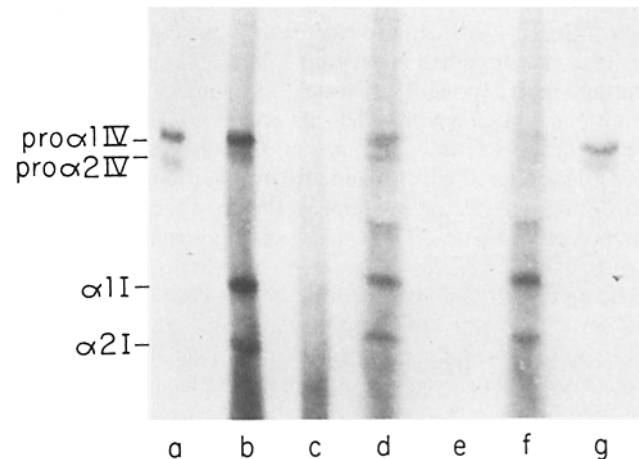


Figure 1. Immunological identification of rat lung procollagen IV. Aliquots of newly synthesized, DEAE-cellulose-purified procollagen IV were reduced and electrophoresed in SDS 4.5% polyacrylamide slab gels either before (b) or after (c) treatment with highly purified bacterial collagenase. Other aliquots were reacted with either preimmune serum or rabbit anti-mouse procollagen IV antiserum and the complexes were precipitated with formaldehyde-fixed *Staphylococcus aureus* (d–g). (d) Preimmune serum supernatant; (e) preimmune serum precipitate; (f) immune serum supernatant; (g) immune serum precipitate. Lane a is mouse procollagen IV prepared from the culture medium of PF-HR9 cells.

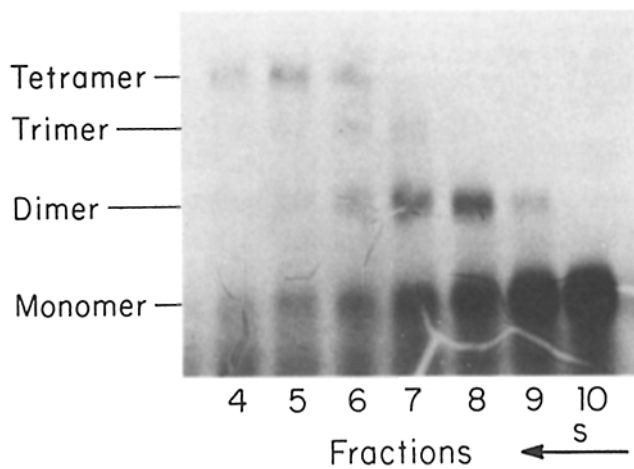


Figure 2. Velocity sedimentation of newly made, native rat lung procollagen IV monitored by SDS agarose gel electrophoretic analysis. A 200- μ l aliquot of DEAE-cellulose-purified procollagen IV, labeled with [3 H]proline and [3 H]leucine for 4 h, was sedimented at 22°C on a 5–20% sucrose gradient containing buffer A. Fractions were collected and electrophoresed on an SDS–1.5% agarose slab gel. The fluorogram is shown. Sedimentation was from right to left. The electrophoretic mobilities of identified, covalently linked oligomers of mouse procollagen IV are indicated.

Covalently linked tetramers, trimers, dimers, and monomers of rat procollagen IV molecules were resolved by velocity sedimentation (Fig. 2). Their sedimentation properties corresponded to the predicted values obtained with covalently linked mouse procollagen IV molecules. The equivalent sedimentation behavior of chick gizzard procollagen IV monomers and of tetramers are illustrated in Fig. 3. The top panel shows densitometric evaluation of the total pro α (IV) chains from electrophoretic analyses of aliquots of successive sedimentation fractions. The ratio pro α 1(IV)/pro α 2(IV) for these electrophoretic bands was (58–62):(42–38) (data not shown). The fractions indicated by bars A and B were pooled, resedimented in separate tubes, and gave the distributions shown in the lower panel. The small trailing foot of peak A could either be due to inherently present dimers of molecules or to some dissociation of tetramers which were only partly covalently linked. Electrophoretic analysis on SDS–1.5% agarose gels confirmed the presence of tetramers, monomers, and small amounts of dimers (data not shown).

Supramolecular Association of Procollagen IV

This section deals with the progressive cross-linking of procollagen IV molecules in tissues, which took several forms. Material that was soluble without reduction was covalently associated into oligomers of increasing size. With time, most of the material required reduction for extraction. Tissues were incubated with radioactive amino acids for 3 or 4 h and were analyzed after chase periods of up to 22 h. Immediately at the end of the labeling period several sequential extractions with buffered 4 M guanidine-HCl solubilized 90% of the procollagen IV. The remaining 10% of extractable procollagen IV was obtained subsequently with the same solvent, but in the presence of 50 mM DTT. The total radioactivity which was extractable only after reduction increased slightly more rapidly than linearly with chase time. Only a minor part of the initial radioactivity remained in the

small residues that were left after reductive extraction. The following experiments indicated that both disulfide bonds and other covalent bonds progressively linked the procollagen IV assemblies.

The procollagen IV that was extracted without reduction from chick gizzard or rat lung showed changes in covalent linkage with chase time, upon SDS PAGE analysis under reductive conditions (data not shown). While at the end of the labeling period most of this material was resolved as individual pro α 1(IV) and pro α 2(IV) chains, higher covalently-linked forms successively appeared later. Progressively, radioactive bands occurred in the positions of two pro α chains linked to each other, at the interface of the 4.4% running gel and 3% stacking gel of polyacrylamide, and as material which could not enter the stacking gel.

The following results indicate that disulfide bridges progressively stabilize the amino junctional complex, and only later, and slowly, the carboxyl junctional complex. At the end of a 3- or 4-h labeling period most of the radioactive material was extracted without reduction. It was partly associated into oligomers of procollagen IV molecules, and these were incompletely linked, internally, by disulfide bonds. Velocity sedimentation in 2 M urea at 4°C disrupts some, or all, of the noncovalent interactions of such molecules (1). Under these conditions, extracts of chick gizzard gave mostly monomers and some tetramers of procollagen IV (Fig. 3). Velocity sedimentation analysis of extracts

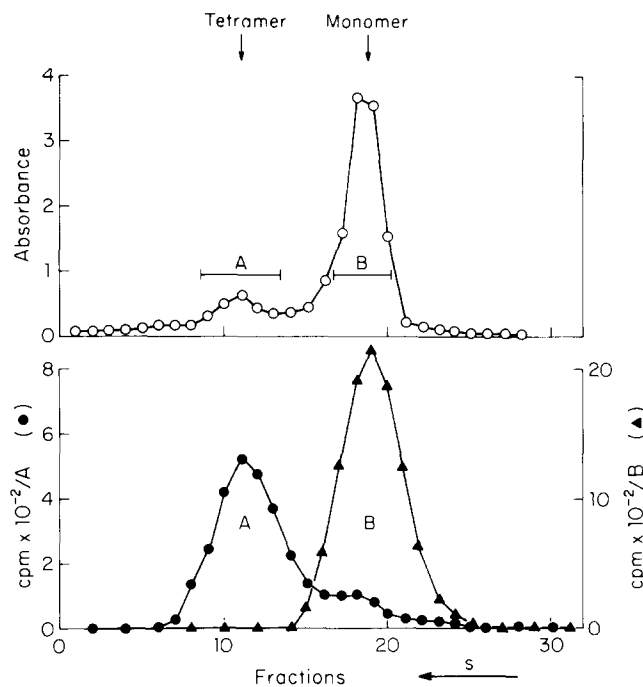


Figure 3. Resolution of associated forms of chick smooth muscle procollagen IV by repeated velocity sedimentation. A 200- μ l aliquot of DEAE-cellulose-purified, radioactive chick gizzard procollagen IV was sedimented at 4°C on a 5–20% sucrose gradient in buffer A, fractions were collected, and aliquots were analyzed electrophoretically on an SDS–4.5% polyacrylamide gel and quantitated by densitometry. Aliquots of the fractions spanning the tetramer peak (A) and the monomer peak (B) were pooled and resedimented separately. The sedimentation profile is shown in the bottom panel. The arrows indicate the peaks of mouse procollagen IV tetramers and monomers.

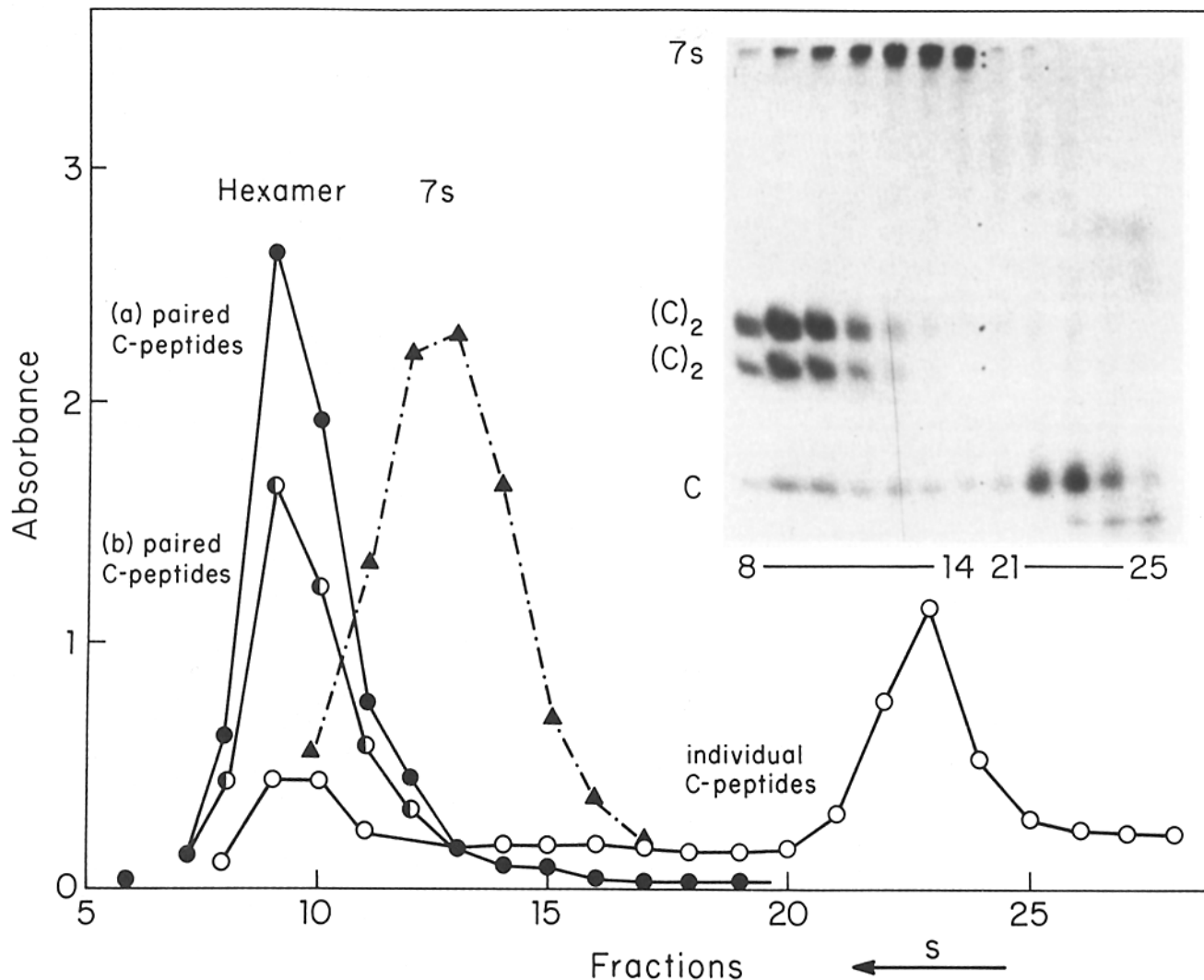


Figure 4. Carboxyl and amino peptides of procollagen IV released by collagenase digestion of rat lungs. Lungs were labeled for 4 h, chased for 19 h, and extracted with 4 M GuHCl. The residue was digested with collagenase and the solubilized material was sedimented through a 25–95% D₂O gradient. Successive fractions were analyzed by SDS-12% PAGE. A fluorogram is shown together with the densitometric measurements of the following bands for each fraction: ○ and C, individual C-peptides; ▲ and 7s, 7S collagen; ● and C₂, slower migrating, paired peptides (a); and ◐ and C₂, faster electrophoresing peptides (b). Direction of sedimentation was from right to left.

of freshly labeled rat lung in 2 M urea at 22°C gave a range of sedimentation velocities, and subsequent analysis of sedimentation fractions by SDS-agarose electrophoresis showed the presence of covalently linked tetramers, trimers, and dimers, as well as individual procollagen molecules (Fig. 2). Some of these may have been in noncovalent associations with other procollagen IV molecules in the tissue. The dimers of procollagen IV molecules in Fig. 2 could be covalently linked either through their carboxyl or their amino ends. We were unable to recover covalently linked carboxyl peptides from this electrophoretic band after digestion with bacterial collagenase. As described below, covalently linked carboxyl peptides only appeared relatively slowly in the insoluble residues. We conclude that the dimeric material of Fig. 2 was probably linked through the amino ends, and represents an incompletely linked junctional complex that was still susceptible to bacterial collagenase.

To obtain further information on all the procollagen IV that resisted extraction with buffered 4 M guanidine without

reduction, the residue was digested with bacterial collagenase. This set free the noncollagenous amino and carboxyl ends of the procollagen IV molecules and we examined their states of association. Samples were sedimented at 35°C on 5–20% sucrose or 25–95% D₂O gradients and successive sedimentation fractions were analyzed by SDS PAGE. Fig. 4 shows one of the fluorograms and the densitometric measurements of the bands of individual carboxyl peptides (C), the 7S tetrad of amino peptides, and two sets of dimerically linked carboxyl peptides denoted as (C)₂, a and b. We ascribe the slower sedimentation peak to individual, monomeric carboxyl peptides and the faster one to hexamers of the carboxyl peptides in the “knob-to-knob” junction between the knob-like carboxyl ends of two molecules. In sucrose gradients, this faster peak had a corrected sedimentation velocity of 8.6S, in agreement with that measured for chemically purified hexamers (33). When the same sedimentation fractions were reduced before electrophoresis, some but not all of the dimerically linked carboxyl peptides resolved into

Table I. Rate of Covalent Bond Formation in Insoluble Type IV Procollagen Networks

Total incubation time	4 h	8 h	18 h
	%	%	%
Amino peptides			
covalently cross-linked	100	100	100
Carboxyl peptides			
covalently cross-linked	13	21	71
noncovalently associated	87	79	29

Lungs were labeled with radioactive amino acids for 4 h, chased for an additional 4 h and 14 h, and then analyzed as described for Fig. 4 and in the text.

individual peptides (not shown). Therefore, some are linked by disulfide bridges. We have not characterized the different dimers (a) and (b), but they closely resemble corresponding variations of dimers of the carboxyl ends of bovine renal glomerular basement membrane collagen (7) and collagen IV from EHS tumors (33). Reduction also slowed the migration of the monomeric carboxyl peptides, as previously described (11) due to opening of intrapeptide disulfide bonds (not shown). The proportion of carboxyl peptides in the hexamer peak which are covalently linked as dimers increased with incubation time, as illustrated by the results of Table I, whereas all of the 7S collagen was covalently linked at the corresponding times. At early incubation times, most of the carboxyl peptides in the hexamer peak were not covalently linked. We have not examined how far the distribution of individual carboxyl peptides changes between hexamer and monomer peaks with experimental conditions, but the total peptides in the hexamer peak increased with incubation time. We conclude that time-dependent maturation processes influence the association, covalent linkage, and electrophoretic migration as dimers of the carboxyl peptides.

Specificity of the cDNA Probe

The following experiments established the ability of the mouse type IV collagen cDNA probes to cross-hybridize to rat RNAs, and demonstrated the lack of cross-hybridization of the probes to type I collagen RNAs. The specificity of the mouse cDNA probes PE123 and PE18 for, respectively, *pro α 1(IV)* and *pro α 2(IV)* was demonstrated by hybridization to total cellular RNA which had been denatured and electrophoresed on 0.8% agarose-formaldehyde gels (24) and electrophoretically transferred to Gene Screen Plus membrane (Fig. 5). PE123 encodes the carboxyl-most 204 amino acids of the carboxyl (NCl) domain and the entire 3' untranslated region of mouse *pro α 1(IV)*². PE18 encodes a portion of the triple-helical domain of mouse *pro α 2(IV)* (21). PE123 hybridized to a major band of \sim 6.8 kb and a minor band of 6.2 kb in both mouse and rat total RNA (Fig. 5 A). PE18 hybridized to a band of 6.4 kb in both mouse and rat total RNA (Fig. 5 B). Neither probe hybridized to total RNA from rat calvaria which make procollagen I but not procollagen IV; thus these probes measured the levels of procollagen IV mRNA independently of type I collagen mRNA. In both cases, hybridization and washing were carried out stringently at $T_m - 10^\circ\text{C}$ for the RNA-DNA duplex (5). Under these conditions no cross-hybridization of the probes was seen to either the mRNA encoding the other chain or to any other RNA.

Our subsequent use of these probes to quantitate the levels of rat lung procollagen IV RNAs is strengthened by the results of Fig. 5, lanes *a* and *b*. These show the reported increase of mouse procollagen IV RNAs upon differentiation of F9 teratocarcinoma cells to parietal endoderm-like cells (20). The detection of procollagen IV RNAs in mouse and rat parietal endoderm (Fig. 5, lanes *c* and *d*), is consistent with the known elaboration of procollagen IV by these tissues. However, as the relative concentration of these RNAs are not known in the mouse and rat samples comparison of lanes *c* and *d* is not an assured index of the degree of interspecies cross-hybridization of these probes. Parietal endoderm has one of the highest rates of procollagen IV production. The content of procollagen IV RNA in 3-d-old rat lung is less than in parietal endoderm, but is still comparable with it (Fig. 5, lanes *d* and *f-h*). This supports the conclusion that neonatal rat lung intensively synthesizes basement membrane collagen.

Developmental Expression of Procollagen IV and Collagen I

The following data show that both types I and IV procollagen syntheses decrease drastically at birth and then, after a minimum at \sim 2 d postnatal, increase again. The decline and return of procollagen IV RNA level is similar, but not identical. Developmental expression of procollagen IV was monitored both at the level of protein synthesis and of mRNA. A measure of the total radioactivity in procollagen IV or collagen I recovered from *in vitro*-labeled lungs was obtained by electrophoresis of DEAE-cellulose effluent fractions followed by fluorography and densitometry of the fluorograms. The percent of radioactivity in procollagen IV was expressed as densitometer units normalized to total TCA-precipitable cpm and plotted as a function of time before or after birth (Fig. 6 A). The percentage of radioactivity hybridized to *pro α 1(IV)* mRNA was expressed in densitometer units normalized to total input RNA and plotted as a function of time from birth (Fig. 7). There were coordinate decreases in the relative levels of procollagen IV polypeptides and *pro α 1(IV)* mRNA which reached a minimal level at \sim 2 d after birth. Subsequently, the level of procollagen IV and *pro α 1(IV)* mRNA increased sharply until \sim 5 d after birth, at which time the mRNA levels began to decrease but the protein levels continued to increase until 7 d before decreasing. *Pro α 2(IV)* mRNA levels followed a similar profile (not shown). Collagen I protein levels exhibited essentially the same pattern as procollagen IV (Fig. 6 C). This suggests that the two collagens may be coordinately regulated. Unfortunately, no DNA probe was available to us which could hybridize exclusively to procollagen I message in this system.

The developmental expression of both collagens was also graphed as radioactivity in the extracted collagen, normalized to the mass of DNA in the total cell extract (Fig. 6, B and D). A sharp drop in the levels of both procollagen IV and collagen I followed birth. The minimal levels were reached at 1-3 d after birth and increased rather sharply thereafter. These measurements were made on groups of animals of different litters with an uncertainty of half a day in their age. For more precise determinations, sibling rats were killed at successive ages at half-day intervals over a 3-d period and the lungs were removed and divided into two por-

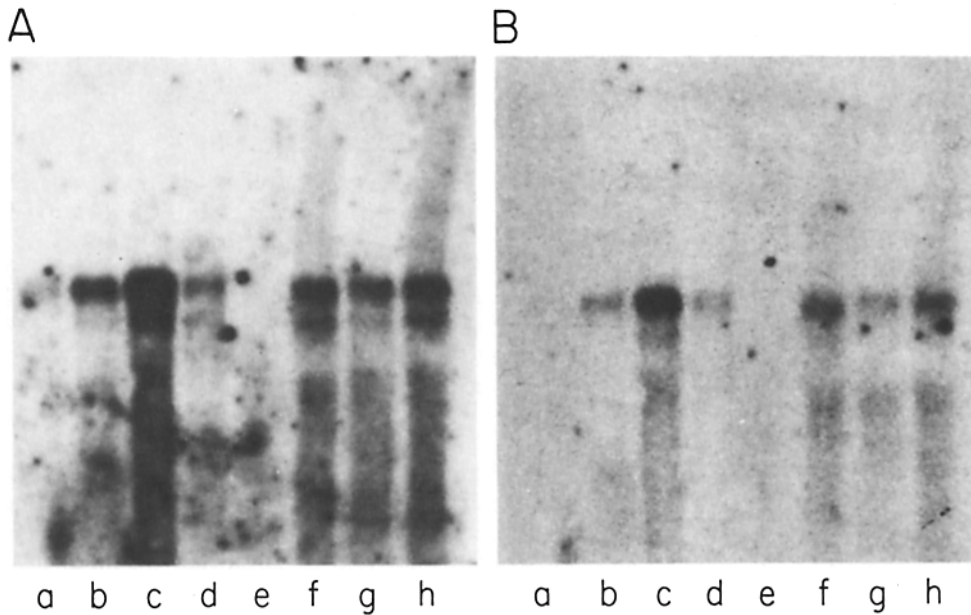


Figure 5. Hybridization of rat lung RNA with cDNA probes for mouse pro α 1(IV) and pro α 2(IV). Aliquots of total cellular RNA were denatured with formamide and formaldehyde and electrophoresed in an 0.8% agarose formaldehyde gel. The RNA was electrophoretically transferred to Gene Screen Plus membrane. (a) 2 μ g undifferentiated F9 mouse embryonal carcinoma RNA; (b) 2 μ g differentiated F9 RNA; (c) 2 μ g mouse parietal endoderm RNA; (d) 2 μ g rat parietal endoderm RNA; (e) 20 μ g rat calvaria RNA; (f-h) 20 μ g rat lung RNA from different 3-d-old rats. (A) Hybridization with probe PE123 which is specific for pro α 1(IV). (B) Same blot rehybridized with PE18 which is specific for pro α 2(IV). Exposure time was 18 h at -70°C with 2 Dupont Cronex lighting plus intensifying screens.

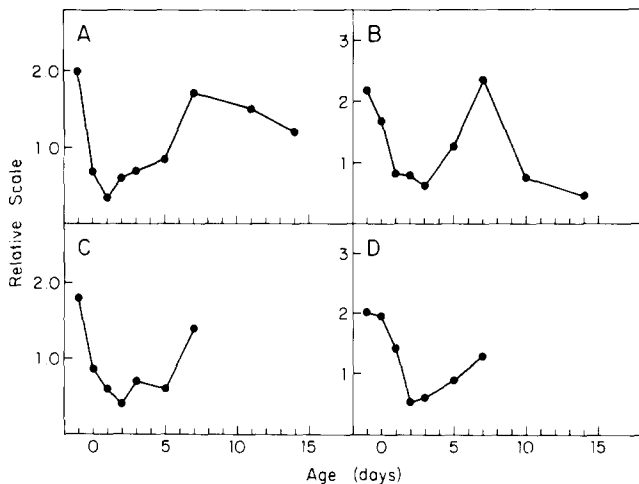


Figure 6. Rates of synthesis of procollagen IV and collagen I in neonatal rat lung. Aliquots of DEAE-cellulose effluent fractions containing radioactively labeled procollagen IV and collagen I were pooled and analyzed by SDS-4.5% PAGE. The amounts of each protein were quantitated by densitometry and normalized to total DNA in ng or TCA-precipitable counts. (A) Procollagen IV normalized to total TCA-precipitable cpm. (B) Procollagen IV normalized to total DNA. (C) Collagen I normalized to TCA-precipitable cpm. (D) Collagen I normalized to total DNA in ng.

tions: $\frac{3}{4}$ of each lung was incubated to measure protein synthesis and total DNA and $\frac{1}{4}$ was used for RNA extraction. Under these conditions, the minimal levels of both procollagen IV and of collagen I were reached at 2 d after birth when normalized to total DNA concentration. The minimal levels of synthesis of both procollagen IV and of collagen I were reached at 1.5 d after birth normalized to total TCA-

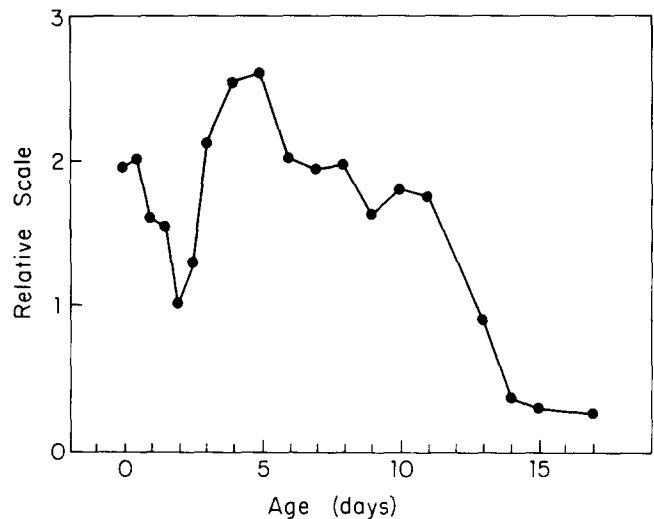


Figure 7. Variation of mRNA for procollagen IV with age in neonatal rat lung. Total cellular RNA from animals of different ages was denatured and attached to nitrocellulose filters as described. The filters were probed with PE123, autoradiographed, and densitometrically evaluated. The ordinate values are the variation of autoradiographic density with mass of RNA.

precipitable cpm as was the level of pro α 1(IV) mRNA normalized to total RNA (data not shown).

Discussion

This study combines measurements of mRNA levels, newly produced protein, and supramolecular assembly of basement membrane collagen over a developmental time period. Birth subjects the neonate to great physiological changes. Our

results demonstrate that birth is accompanied by substantial, temporary decreases in the synthesis of lung procollagens I and IV, which are initiated before birth. Complex hormonal changes that occur at birth will influence protein synthesis. The drop in lung collagen synthesis is seen equally whether the results were normalized to total protein synthesis or to DNA content. As the RNA for procollagen IV, expressed as a fraction of total lung RNA, also drops at this time the results suggest a causal relationship of changes in message level and procollagen IV synthesis. The RNA level is controlled both by transcription and message stability, which were not determined. While our investigation was focused on neonatal events, measurements at ages approaching 2 wk indicated increasingly more procollagen IV biosynthesis in proportion to specific RNA at this later age. Thus during the first 2 wk of life, the production of rat lung procollagen IV is influenced both by changes in the level of the RNA which codes for it, and by the efficiency with which this RNA is used.

Tolstoshev et al. (30) compared the use of mRNA for procollagen I in neonatal sheep lung and skin. In skin, the procollagen I translated in vitro from extracted mRNA was not proportional to the mRNA levels measured by dot-blot hybridization. However, for lung the cell-free production of pro α peptides was correlated with the extractable RNA. This suggests that the drop in procollagen I synthesis which we observed in neonatal rat lung at birth may also have been accompanied by a decrease in the level of the RNA coding for it. The coordinate decreases in the synthesis of procollagens I and IV may have been mandated by the intense cell replication that occurs at this time, as indicated by measurements of DNA concentration. However, total protein synthesis as measured by total TCA-precipitable radioactivity incorporated from radioactive amino acids did not decrease. The results suggest that in the expansion of epithelial cell sheets, cell replication may precede the actual increase of basement membrane matrix which must eventually accompany the basal cell surfaces. Electron microscopic studies of the developing rat lung show a discontinuous basement membrane during early lung development in accord with our biochemical data (3, 12).

Neonatal rat lung synthesizes a remarkably high proportion of basement membrane collagen, as judged by the ratio of radioactive amino acids incorporated into procollagen IV relative to collagen I, thus it is a good experimental model. A steady synthetic rate and good supramolecular association are continued for several hours. Our isolation of radioactive tetrads of procollagen IV molecules, linked through their amino ends, shows that these tetramers are assembly intermediates of the basement membrane collagen scaffold. Monomeric, three-chained molecules appeared first and are the precursors for these tetramers, as well as for dimers and trimers. The covalently linked dimers and trimers that we found may in part be dissociation products of only partly covalently linked tetramers. Similar tetramers were isolated from chick embryo gizzard as well as chick lungs, supporting the view that tetramers are functional assembly intermediates. Kefalides et al. (16) have shown that in anterior lens capsule, tetramers of procollagen IV are the predominant acid-extractable form. While some tetramers could have been set free from partly assembled networks, our pulse-chase experiments also show that the covalent linkage of the

amino ends of pulmonary procollagen IV proceeds significantly faster than that of the carboxyl ends.

A remarkable noncovalent association of the carboxyl ends of two molecules (six chains) occurs. This may be important at an early point of assembly. It increases with time and eventually covalently linked pairs of peptides appear. There is a maturation process, and two different kinds of paired peptides appear, similar to different forms of carboxyl peptides extracted from renal basement membranes and EHS sarcoma (7, 33). It seems that maturation of the carboxyl-carboxyl junction proceeds relatively slowly in the systems that we examined. Thus when general cross-linking had produced an insoluble matrix that required digestion with bacterial collagenase, a substantial portion of the carboxyl peptides that were set free were not part of covalently closed hexameric junctions. In contrast, most of the basement membrane collagen IV extracted from the EHS tumor consists of dimers of molecules linked by disulfide bridges and unknown covalent links between their carboxyl ends. In vitro reconstitution experiments, based on this dimeric material, led Yurchenko and Furthmayr (34) to reject the earlier evidence (1, 10) that tetramers, joined at the amino ends, could be assembly intermediates. However, the preferred method of extraction of EHS tumor-derived material includes DTT (34). This will change the complex of disulfide linkages at the amino ends of procollagen IV molecules, and, in the urea-containing solvent, the amino ends may acquire conformations that do not readily reassociate to tetrameric junctions. There are also likely to be differences in structure and assembly among different basement membranes. The assembly of all networks could proceed by several parallel paths in which both amino-linked tetramers and carboxyl-linked dimers participate to varying degrees.

From the current studies we conclude that in lung and gizzard basement membranes the assembly steps are analogous to those which we found in vitro. Initial, strong noncovalent interactions at both amino and carboxyl ends of procollagen IV molecules are first reinforced by disulfide links between the tetrameric amino ends. Covalent linkages between the carboxyl ends are formed more slowly, as well as additional, unidentified cross-links.

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