Basement Membrane Structure In Situ: Evidence for Lateral Associations in the Type IV Collagen Network

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Abstract. To determine molecular architecture of the type IV collagen network in situ, the human amniotic basement membrane has been studied en face in stereo relief by high resolution unidirectional metal shadow casting aided by antibody decoration and morphometry. The appearance of the intact basement membrane is that of a thin sheet in which there are regions of branching strands. Salt extraction further exposes these strands to reveal an extensive irregular polygonal network that can be specifically decorated with gold-conjugated anti-type IV collagen antibody. At high magnification one sees that the network, which contains integral (9-11-nm net diameter) globular domains, is formed in great part by lateral association of monomolecular filaments to form branching strands of variable but narrow diameters. Branch points are variably spaced apart by an average of 45 nm with 4.4 globular domains per micron of strand length. Monomolecular filaments (1.7-nm net diameter) often appear to twist around each other along the strand axis; we propose that super helix formation is an inherent characteristic of lateral assembly. A previous study (Yurchenco, P. D., and H. Furthmayr. 1984. Biochemistry. 23:1839) presented evidence that purified murine type IV collagen dimers polymerize to form polygonal arrays of laterally as well as end-domain-associated molecules. The architecture of this polymer is similar to the network seen in the amnion, with lateral binding a major contributor to each. Thus, to a first approximation, isolated type IV collagen can reconstitute in vitro the polymeric molecular architecture it assumes in vivo.

The structural scaffolding of basement membranes is formed by a polymerized network of type IV collagen (26, 29, 31) whose molecular architecture in situ has not been previously defined.

Our concepts about this polymeric structure have been derived principally from biochemical studies. The monomeric unit of the network is a 400–426-nm-long (26, 29) threadlike molecule with a globular domain at the COOH terminus (8). Proteolytic extraction studies (19, 26) on the Engelbreth-Holm-Swarm (EHS)† tumor matrix led to the identification of a binding site at each end of the monomer, each mediating the formation of a covalently stabilized complex: the amino termini of four monomers join together by a 30-nm end-overlap (1, 6, 17, 26) to form a four-armed tetramer (7S) and the COOH termini of two monomers join end-to-end to form a linear dimer (26). Timpl et al. (26) postulated that NH₂- and COOH-terminal binding accounts for the formation of an open polymeric network of type IV collagen. Yurchenco and Furthmayr (29), on the other hand, proposed that purified type IV collagen dimers self-assemble into a tighter polygonal network held together prominently by lateral (side-by-side) interactions as well as the end-domain interactions identified earlier. The in vitro self-assembled network had the appearance in rotary-shadowed replicas of an irregular polygonal array whose sides were from one to several triple helical strand thick and whose vertices were frequently occupied by the COOH-terminal globular domains. More recently evidence has been advanced (27) that the globular domain will itself bind type IV collagen along its chain and that this domain is important for lateral assembly.

Verification of polymer structure proposed from in vitro work has not been previously provided by structural analysis of basement membranes in tissue. In the electron microscope, basement membranes are seen to consist of a lamina densa bounded by one or two lamina rarae (7) with type IV collagen found in all layers (20). Some basement membranes have been described as having a fine meshwork of fibrils ~4 nm in diameter (7). Reichert’s membrane has been reported to consist of parallel layers, each composed of 3–8-nm-thick cords, which, on plasmin digestion, become a meshwork of 1.5–2-nm filaments (12). Descemet’s membrane, on the other hand, contains a regular collagenous hexagonal lattice within which can be found a finer irregular collagenous mesh (25). These and other studies, while providing important information on basement membrane morphology, have not revealed the molecular detail needed to validate competing structural models developed from in vitro data.

To study the molecular architecture of a type IV collagen...
network in situ, we have examined the basement membrane of the human amnion en face using a stereoscopic high resolution freeze-dry, unidirectional platinum replication technique for transmission electron microscopy (21-24), which has been used to visualize the molecular architecture of other biopolymers (21, 24). The amnion possesses a simple, continuous basement membrane that lies between a superficial cuboidal epithelial layer and a deeper collagenous stroma (13, 15, 16). This basement membrane can be exposed as a flat surface for replication analysis (13) and soluble components can be extracted, further exposing the collagen network. In this study we present evidence that this network, visualized in situ, is formed in significant part by lateral associations, and that supramolecular filament twisting is a characteristic of these associations.

Materials and Methods

Preparation of Amniotic Basement Membrane Surfaces

Normal term human placentas, obtained in a fresh state within 2 h after delivery, were rinsed in cold 10 mM sodium phosphate, pH 7.4, 127 mM NaCl (PBS). The amnion was blunted dissected from the chorionic plate. Sections of the amnion were mounted and oriented on plastic rings and stripped of chorionic plate and mesenchymal elements. Based upon the method of Liotta et al. (13), briefly, the tissues were washed through several changes of cold 5 mM Tris-HCl, pH 7.4, with 0.5 mM diisopropylfluorophosphate, 1 mM EDTA, 1 mM N-ethylmaleimide (NEM) over a period of several hours and then incubated at 37°C in 4% sodium deoxycholate with 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM EDTA, and 1 mM NEM for 90 min. The samples were then washed with PBS, intact, salt-extracted, and antibody-decorated amnions were fixed over a period of several hours followed by incubation at 10°C for 68-72 h in a rotor (Ty65; Beckman Instruments, Inc., Palo Alto, CA). The central peak was washed with solid CsCl to a density of 1.4 g/ml and centrifuged at 50°C for 6-8 h in a rotor (Ty65; Beckman Instruments, Inc., Palo Alto, CA). The central peak was mixed with solid CsCl to a density of 1.4 g/ml and centrifuged at 50°C for 6-8 h in a rotor (Ty65; Beckman Instruments, Inc., Palo Alto, CA). The central peak was mixed with solid CsCl to a density of 1.4 g/ml and centrifuged at 50°C for 6-8 h in a rotor (Ty65; Beckman Instruments, Inc., Palo Alto, CA). The central peak was then adjusted to 0.05% polyethylene glycol (MW 15-20,000), 0.27 M NaCl, pH 8.3, and 0.1% BSA, centrifuged for 20 min at 8,000 rpm in an SS34 rotor (Sorvall Instruments, Wilmington, DE), and the remaining suspended gold pelleted at 34,000 rpm in a Ty65 rotor for 45 min. The pellet was resuspended in several milliliters of PBS containing 0.1% BSA, 0.05% sodium azide and layered onto a 10-35% linear glycerol gradient in the above buffer. The samples were centrifuged in a rotor (SW40Ti; Beckman Instruments) in the cold at 30,000 rpm for 45 min. The dispersed antibody-bound gold was recovered from the middle third of the centrifuge tube, diluted in the above buffer, and adjusted to an A400 of 0.35.

Immunolocalization of Antibody by Light Microscopy

5-μm-thick frozen sections of human amnion and kidney (mouse and human) were prepared on albumin-coated glass slides. Sections to be treated with antibody-bound gold were incubated in 4% formaldehyde in PBS on ice for 1 h, washed in PBS, and then transferred to 503 or 50% ethanol on ice.

Preparation and Evaluation of Type IV Collagen Antibody

Type IV collagen was extracted and purified from lathyritic murine EHS tumor as described (29). This protein, after dialysis into PBS at a concentration of 0.5-1 mg/ml, was mixed with complete Freund’s adjuvant and used to immunize New Zealand white rabbits. Antiserum was collected and type IV collagen-specific antibody was purified by affinity chromatography using the collagen coupled in 0.2 M sodium bicarbonate to cyanogen bromide (CNBr)-activated Sepharose CL-4B (Pharmacia Fine Chemicals, Piscataway, NJ). The antibodies were affinity-purified type IV antibody and preimmune IgG were coupled to 5 nm gold particles based upon a method recommended by Jansen Pharmaceuticals (Piscataway, NJ) and modified by Dr. David Birk (personal communication). Several milligram of antibody were dialyzed into 5 mM sodium phosphate buffer, pH 8.6, and then mixed with saturating amounts of 5-μm colloidal gold suspension (Jansen Pharmaceuticals). The mixture was then adjusted to 0.05% polyethylene glycol (MW 15-20,000), 0.27 M NaCl, pH 8.3, and 0.1% BSA, centrifuged for 20 min at 8,000 rpm in an SS34 rotor (Sorvall Instruments, Wilmington, DE), and the remaining suspended gold pelleted at 34,000 rpm in a Ty65 rotor for 45 min. The pellet was resuspended in several milliliters of PBS containing 0.1% BSA, 0.05% sodium azide and layered onto a 10-35% linear glycerol gradient in the above buffer. The samples were centrifuged in a rotor (SW40Ti; Beckman Instruments) in the cold at 30,000 rpm for 45 min. The dispersed antibody-bound gold was recovered from the middle third of the centrifuge tube, diluted in the above buffer, and adjusted to an A400 of 0.35.

Decoration of Amniotic Basement Membrane En Face with Gold-coupled Antibody for Platinum Replication

PBS-washed salt-extracted amniotic samples were prefixed in freshly prepared 4% formaldehyde in PBS on ice for 1 h, washed in PBS, and then treated with 0.5 mg/ml sodium borohydride in PBS in the cold for 1 h. After washing with PBS, treated with freshly prepared 4% formaldehyde in PBS for one to several hours followed by incubation in 0.5 mg/ml of sodium borohydride in PBS. After washing in PBS, the sections were overlayed with 0.2 ml of antibody (5 μg/ml) or gold-coupled antibody (0.4 dilution) in PBS containing 0.1% BSA and 0.05% Tween 20 (Jansen Pharmaceuticals) for 1 h and then washed. Free antibody-treated specimens were overlaid with a 1:30 dilution of fluorescein conjugated sheep anti-rabbit IgG (Cooper Biomedical, Malvern PA), incubated at room temperature for 1 h, washed, mounted with a coverslip, and examined in a Zeiss microscope fitted for epifluorescence. Slides treated with gold-coupled antibodies were enhanced with a silver stain using a commercial kit (Intense; Jansen Pharmaceuticals), counterstained with hematoxilin, dehydrated, and mounted.

Purification of Macromolecules for Competition Binding Assays

Laminin and the 150,000-D form of nidogen were purified from lathyritic EHS tumor as described (18). The low density, high molecular mass form of basement membrane heparan sulfate proteoglycan was purified from lathyritic EHS tumor (30). This proteoglycan, similar in size to the low density form described by Hassel et al. (10), has a core protein of ~450,000 D and generally three heparan sulfate chains, each ~80,000 D. Briefly, tumor residue, after preextraction with 3.4 M and 1.7 M NaCl (29), was extracted with 5 M urea, 125 mM Tris-HCl, pH 7.4, with 0.5 mM DFP, 1 mM EDTA, 8 mM NEM. After centrifugation, the supernatant was bound onto DEAESephasil (Sigma Chemical Co.) equilibrated in the same buffer containing 0.1% 3-[3-cholamidopropyl]-dimethylammonio]propane sulfonate and was eluted with a linear 0.8 M NaCl gradient. The large bound peak was mixed with solid CsCl to a density of 1.4 g/ml and centrifuged at 10°C for 68-72 h in a rotor (Ty65; Beckman Instruments, Inc., Palo Alto, CA). The central peak was pooled, dialyzed against 4 M GuHCl in Tris buffer, chromatographed on a Sephacryl S400 column, and stored in liquid nitrogen. Human types I, III, and V collagen were isolated from placenta and purified by salt fractionation in acetic acid and ion exchange chromatography (48). Type V collagen was compared by SDS-PAGE with a chick standard kindly provided by Dr. David Birk (Dept. of Pathology, UMDNJ-Robert Wood Johnson Medical School, Piscataway, NJ).

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washed in PBS, transferred to 30% aqueous ethanol, and prepared for platinum replication as described below.

**Freeze-drying and Pt/C Replication**

The amniotic specimens, oriented and placed on one-half-inch filter paper disks, were rapidly plunged into a cup of liquid propane cooled in a Dewar flask of liquid nitrogen. Soluble type IV collagen in 0.1 M acetic acid was diluted to ~15 μg/ml, sprayed onto freshly cleaved mica disks, and then frozen as described above. Each disk was mounted on the precooled (~150°C) stage (23) of a modified freeze-etch unit (model 300; Balzers, Hudson, NH) fitted with electron beam guns, quartz crystal monitor, and cryopump. The chamber was evacuated and the tissue samples were freeze-dried at ~−80°C for several hours. Soluble collagen samples on mica, on the other hand, were generally only freeze-dried for 30–45 min. Each sample was then replicated without rotation with 1.0 nm of Pt/C at a 45° angle with a stage temperature of −178°C in a 5 × 10⁻⁴-torr vacuum. The stage was then rotated during the application of 12–15 nm of carbon support backing. Tissue-derived replicas were treated with 80% sulfuric acid to remove adherent tissue, soluble collagen-derived replicas were separated from mica with hydrofluoric acid, transferred to water, and replica pieces were picked up onto 300-mesh copper grids.

**Electron Microscopy**

Replicas were examined in either a Phillips 420 at 80 kV with a 30-μm objective aperture or JEM 100CX at 80 kV with a 40-μm objective aperture (0.66 nm point to point resolution) at magnifications calibrated with a diffraction grating. Freeze-dried replicas were photographed, using a goniometer, in a tilt series at 10° angle intervals. Plates from rotary shadow replicas and freeze-dried replicas were contrast-reversed (22) by contact printing onto Kodak 7512 fine grain positive film before final printing. In contrast-reversed images the platinum metal appears light and regions without metal appear dark.

**Results**

**Basement Membrane Surface**

The amniotic basement membrane was exposed after detergent-aided removal of the overlying cell layer. As judged by light microscopy of stained frozen amnion sections over 95% of the cell layer was removed. The basement membrane was judged to be intact (with occasional small disruptions) by light immunofluorescence microscopy using rabbit anti-type IV collagen antibody. The Pt/C replicated surface image is that of a relatively flat and dense textured matrix (Fig. 1 A). Scattered on the surface are occasional holes penetrating to the underlying stroma and many small regions in which one can identify portions of a fine branching strands.

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**Figure 1.** Pt/C replicas of amniotic basement membrane and polymerized type IV collagen. (A) En face view of amniotic membrane surface after removal of epithelial cell layer. Arrow indicates exposed region of fine branching filaments. On left side is a hole revealing underlying stroma. Example of interstitial fiber identified with arrowhead. (B) Amniotic basement membrane after salt extraction is seen as a sheet of delicate branching strands forming a network. (C) Purified EHS type IV collagen was incubated in neutral phosphate buffer, diluted into ammonium acetate buffer, sprayed onto mica, and rotary shadowed. Small thin arrows mark examples of fusion branch points and larger arrows indicate examples of globular domains. (D) Stereo, higher magnification view of a region of salt-extracted amniotic basement membrane network. Small thin arrows indicate examples of fusion branch points, larger arrows mark examples of globular domains, and longer thin arrow identifies two strands crossing over. The pale film around each Pt/C-coated filament is the carbon support sheath. Bars: (A–C) 250 nm; (D) 50 nm.
Figure 2. Type IV collagen antibody characterization and network decoration. (A) Competition ELISA assay in which wells were each coated with 1 μg of type IV collagen. Soluble proteins were evaluated for inhibition in twofold serial dilutions, starting at 11 μg/ml. (Solid circles) EHS type IV collagen; (asterisks) EHS low density heparan sulfate proteoglycan; (open triangles) EHS nidogen, 150 kD; (open squares) EHS laminin; (solid triangles) placental type V collagen; (open circles) placental types I and III collagen; (crosses) BSA. (B and C) Immunofluorescence pattern for frozen sections of human amnion incubated with either anti-type IV collagen (B) or preimmune rabbit IgG (C) followed by fluorescein-conjugated sheep anti-rabbit IgG. (D and E) Silver-enhanced pattern of 5-nm gold-tagged anti-type IV collagen (D) and preimmune IgG (E) incubated with cell-stripped salt-extracted human amnion frozen sections. (F and G) Electron micrograph of Pt/C replica of salt-extracted amniotic basement membrane network decorated with 5-nm gold-conjugated anti-type IV collagen (F) and 5-nm gold-conjugated preimmune IgG control (G). Gold particles (small thin arrows) conjugated to specific antibody are seen in association with the network of fine filaments. Small arrowheads mark examples of less frequent larger globular domains. Bar, 100 nm.

(Fig. 1 A, arrow). In the larger holes one can identify interstitial collagen fibers, ~15–40 nm in diameter (Fig. 1 A, left). Treatment of the amnion with a series of dissociative salt solutions, which in the EHS tumor are routinely used to extract noncollagenous components, appears to remove much of the dense matrix, leaving behind an extensive branching polygonal network of strands (Fig. 1 B). This extensive sheet is not seen on the stromal side (data not shown). We are reasonably confident that salt treatment and fixation have not induced substantial rearrangements of the basic network structure. First, although only small regions of network can be seen without extraction, these regions appeared to be similar to the network seen after extraction. Second, similar regions of branching network can be seen in the absence of fixation. Third, examination of plasmin-treated amnion (10 μg/ml, 37°, 2 h) exposes the same network.

Examination of the network in situ at higher magnification and in three dimensions reveals further structural detail (Fig. 1 D, stereopair). The network is seen as a volume-filling array of strands forming a complex irregular lattice in which the strands frequently ascend or descend from one plane to the next. These strands, forming the “sides” of the irregular polygons, are of variable but narrow metal-coated diameter ranging from 2.5 to ~7 nm; between a pair of polygonal vertices the diameter is found to be relatively constant. We interpret this to reflect variable numbers of triple-helical chains laterally associated to each other to form thicker strands. At the polygonal vertices the strands join to form “branch points,” most often with three radiating arms (small thin arrows). One can also identify branch points with four radiating arms; while some of these may be produced by the contacting intersection of two straight strands (Fig. 1 D, small longer arrow), others, whose arms are of different diameters, are interpreted to be formed by lateral associations. Sometimes more complex branches are also identified. Structures that strictly meet morphological criteria for “naked” NH₂-terminal tetrameric domains (i.e., a thickened 30-nm-long chord with “Y” branches at each end) are rarely seen. The NH₂-terminal domains might be contained in some of the branching structures that have greater complexity (e.g., a five-arm branching structure, Fig. 4 A) or the four arms as pairs might even run parallel to each other.

In examining the network one can identify a carbon support sheath that surrounds each free-standing strand (e.g., Fig. 1 D): it is darker (less electron dense) than the platinum replica, and, because it was applied in a rotary fashion, often identifies the location of network at deeper levels even when platinum has failed to coat the filaments.

When purified type IV collagen, isolated from lathyritic EHS tumor and polymerized in vitro, is examined in low angle Pt/C rotary-shadowed replicas (Fig. 1 C), it is seen as an irregular (two-dimensional) polygonal network in which
Figure 3. Comparison between globular domains of network with COOH-terminal globular domains of purified type IV collagen dimers in unidirectional Pt/C replicas. (a) Selected globular domains from guanidine-treated amniotic basement membrane networks. (b) COOH-terminal globular domains of purified EHS type IV collagen dimers sprayed in acetic acid onto mica. Arrows identify globular domains.

lateral associations are prominent contributors to the visualized structure, and is similar to the network observed in situ with strand branching (Fig. 1 C, small thin arrows), variable polygonal side thickness, and globular domains (larger arrows) often located at or near the branch points. While the in situ and in vitro networks possess the same basic structural features, the network formed in vitro appears about twofold less tightly meshed. A measurement of 300 in situ polygonal vertex to vertex contour distances yielded an average of 45 ± 33 nm (SD) while a measurement of 174 in vitro distances yielded an average of 109 ± 51 nm.

Antibody Characterization and Decoration of the Basement Membrane Network

Affinity-purified polyclonal rabbit anti-mouse type IV collagen antibody was evaluated for specificity of binding by competition ELISA assay with purified extracellular matrix components (Fig. 2 A) and by light immunomicroscopy (Fig. 2, b–c). Purified mouse laminin, low density heparan sulfate proteoglycan, and nidogen as well as human placental collagen types I, III, and V did not bind antibody (Fig. 2 A). The antibody was seen to bind both human amniotic basement membrane (Fig. 2 B; immunofluorescence) as well as bind the basement membranes of the glomerulus, tubules, and vessels of human and mouse kidney (not shown). Antibody coupled to 5-nm colloidal gold labeled the amniotic basement membrane. This can be seen in sections of cell-stripped, salt-extracted membranes by light microscopy (Fig. 2 D). Both immunofluorescence and gold-conjugated antibody staining revealed the cell-stripped basement membrane to be almost entirely intact.

When gold-conjugated antibody was incubated with amniotic basement membranes and examined as freeze-dried platinum replicas en face in the electron microscope (Fig. 2 F), the gold was seen to decorate the fine network of branching strands (small arrows indicate examples of gold particles). Background was seen to be uniformly low (Fig. 2 G). Accumulations of gold did not decorate the larger fibers but did decorate fine network strands that course over these fibers (not shown). These gold particles range in diameter from ~4–8 nm, consistent with the size distribution analysis provided by Janssen Pharmaceuticals for the colloidal gold (plus 1 nm of deposited Pt/C). The less frequent globular domains, integral to the network (Fig. 2, f and g, small arrowheads; see Fig. 3 for higher resolution images) were noted to be larger (10–12 nm) and could be distinguished from the gold.

Structural Elements of the Type IV collagen Network

COOH-Terminal Globular Domains. Globular structures seen in the network (Fig. 3 a) had essentially the same shape (slightly spheroid) and size (long axis 12 ± 2 nm; short axis 10 ± 1 nm; n = 22 metal-coated dimensions) as the dimeric COOH-terminal globules (Fig. 3 b) of freeze-dried purified type IV collagen (long axis 12 ± 1 nm; short axis 10 ± 1 nm; n = 30 metal-coated dimensions). The net dimensions are considered to be 9–11 nm after subtraction of the metal coat contribution (see below). We interpret the structural similarity as a morphological identification of the COOH-terminal domain of the network in situ. Compared with the globular domains replicated unidirectionally at high angle, the globules seen after low angle rotary shadow (Fig. 1 C) were more contrasted and exaggerated in size (14–15 nm) and therefore appear superficially more prominent.

An initial quantitative characterization of the in situ and
Figure 4. Macromolecular architecture of network branch points and strands. (A) Three-frame stereo view (each frame 10° apart) of a portion of the type IV collagen network. Three narrow filaments (2.6-nm unit filaments, see text) labeled 1-3, join to form a thicker laterally associated triple filament (~6-nm diameter). When viewed in stereo, filament 2 appears to pass under 1 and twist around it. Filament 3 then passes over 1 and 2 and twists around them. Below the second Y branch the filaments continue to twist around each other for a series of turns. In addition, a helically wrapped strand derived from two unit filaments is seen below the globular domain (small horizontal arrow). Small vertical arrow indicates five-arm branch point and small arrowhead marks free filament. (B) Detail of double filament that appears to twist around itself along strand axis. Oblique arrows mark imperfect helical crossover periodicity and horizontal arrow identifies globular domain. (C) Detail of region where three filaments join and appear to helically twist. (D) Unit filament, or thinnest filament (2.6-nm average metal-coated diameter) that is seen to fuse with other filaments to form thicker strands. (E) Detailed view of two unit filaments that join (just above pair of arrows) to form thicker double filament. Filament to left appears to join vertical filament from behind and then crossover ~15 nm below junction.

In vitro networks was performed. Globular domains were not regularly spaced in situ or in vitro. They could be identified along the strands of both networks with similar overall frequency. In a replicated amniotic network 4.4 globular domains were detected per micrometer of summed strand length (59 globules were counted along 13.5 μm of measured strands), whereas in a representative region of low angle-shadowed in vitro network (Fig. 1 C), 3.9 globular domains were identified per micrometer strand length (74 globules along 19.0 μm of strands). In comparison, one
would expect 1.2 globules per micrometer in a network held
together only by end-domain interactions. The globules were
often located at or near the vertices of the network; in the
same amniotic network 83% (n = 59) of globular domains
were noted to be within one-half diameter from a branch
point compared with 81% (n = 74) in the purified type IV
collagen network. In contrast, while only 20% (n = 246)
of amniotic network branch points were occupied by globular
domains, 50% (n = 120) of the purified type IV collagen
polymer branch points were similarly occupied. These mea-
urements suggest that the increased mesh tightness of the in
situ network is due to more frequent strand branching as well
as to higher two- (and presumably three-) dimensional den-
sity of COOH-terminal domains.

Lateral Associations. In many regions (Figs. 1 D, small
short arrows, and 4, A and C), because the Pt/C-coated net-
work is sufficiently continuous and the orientation correct,
one can trace the joining of filaments to form lateral associa-
tions with a resulting increase in overall diameter distal to
the fusion vertex. In Fig. 4 A, shown in stereo (+10°, 0°,
−10°), three filaments join laterally to form a 6-nm strand.
These filaments (marked 1–3) are examples of the narrowest
filaments seen to laterally bind to form thicker strands and
are referred to here as "unit filaments." A detailed view of a
unit filament is shown in Fig. 4 D: it has essentially the same
metal-coated thickness (2.6 ± 0.3 nm; n = 99) as the triple
helical strands of purified dimers (2.8 ± 0.5 nm; n = 51)
replicated in the same manner after spraying of the collagen
onto mica (portions adjacent to globular domains shown in
Fig. 3, bottom). The net unit filament diameter is calculated
(1.7 ± 3 nm) by subtracting the thickness of the metal coat
(1.0 nm minus a previously determined [24] correction factor
of 0.15 nm) from the measured thickness of metal-coated fila-
ments oriented in the same direction at the Pt/C beam, a
value in agreement with the 1.5 nm expected for a triple he-
lix. For these two reasons we conclude that the unit filament
is a monomolecular filament. In Fig. 4 E we can trace two
unit filaments that laterally join to form a thicker double
filament.

Supramolecular filament twisting along the strand axis ap-
peared to be present in many regions of the network when
examined at very high magnification (Fig. 4, A–C). In Fig.
4, A–C, individual unit filaments are seen to twist around
each other upon lateral association to produce double fila-
ment–twisted (Fig. 4 B) and triple filament–twisted (Fig. 4
C) structures. In the regions shown (and elsewhere) there is
an imperfect crossover periodicity of ~5 nm. In the figure
shown the filaments appear to twist in a right-handed man-
ner; however, this must be considered a relative handedness
since the absolute specimen (and thus handedness) orienta-
tion may have been lost during specimen processing. While
many regions had obvious helixlike regions, many other
regions were only suggestive of these patterns; sometimes
laterally associated strands (Fig. 4 E) had a long twist cross-
over pattern. It is our overall impression, however, that
strand twisting is widespread and a characteristic of lateral
assembly.

Model
A three-dimensional wire and bead model of the network
was constructed, photographed, and traced in stereo (Fig. 5).
This model reflects essential binding structural features and
should be regarded as an approximation of the structural
complexity present in the amnion.

Discussion
In this study we have analyzed, in situ, molecular architec-
tural features of the amniotic basement membrane collagen
network using a high resolution metal shadow casting tech-
nique coupled with selective extraction, decoration with
gold-conjugated polyclonal antibody, and morphometry.
This network is a layered polygonal mesh of laterally (side
by side) associated branching monomolecular filaments.
Supramolecular filament twisting can be detected, and glob-
ular structures, which we interpret as the COOH-terminal
dimeric domains, are integral to this network.

Basement Membrane Collagen Network
In Situ and In Vitro
The structure visualized in situ supports essential features of
the lateral and end-domain association model derived from
in vitro data and proposed earlier (29, 31). Both in situ and
in vitro networks have extensive lateral associations of single
molecular filaments and are seen to frequently branch to
form an array with sides from one to several triple helical
filaments thick. In both networks the globular domains, sites
of COOH-terminal linear dimerization, are often noted to be
located at or near the polygonal vertices. While structures
identical to the isolated NH2-terminal tetrameric region are
only infrequently observed, we suspect that in most cases
this domain is present with greater structural complexity
produced by superimposed lateral associations. Using do-
main-specific antibody, it should be possible to better char-
acterize the in situ architectural relationships of this unique
structure.

On the other hand, the appearance of the network in the
amnion is not compatible with a model (26) in which type
IV collagen monomers are connected only at their amino
tetrameric and carboxyl globular domains. With such a net-
work one would expect to find that the branch point to branch
point (here NH2-terminal to NH2-terminal tetrameric do-
main) contour distance would be ~800 nm (26), the only Y
branches seen would be 30-nm-spaced pairs (78) and all fila-
ments would be of the same diameter equal to the unit
filament.

The similarity between the network identified in the am-
nion with that formed in vitro indicates that type IV collagen
dimers, when incubated in neutral phosphate buffer, can
recapitulate by mass action, to a first approximation, the as-
sembled architecture they normally assume in a basement
membrane and that the basic assembly information is en-
coded in the dimers. It can be argued that a structurally cor-
correct or nearly correct basement membrane scaffolding
and that the basic assembly information is en-
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in most (although not all) fields examined, has a tighter mesh than the latter. In this study most amniotic intervertex distances appear to be closer to ~45 nm, while those of a reconstituted polymer are closer to 100 nm. At present we can only speculate on the reason for these spacing differences. An analogy can be made with the interstitial collagens where heterogeneity of subfibril spacing, reflected in banding pattern, has been observed in reconstituted fibers (4, 5), perhaps produced by alterations of self-assembly environmental conditions. Similarly, in type IV collagen, network spacing may be dependent on pH, salts, protein concentration or, in the case in situ, the modulating action of other matrix macromolecules or even the cell surface. In examining the network of the amnion we have on occasion found regions to be considerably more open with increased vertex to vertex spacing. While representing a preliminary finding, this observation raises the possibility that regional mesh size heterogeneity exists in vivo and that there might be specific mechanisms by which a cell can regulate mesh tightness.

**Network Geometry In Situ**

The three-dimensional structure of the network in situ is complex and the irregularity of polygonal dimensions indicates that each dimer has more than a single allowed spatial relationship with a neighboring dimer. The binding rules that determine these relationships are not yet known; we speculate that the branch point locations may be determined by some of the irregularly spaced interruptions of the gly-x-y repeat sequence (28) related to sites of flexibility (11) along the collagen chain. Previously (29, 31) we suggested that type IV collagen might form a crystalline-like lattice using lateral and end-domain interactions. The study reported here does not substantiate this since the interglobular and intervertex distances are not uniform.

More regular order exists at the level of the laterally associated filament. First filament thickness is found to lie in a fairly narrow range, reflecting a relatively stringent limitation in the number of unit molecular filaments that can laterally associate. Second, we have evidence for double and higher order unit filament twisting. We are curious as to whether there might be a relationship between a 10-nm repeat along the filament axis reported in a recent x-ray diffraction study of stretched lens capsule (2) and the helixlike strands detected in the amnion (realizing the actual strand twist periodicity might have been altered by stretching). Of the other collagens, type VI has also been found to possess a superhelicity in its double-stranded rodlike regions (9).

**Network Assembly**

The proposal that supramolecular filament twisting along the strand axis is an inherent property of lateral assembly has important consequences for overall network structure and assembly. First, lateral associations in which supramolecular helices form, even if produced by relatively weak noncova-
lent interactions, would lead to a stable structure once the amino and carboxyl end-domains were "locked" into the network to form what can be regarded as intertwined "closed loops"; the laterally associated monomolecular filaments could not be undone unless the covalently bound chains or the ends of the collagen were broken apart. Second, where this twisting is present, lateral assembly would have occurred with one or more free collagen ends that must rotate around each other to form the supramolecular helices before closure of the loops. This bears on the question of bonding sequence in network formation (3, 6, 17, 29). Examination of the self-assembly of collagen dimers derived from EHS tumor (29) led to the proposal that COOH-terminal dimer formation occurs first, lateral association second, and NH2-terminal tetramer formation last, while studies on tissue culture-derived collagen monomers (6, 17) and collagen extracts from developing tissue (3) led to the proposal that tetramer formation is an initial event in network formation. These apparent discrepancies might be resolved by the consideration (3) that assembly of the network could proceed by several parallel pathways in which steps of assembly (before covalent cross-linking) are path independent. In those regions where NH2- and COOH-terminal bonds have first formed closed loops we would expect to find an absence of supramolecular filament twisting.

**Universality of Model**

Electron microscopic studies of a variety of basement membrane have revealed architectural heterogeneity with respect to thickness, number of layers, and the presence, in some, of geometric structure. Furthermore, biochemical studies have revealed differences in the relative amounts of basement membrane components (reviewed in reference 31). While we do not know if all basement membrane collagen networks display the same basic architectural features seen in this study, we predict that the network of the amnion will prove to be typical of many basement membranes. First, the basement membrane studied has a cross-sectional morphology that can be regarded as representative of many simple basement membrane elements. Second, the same network is seen in the mouse EHS tumor matrix (Yurchenco, P. D., and G. C. Ruben, manuscript in preparation). Third, the overall pattern of the plasmin-exposed network of Reichert's membrane (12) as well as the fine irregular (as opposed to the larger hexagonal) network seen in Descemet's membrane (25) are suggestive of simple basement membranes. Fourth, there is x-ray diffraction evidence of lateral association of triple helical filaments to form strands in the basement membrane network of lens capsule (2). Fifth, basement membranes contain the same basic type IV collagen molecular "building block." If there is heterogeneity of the basement membrane molecular architecture, it may instead be found in the other components. Using the replication technique described in this study along with differential extraction and domain-specific antibody decoration it should be possible to study the structural relationships of these other components to address this and other questions.

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