Extracellular Compartments in Tendon Morphogenesis: Collagen Fibril, Bundle, and Macroaggregate Formation

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Abstract. The formation of collagen fibrils, fibril bundles, and tissue-specific collagen macroaggregates by chick embryo tendon fibroblasts was studied using conventional and high voltage electron microscopy. During chick tendon morphogenesis, there are at least three extracellular compartments responsible for three levels of matrix organization: collagen fibrils, bundles, and collagen macroaggregates. Our observations indicate that the initial extracellular events in collagen fibrillogenesis occur within narrow cytoplasmic recesses, presumably under close cellular regulation. Collagen fibrils are formed within these deep, narrow recesses, which are continuous with the extracellular space. Where these narrow recesses fuse with the cell surface, it becomes highly convoluted with folds and processes that envelope forming fibril bundles. The bundles laterally associate and coalesce, forming aggregates within a third cell-defined extracellular compartment. Our interpretation is that this third compartment forms as cell processes retract and cytoplasm is withdrawn between bundles. These studies define a hierarchical organization within the tendon, extending from fibril assembly to fascicle formation. Correlation of different levels of extracellular compartmentalization with tissue architecture provides insight into the cellular controls involved in collagen fibril and higher order assembly and a better understanding of how collagen fibrils are collected into structural groups, positioned, and woven into functional tissue-specific collagen macroaggregates.

THE organization of collagen fibrils, with respect to diameter, packing density, bundle size, and net uniaxial orientation, is an important determinant of tendon function. Collagen fibrillogenesis, as a general process, has been shown to be a multistep event involving both intracellular and extracellular assembly reactions (29). That fibroblasts synthesize collagen and other matrix components is well-established (17, 22). That fibroblasts exert control over collagen fibril formation and other extracellular assembly events is less well-established.

Cells control the stoichiometry of mixing of different matrix components during intracellular packaging and/or at the site of discharge (29, 30, 33). In this relatively simple manner the cell provides a mechanism in which various biosynthetic products can be combined in a number of different ways to produce a diversity of connective tissue structures.

Fibroblasts also regulate a series of extracellular compartments during the deposition and assembly of the newly synthesized matrix components, particularly at the sites of matrix assembly (1, 30, 31). The chick embryo corneal fibroblast partitions the extracellular space forming at least three compartments (1). This hierarchy of compartments provides a series of controlled spaces within which the extracellular events of collagen fibrillogenesis and development of fibril bundles and lamellae occur sequentially. The formation of the structural elements of the developing matrix in specific cell-defined extracellular compartments also serves to physically position these elements within the developing matrix.

Understanding the factors that regulate the deposition of collagen fibrils into specific spatial patterns represents a major problem in morphogenesis. In recent years, attention has focused on the process of collagen fibril formation per se. However, it is not the collagen fibril, but groups of fibrils organized as bundles, that represent the major morphogenetic unit in most tissues (1, 30). These fibril bundles are interwoven into patterns that define tissues, such as the large bundles of fibrils characteristic of tendon, the interwoven layers in cornea and intervertebral disc, or the layers in bone (30, 32).

The tendon is a uniaxial connective tissue which functions in the transmission of forces from the muscular to the skeletal system. Tendons are composed of highly aligned collagen fibrils, grouped as bundles. The fibril bundles together with the tendon fibroblasts are organized into fascicles, and the fascicles are bound together in a connective tissue sheath to form a tendon. The orderly development and maintenance of this hierarchy is required for mechanical integrity and normal function (6, 16, 23).

Tendons develop from local accumulations of mesenchyme cells arranged as columns. The arrangement of the cells and newly formed collagenous matrix becomes increas-

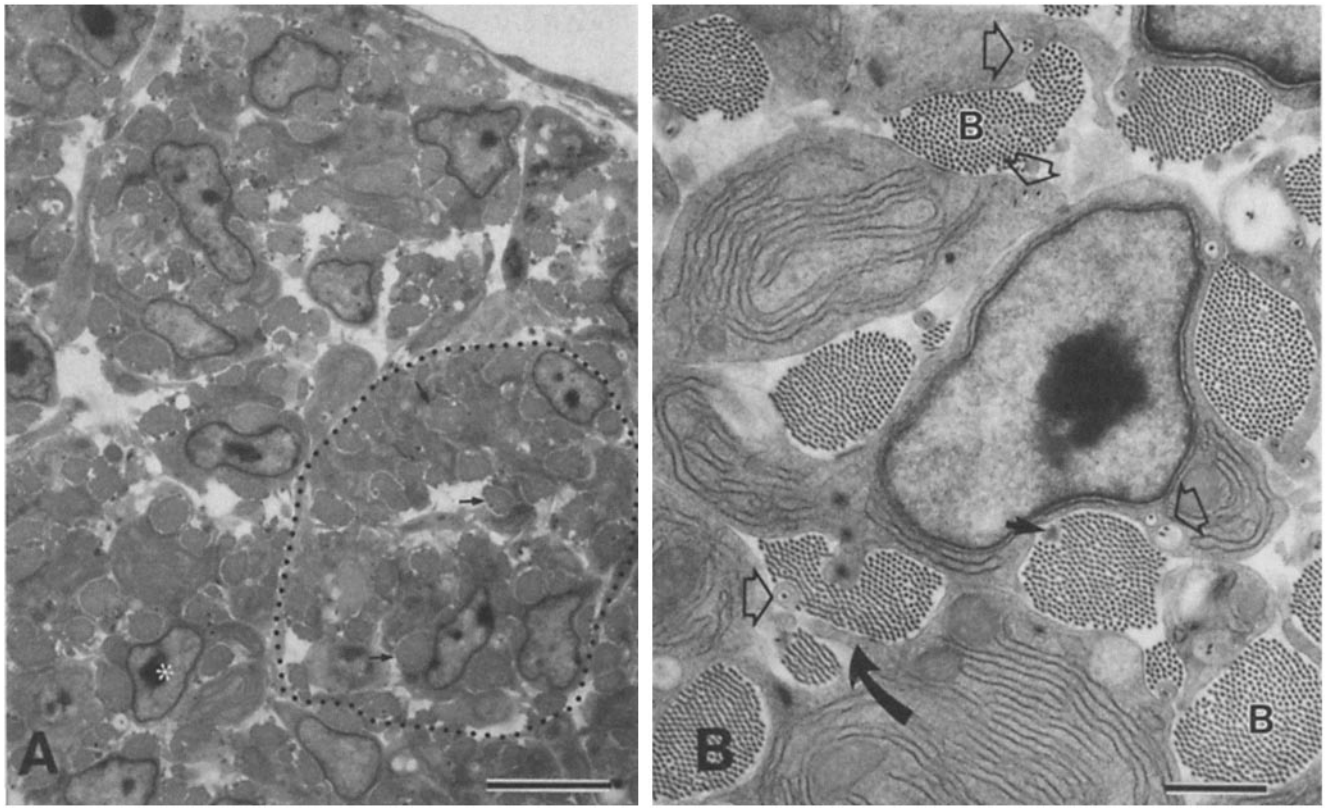


Figure 1. High voltage transmission electron micrographs of 0.5–0.75-μm thick sections from 14 d (stage 40) chick embryo tendons cut perpendicular to the tendon axes. (*A*) A low magnification micrograph showing the fascicular structure of the tendon. A single fascicle is indicated (dotted line). This fascicle contains at least three fibroblasts and abundant collagen fibrils grouped as discrete bundles (*arrows*). The cell indicated (*) is shown at higher magnification in *B*. Bar, 5 μm. (*B*) In this micrograph the relationship of a single fibroblast to the developing matrix is apparent. Numerous collagen fibrils are present within membrane delimited recesses (*open arrowheads*). These recesses most commonly contain single collagen fibrils, but recesses containing two to five fibrils also are observed. Also present in this micrograph are numerous fibril bundles (*B*) intimately associated with the fibroblast surface. Cytoplasmic processes are often seen separating fibril bundles (*curved arrow*). Small bundles of densely staining filaments, presumably elastin-associated microfilaments, are commonly seen at the periphery of fibril bundles (*arrows*). Bar, 1 μm.

ingly fascicular as development proceeds, with fascicles being easily recognizable by day 14 of development. As development continues collagen fibril bundles are continuously added to the extracellular space where they coalesce to form the large aggregates typical of the mature tendon, and the fibroblasts become increasingly attenuated. With continued growth of the tendon, large collagen fibril bundles fill the extracellular space, and only thin cellular processes are seen interdigitating between the fibril bundles (12, 19).

In this study we examine the relationship between the fibroblast cell surface and the developing collagenous matrix using conventional and high voltage electron microscopy, and evaluate the role of the fibroblast in the control of fibril assembly and aggregation of fibrils into ordered structures during tendon morphogenesis.

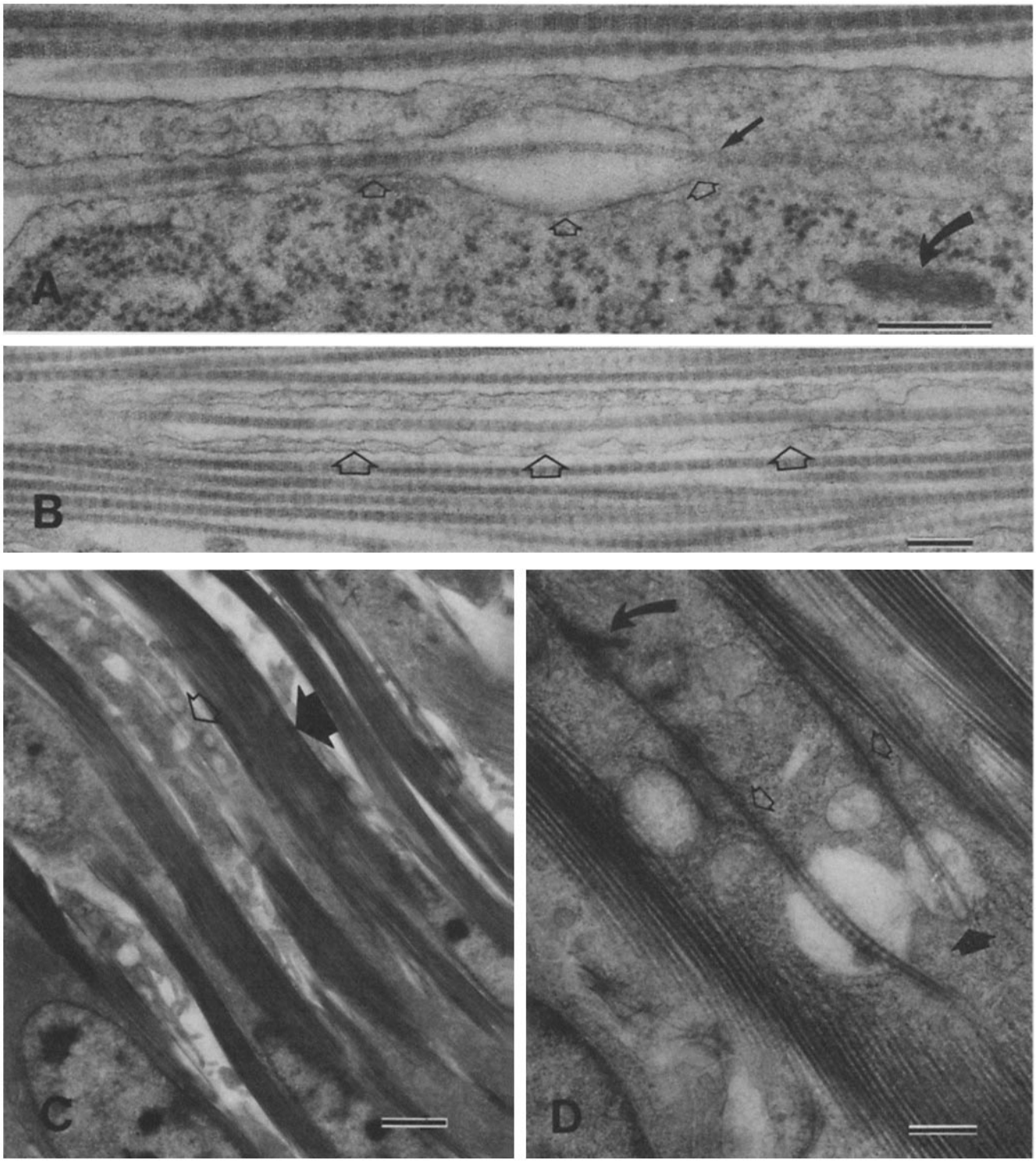
Materials and Methods

White leghorn chick embryos were incubated at 37.5°C in a humidified atmosphere and staged according to Hamburger and Hamilton (13).

Limbs at different stages in development were dissected and fixed in 4% paraformaldehyde, 2.5% glutaraldehyde, and 0.1 M sodium cacodylate, pH 7.4 containing 8 mM $CaCl_2$ for 5 min at room temperature followed by 30–60 min at 4°C. The metatarsal tendons were carefully dissected, washed in 0.1 M cacodylate buffer, and postfixed in 1.33% osmium tetroxide in collidine buffer, pH 7.4 for 1 h at 4°C. After fixation, the tendons were washed and dehydrated in a cold graded ethanol series followed by propylene oxide. The tissue was infiltrated with increasing amounts of final embedding mixture (Polybed 812, nadic methyl anhydride, and dodecenylsuccinic anhydride, Polysciences, Inc., Warrington, PA) in propylene oxide with constant mixing. The infiltration was as follows: 1:1 for 4 h, 1:5 for 12 h, final embedding mixture for 8 h. Finally, the tissues were embedded in fresh embedding mixture, polymerized at 68°C, and sectioned either parallel or perpendicular to the tendon axis (1).

Thick sections (0.5–0.75 μm) were cut for high voltage transmission electron microscopy and picked up onto grids with a carbon-stabilized formvar film. Serial 0.5-μm thick sections were cut and picked up onto 1 × 3-mm slot grids with a carbon-stabilized formvar film. The sections were stained with 2% aqueous uranyl acetate for 1 h at room temperature followed by 30–45 min with 0.2% lead citrate in 0.1 N NaOH. Thick sections were examined and photographed at an accelerating voltage of 1 million volts using the AEI EM 7 high voltage electron microscope at the New York State Department of Health Laboratories in Albany, New York. Thin sections and 0.25-μm thick sections were examined using a Philips 420T electron microscope.

Figure 2. Electron micrographs of sections cut parallel to the axes of 14 d (stage 40) chick embryo tendons. (*A*) In this conventional transmission electron micrograph, a portion of a tendon fibroblast with a narrow cytoplasmic recess containing a collagen fibril (*solid arrow*) is apparent. This recess is membrane delimited (*open arrowhead*) and contains a dilated portion. Secretory vacuoles (*curved arrow*) are often



observed associated with these regions. Bar, 300 nm. (*B*) In this conventional transmission electron micrograph, a fibroblast process or outfolding of the cell surface is cut in longitudinal section. A narrow cytoplasmic recess containing a single collagen fibril (*arrows*) courses almost the entire length of this micrograph. The membrane delimiting the recess is dimpled and gives the impression of arising as a result of the fusion of numerous secretory vacuoles. Bar, 300 nm. (*C*) In this high voltage electron micrograph, portions of at least four adjacent fibroblasts are seen. The cells are intimately associated with the collagenous matrix. Numerous narrow surface recesses containing single collagen fibrils are apparent. At the open arrowhead one such recess is observed in association with numerous secretory vacuoles. Larger bundles of fibrils also are seen in close association with the fibroblast surface (*closed arrowhead*). Bar, 2 μm. (*D*) This high voltage electron micrograph illustrates several narrow, deep cytoplasmic recesses (*open arrowheads*). Numerous vacuoles are seen associated and sometimes are continuous with these recesses. The solid arrowhead indicates a less-ordered region containing a hairpin loop in the collagen fibril. These less-ordered regions are very commonly observed and may be sites of fibril growth. The curved arrow indicates a narrow, dense, secretory vacuole containing electron dense material. This vacuole length is sufficient to contain two parallel associated O-D staggered aggregates. Bar, 500 nm.

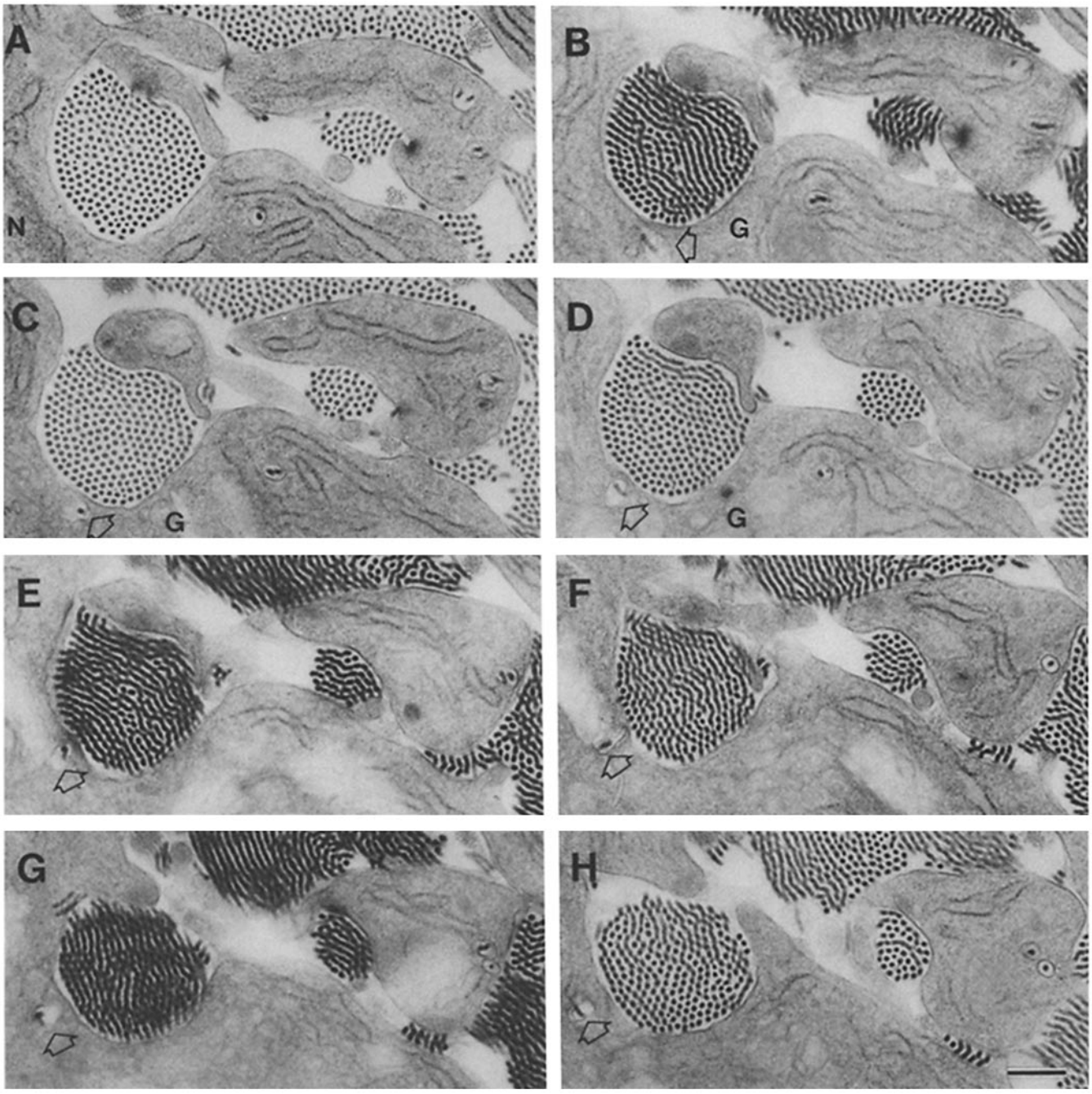
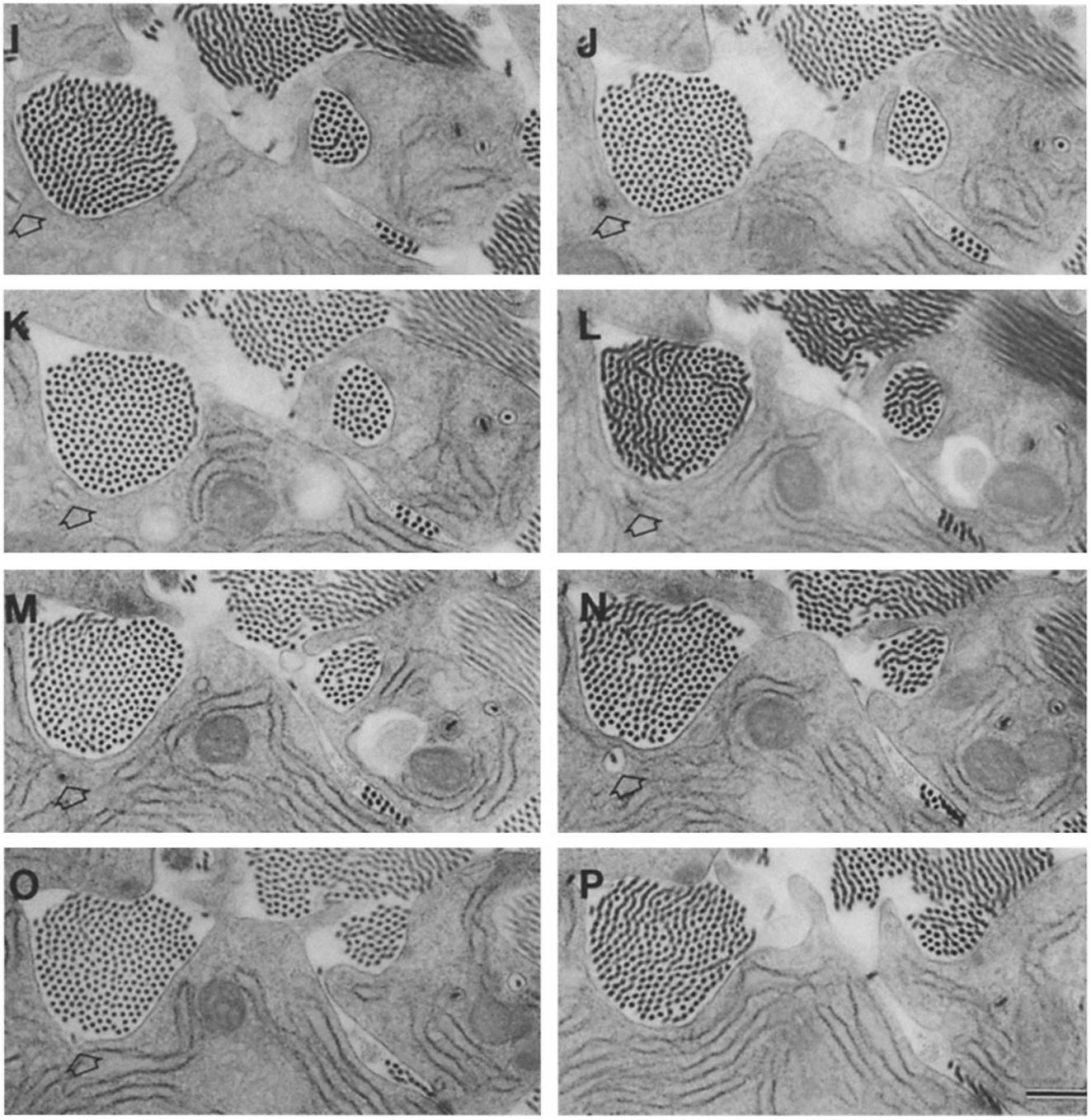


Figure 3. Serial high voltage electron micrographs of thick sections cut perpendicular to the axis of 14-d chick embryo tendons. In this series of consecutive micrographs, a collagen fibril with a narrow recess (*open arrowhead*) is followed from its initial appearance (*B*) in a perinuclear region surrounded by Golgi elements to its opening to the extracellular space (*O*) where it is seen to join a fibril bundle.

Results

Thick sections (0.5–0.75 μm) cut perpendicular to the axes of 14-d (stage 40) chick embryo tendons demonstrate the complexity of the fibroblast cell surface. Specialization of the fibroblast cell surface is responsible for extracellular partitioning with at least three compartments recognizable. The first compartment consists of a series of narrow, deep recesses containing single or sometimes, small groups (two to five) of collagen fibrils. These fibrils are seen entirely enveloped within the cell membrane and are present deep within the cytoplasm (Figs. 1 and 2). Serial sections reveal that these narrow recesses originate deep within the fibroblast cytoplasm often in a perinuclear position associated with the Golgi region and are open to the extracellular space (Fig. 3). These narrow, deep recesses fuse with each other laterally and add to the second fibroblast delimited compartment where bundle formation occurs (Fig. 3). The second extracellular compartment consists of collagen fibrils grouped as fibril bundles surrounded by the tendon fibroblast and in close association with the cell surface (Figs. 1 and 2). The third compartment consists of laterally associated fibril bundles, tendon macroaggregates, which are seen partially sur-



This small region of the original micrographs was a single example of a common observation. These micrographs have been cropped for presentation. In the original micrographs there was an unrestricted view of the individual fibril. *N*, nucleus; *G*, Golgi complex. Bar, 500 nm.

rounded by cell processes and foldings of a single cell's surface and by the surfaces of two to three adjacent fibroblasts (Figs. 1 and 2).

In sections cut parallel to the tendon axis (Fig. 2), dilations are seen associated at the ends and along the narrow, deep recesses. These dilations are most often electron lucent and sometimes are seen to contain fibril ends. The fibril ends associated with these regions are curled and may execute nearly 180° turns. The dilated regions are present at multiple sites along a deep cytoplasmic recess and in some cases vacuoles, previously identified as collagen secretory vacuoles, are found in close association with the recesses.

A more detailed understanding of the fibril–cell relationship is obtained from studying consecutive serial thick sections (Fig. 3), stereo pair electron micrographs (Figs. 4 and 5), and thick sections cut perpendicular to the tendon axis and studied at a variety of tilt angles (Figs. 6 and 7).

The three-dimensional course of a fibril within a narrow cytoplasmic recess can be readily traced in serial thick sections (Fig. 3, *A–P*) and shown to extend from the perinuclear Golgi region (Fig. 3 *B*) to the cell surface (Fig. 3 *O*). These micrographs are taken from a small portion of 36 serial micrometers of chick metatarsal tendon. The content, of the recess indicated, consists of only a single collagen fibril that

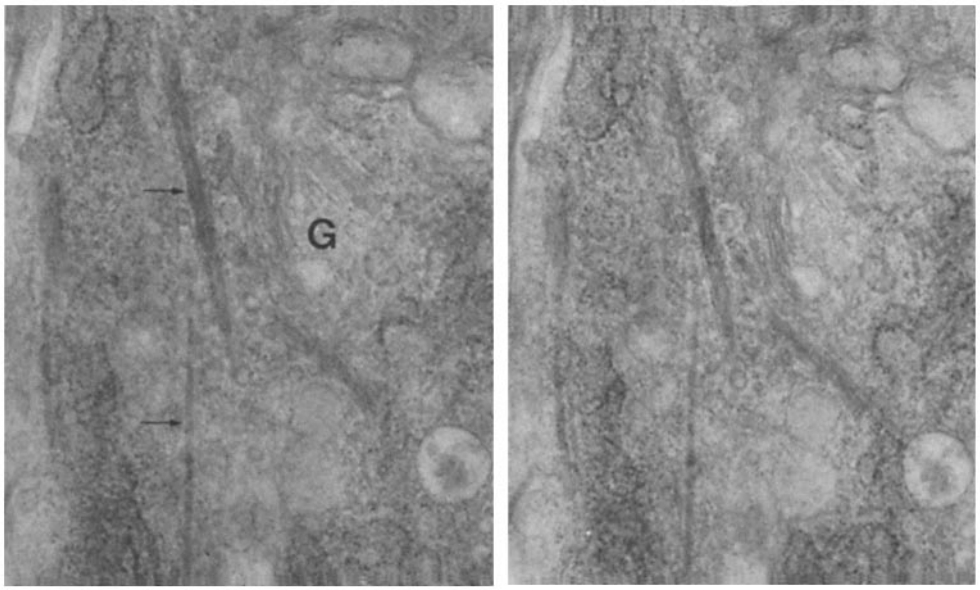


Figure 4. Stereo pair of high voltage electron micrographs from a section cut parallel to the axis of a 14-d chick embryo tendon. Two narrow recesses containing collagen fibrils are seen at the arrows. These narrow recesses are seen deep within the cytoplasm surrounded by Golgi elements and secretory vacuoles. 0.5-μm thick section presented at ±8° of tilt.

undergoes a variety of tilts along the length of the compartment. The end deepest within the cell terminates or begins within a swelling such as those illustrated in Fig. 2. The opposite end of the fibril appears to be present close to or at the site of fusion between the recess and the cell surface.

In stereo pair electron micrographs of sections cut parallel to the tendon axis, the relationship of the fibrils within narrow recesses and bundles to the cell surface are apparent. Fig. 4 illustrates the relationship of the proximal portion of a narrow cytoplasmic recess containing one to two fibrils to the tendon fibroblast. In these micrographs, the recesses are surrounded by the elements of the Golgi apparatus and numerous secretory vacuoles. Fig. 5 is a stereo pair constructed from transmission electron micrographs. A number of narrow recesses containing collagen fibrils are seen within the cytoplasm adjacent to the nucleus and mitochondria. The close relationship of a fibril bundle to the fibroblast also is apparent.

In high voltage electron micrographs of 0.5–0.75-μm thick sections cut perpendicular to the tendon axis and studied at a variety of tilt angles, the narrow recesses are seen to contain cross-sections of single collagen fibrils completely surrounded by cytoplasm. The fibril-containing recesses are both close to the cell surface and deep within the fibroblast cytoplasm. The recesses and contained fibrils course in a direction similar to that seen in the adjacent fibril bundles (Figs. 5 and 6). As a consequence, when the narrow recesses containing one to three fibrils fuse to generate the bundles, the orientation of the fibrils in the bundle is established.

Single collagen fibrils are an integral part of a fibril bun-

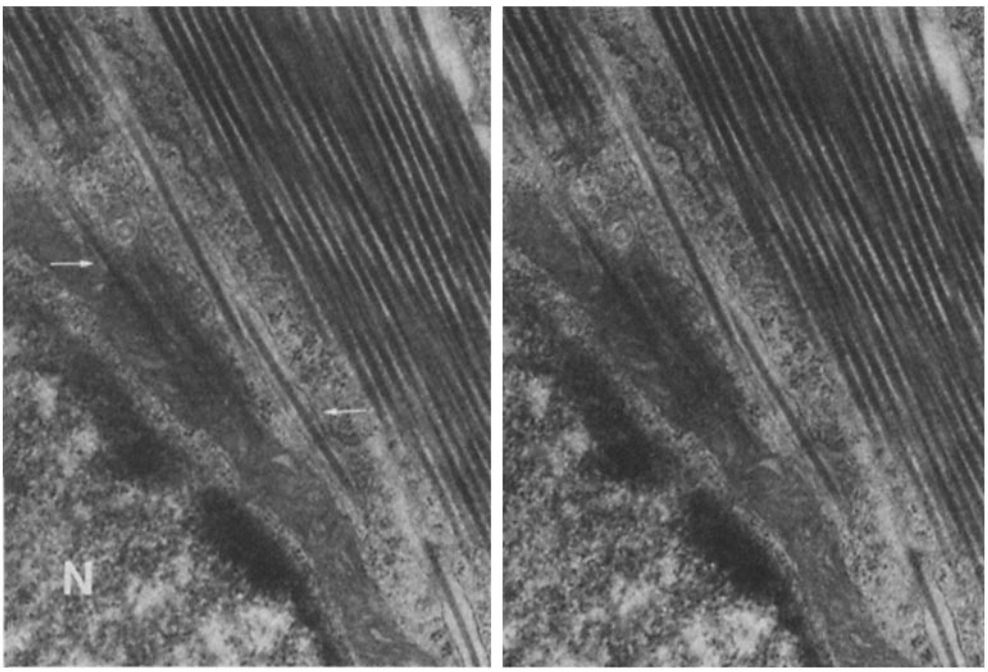


Figure 5. Stereo pair of transmission electron micrographs from a section cut parallel to the axis of a 14-d chick embryo tendon. Several narrow cytoplasmic recesses containing collagen fibrils are indicated by the arrows. 0.25-μm thick section presented at ±8° tilt, examined with an accelerating voltage of 120 kV.

dle, but they are not the primary architectural entity produced by the cells. Single collagen fibrils are only seen within the first narrow, deep extracellular compartment. The major structural and morphogenic unit formed by the tendon fibroblast is the fibril bundle. This major structural unit is found in the second compartment. These fibril bundles then laterally coalesce to form larger aggregates characteristic of the mature tendon.

To estimate the relative number of collagen fibrils within each of these compartments, a series of cross-sections of 14-d (stage 40) chick embryo tendons were studied using high voltage electron microscopy. In these micrographs the different compartments were identified and the number of fibrils within each was determined. Single fibrils in the first compartment constituted 50% (n = 490) of the fibrils. Only an occasional single fibril was seen outside one of the deep, narrow recesses. Groups of two to five fibrils were less numerous (10%) and again were almost always within the narrow recesses. Fibril bundles containing from six to 225 fibrils comprised 26% of the fibril units and were predominantly present in the second extracellular compartment. Larger tendon macroaggregates (226–450 fibrils) comprised 14% of the fibrils and were present within the third compartment or in the general extracellular space. These three levels of organization—collagen fibril, fibril bundle, and tendon macroaggregate—are relatively easily defined by visual inspection of the micrographs and/or by the number of included fibrils. Each level represents a morphological step in the final tendon hierarchy and each has different morphogenetic functions as well as specific structural and biochemical attributes, as discussed later.

Discussion

These studies demonstrate that in developing tendon fibroblasts, extracellular compartments, formed as complex specializations of the cell surface, are important for effecting different levels of matrix organization: collagen fibrils, fibril bundles, and tissue-specific collagen macroaggregates.

The extracellular space should not be perceived as a homogeneous domain, but rather as partitioned for unique functions and conditions. While this partitioning has only recently received attention, its importance in matrix morphogenesis is apparent from the present work as well as earlier studies (1, 31). These extracellular compartments allow the cell's influence to extend into the extracellular space. The cellular control of local factors within these compartments presumably dictates local functions within them.

The functions and specific biochemical attributes of the intracellular compartments in collagen biosynthesis, posttranslational processing, and packaging are well-understood compared to our understanding of the extracellular compartments. We consider these compartments as a structural and functional continuum. In the case of type I collagen, it is our hypothesis that the synthesis, posttranslational processing, packaging, discharge, assembly into fibrils, assembly into bundles, and assembly into collagen macroaggregates are functionally linked and spatially regulated by the cell at each stage.

We do not yet know the specific details of the enzymatic events and assembly steps performed within the three compartments we have identified, but there are a number of interesting possibilities. Tendon cells produce predominantly type I collagen and other minor components including type V collagen (14), type VI collagen (2), and proteoglycans (24, 25, 34). The interactions of the minor components are important in the regulation of fibril structure and/or the interactions of the fibroblasts with the newly formed fibrils. In our model of the tendon cell with intracellular and extracellular compartments, these interactions can be controlled by the cell at the times of synthesis, packaging, discharge, and through the various stages of assembly. The multiple pathways available, in this model, for a limited set of matrix

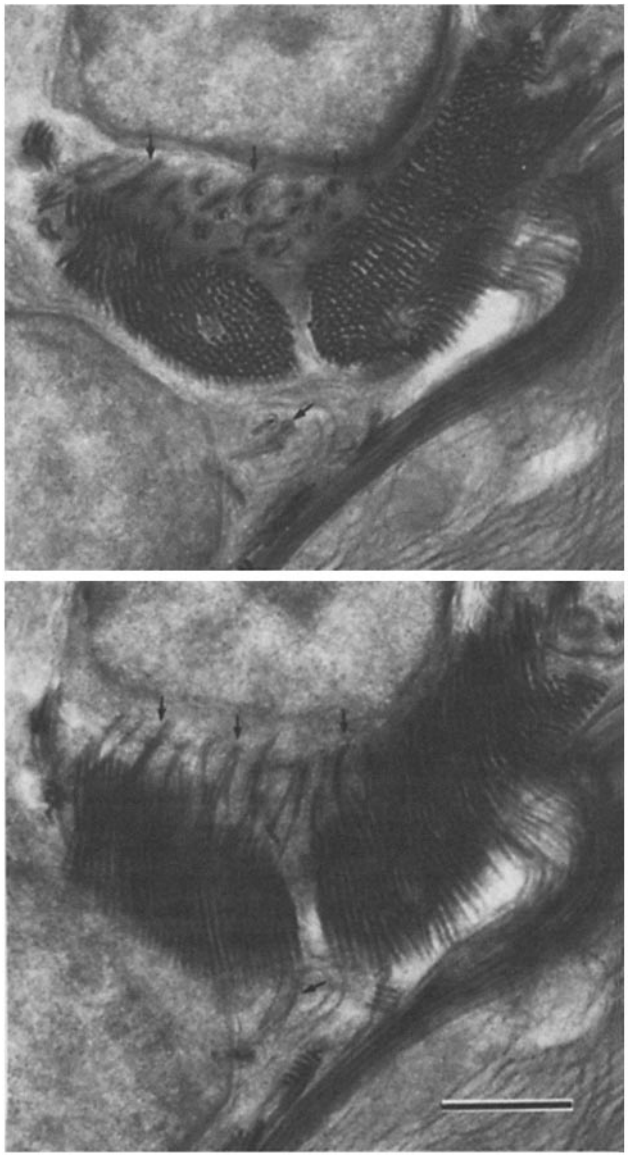


Figure 6. High voltage electron micrographs of a 14-d (stage 40) chick embryo tendon fibroblast cut perpendicular to the tendon axis. This pair of micrographs is from a 0.75-μm thick section photographed at 0° and −20° of tilt. A nucleus is seen at the top and lower left of each micrograph. In the top center, numerous narrow recesses containing single collagen fibrils are apparent (*arrows*). These recesses and their fibrils course in the same direction as the adjacent fibril bundles. The intimate association of fibril bundles with the fibroblast surface and with regions containing numerous narrow recesses also is obvious. Bar, 1 μm.

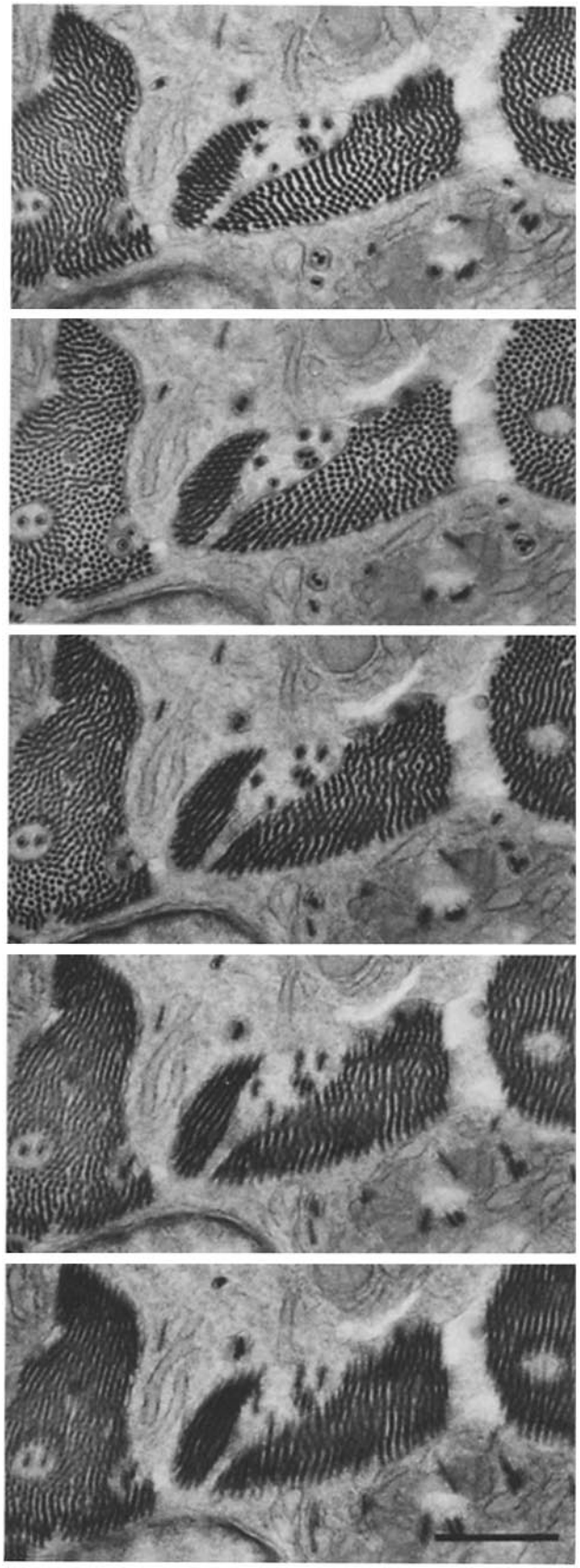


Figure 7. High voltage electron micrographs of a chick embryo tendon fibroblast cut perpendicular to the tendon axis. A 0.75-μm thick section was photographed at −20°, −10°, 0°, +10°, and +20° from horizontal and presented from top to bottom. This series of micrographs clearly demonstrates the intimate association of collagen fibrils and fibril bundles with the fibroblast cell surface. Bar, 1 μm.

gene products provide a means for cells to modulate tissue architecture.

The processing of procollagen is an important event in collagen fibril assembly, and the procollagen propeptides have been implicated in the regulation of collagen fibril formation, as well as the quantity of collagen synthesized (35). In vivo and in vitro the processing of the propeptides has been suggested to be associated with the control of fibril diameter (8, 9, 20, 21). Most data suggest that procollagen processing occurs in the extracellular space. In the present context, we hypothesize that such processing occurs within the narrow fibril-forming recesses, for it is within these recesses that the cells discharge preformed aggregates from the secretory vacuoles. When fibroblasts are placed in culture, the extreme flattening that occurs alters the specialized cellular topography and eliminates the extracellular compartments. Accordingly, in vitro, the procollagen is found in "the medium," where "the medium" represents a sum of the contents of the various extracytoplasmic compartments.

Narrow recesses containing single or small groups of collagen fibrils have been observed by a variety of investigators in a number of different species and tissues (1, 4, 10, 11, 15, 18, 19, 31). We have previously identified the narrow recesses as being the site of earliest fibril labeling by autoradiography and have concluded that the recess is the site of fibril formation (1, 31). In our present studies we have now obtained additional evidence to support this suggestion. The dilations associated with this compartment represent, we believe, sites of discharge of intermediate collagen subassemblies and fibril assembly. The dilations are found at multiple sites along the fibril formation compartment, and since more than one fibril may be present within these compartments, it seems reasonable to expect that the exact site of fibril assembly, i.e., the proximal ends of the fibrils, are present in multiple locations along this compartment. We have not been able to identify an intermediate aggregate form of collagen within these dilations. If SLS or 4-D staggered SLS are intermediate forms in collagen fibril assembly as has been suggested from other studies, it would be helpful to find such aggregates within these regions. However, if fibril assembly involves both the addition of these subunits and some translocation of their relative positions, such aggregates may prove difficult to visualize.

The finding that the ends of newly formed collagen fibrils are present within recesses suggests that an intermediate fibril length is produced which is later coupled, probably by fusion at the ends, with other like segments or with any free end in the bundle-forming compartment. Three-dimensional reconstruction of individual fibrils over a 10–20-μm distance, currently in progress, should resolve the question of whether continuous fibrils or fibril segments are produced.

Covalent cross-linking is a second major postdepositional modification that occurs in collagen (26). This enzymatic process also is thought to occur in the extracellular space. We suggest that during the lateral aggregation of fibrils into bundles, covalent cross-linking driven by oxidative deamination of lysyl residues could occur. This extracellular process

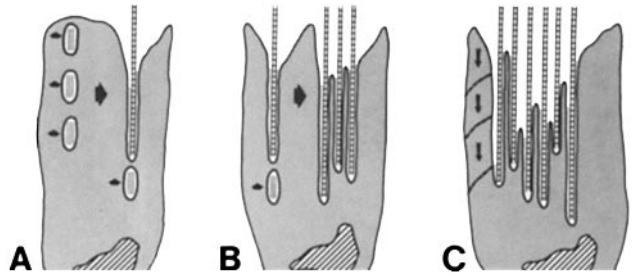


Figure 8. This diagram illustrates a model for the formation of the unique extracellular compartments during tendon morphogenesis. In *A*, secretory vacuoles containing procollagen molecules align tandemly and fuse with the cell surface and adjacent vacuoles. This process of compound exocytosis produces long, narrow recesses, the fibril-forming compartment. In *B*, the lateral fusion of the fibril-forming compartments with the cell surface and one another and the coalescence of their contents produce the bundle-forming compartment. Collagen fibrils are added to the fibril bundles through the continued fusion of fibril-forming compartments with the bundle-forming compartment, possibly as fibril segments. Finally, in *C*, the bundle-forming compartments fuse with adjacent compartments as intervening cytoplasm is retracted and bundles laterally associate. As indicated by the dotted lines and arrows, the processes between adjacent cells are retracted, thus allowing for the coalescence of large bundles within a compartment defined by two to three adjacent fibroblasts. Within this compartment large tissue-specific aggregates form and mature.

also may be carefully regulated by the cells through the specific character of the bundle-forming compartment formed by the fusion of the narrow recesses.

In all of these cell-delimited spaces, the orientation of the fibrils, bundles, and collagen macroaggregates is influenced in a major way by the topography of the compartment. Accordingly, the orientation of the collagen at each morphogenetic step is determined directly by the cell. By this relatively simple mechanism, the three-dimensional orientation of the matrix is initially established.

The relationships among the several extracellular compartments are presented in Fig. 8. The cell labeled *A* contains three elongate vacuoles that contain collagen molecules in a laterally aggregated form which is either unstaggered (an SLS crystallite or 0-D stagger) (3) or in an end-overlapped configuration (4-D stagger) (27). These vacuoles fuse tandemly, similar to compound exocytotic events in other tissues (5, 7), to form the long, narrow fibril-forming recesses, some of which may contain discontinuous fibril segments. In the cell labeled *B*, the long, narrow recesses associate laterally to form a convoluted compartment within which fibril bundles form. Fibril-forming recesses continue to fuse with the bundle-forming compartments, adding fibrils to the periphery of the enlarging bundle. In the cell labeled *C*, the bundle-forming compartments continue to laterally aggregate, the intervening projections of cytoplasm withdraw, and a compartment is generated within which broader bundles are formed. The cytoplasm between adjacent cells also retracts, and tendon macroaggregates are generated from the fusion of large bundles and are defined by two to three adjacent fibroblasts.

These studies define the hierarchical organization within the tendon, extending from fibril assembly to fascicle formation. An understanding of the different levels of extracellular compartmentalization and tissue architecture within the developing tendon provides a basis for continued dissection of the biochemical and cellular controls of collagen fibril and higher order assembly as well as a better understanding of how the newly formed collagen fibrils are collected into structural groups, positioned, woven, and organized into tissue-specific collagen macroaggregates forming a functional tissue.

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