Coalignment of plasma membrane channels and protrusions (fibripositors) specifies the parallelism of tendon

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The functional properties of tendon require an extracellular matrix (ECM) rich in elongated collagen fibrils in parallel register. We sought to understand how embryonic fibroblasts elaborate this exquisite arrangement of fibrils. We show that procollagen processing and collagen fibrillogenesis are initiated in Golgi to plasma membrane carriers (GPCs). These carriers and their cargo of 28-nm-diam fibrils are targeted to previously unidentified plasma membrane (PM) protrusions (here designated “fibripositors”) that are parallel to the tendon axis and project into parallel channels between cells. The base of the fibripositor lumen (buried several microns within the cell) is a nucleation site of collagen fibrillogenesis. The tip of the fibripositor is the site of fibril deposition to the ECM. Fibripositors are absent at postnatal stages when fibrils increase in diameter by accretion of extracellular collagen, thereby maintaining parallelism of the tendon. Thus, we show that the parallelism of tendon is determined by the late secretory pathway and interaction of adjacent PMs to form extracellular channels.

Introduction

The ability of tendon to withstand repeated cycles of tensile force is largely attributable to the presence in the ECM of collagen fibrils that are indeterminate in length and exhibit large diameters. Moreover, the fibrils occur in strict parallel register. It is controversial how the parallelism of tendon arises and how long fibrils are deposited into the ECM. The fibrils, which comprise triple helical collagen molecules that are quarter staggered along the fibril long axis, exhibit a characteristic 67-nm axial periodicity (the ‘D-periodicity’) and are readily observed by EM (Parry et al., 1978). They can be partially solubilized in cold or weak acidic buffers thereby producing a solution of collagen molecules. Upon warming and neutralizing the solutions, the collagen molecules spontaneously self-assemble to form elongated fibrils that exhibit the same D-periodicity as tissue fibrils (Kadler et al., 1996). Reconstituted fibrils form semi-rigid gels in which the fibrils exhibit no preferred orientation. Therefore, although the collagen molecule contains all the necessary sequence information to form D-periodic fibrils it lacks information to direct parallel alignment of fibrils, which suggests a cellular involvement in vivo. However, cells alone are insufficient to specify parallelism of the fibrils; tendon fibroblasts in culture do not assemble a functional tissue, despite synthesizing large amounts of collagen. Here, we tested the hypothesis that the secretory pathway of fibroblasts in situ determines the parallelism of collagen fibrils in tendon.

A primary function of the secretory pathway is to transport macromolecules from their site of synthesis in the ER to the plasma membrane (PM). A critically important node in this pathway is the Golgi complex with its associated TGN consisting of a complex network of anastomosing tubules (Rambourg et al., 1979). The TGN mediates the final modification of N-linked oligosaccharides to the complex form (Roth et al., 1985) and is involved in both the transport and sorting of membrane and secretory proteins (Griffiths and Simons, 1986; Taatjes and Roth, 1986; Orci et al., 1987). Previous works have shown that GFP fusion proteins

Abbreviations used in this paper: 3-D, three-dimensional; ADAMTS, a disintegrin and metalloprotease (reprolysin type) with thrombospondin motifs; BMP, bone morphogenetic protein; dpc, days post coital; GPC, Golgi to plasma membrane carrier; GPC[H11001], GPC containing one or more 67-nm periodic collagen fibrils; pCcollagen, a naturally occurring intermediate in the cleavage of procollagen to collagen that contains the C-propeptides and not the N-propeptides; PM, plasma membrane; pN-collagen, a naturally occurring intermediate in the cleavage of procollagen to collagen that contains the N-propeptides and not the C-propeptides.
are transported from the Golgi to the cell surface in tubular-saccular compartments, which travel along microtubules (Sciaky et al., 1997; Hirschberg et al., 1998; Toomre et al., 1999; Polishchuk et al., 2000; Puertollano et al., 2003). In this paper, the authors distinguish between vesicles and compartments or carriers. The terms “compartment” and “carrier” refer to any membrane-bound transport container within the cell, regardless of its size, position, or function in the secretory pathway. The term “vesicle” refers to any compartment that is spherical or near spherical in shape, regardless of its size, position, or function in the secretory pathway.) These pleomorphic Golgi to PM carriers (GPCs) can be 0.5–1.7 μm in length (Polishchuk et al., 2000) and have also been called transport containers (Toomre et al., 1999) and post-Golgi carriers (Hirschberg et al., 1998). A recent in vitro study has shown that exit from the TGN occurs by the formation of a tubular-reticular TGN domain that is a pre-cursor structure to the release of tubular-saccular GPCs (Polishchuk et al., 2003).

Tendon fibrils are predominately comprised of collagen I, which is the most abundant collagen in vertebrates (Boot-Handford et al., 2003). It is synthesized in the ER as procollagen I, which comprises two proα1(I) chains and one proα2(I) chains folded into an uninterrupted 300-nm-long triple helix flanked by globular N- and C-propeptides. Procollagen molecules are too large to fit into conventional 60–80-nm transport vesicles and traverse the Golgi complex of these cells by cisternal maturation (Bonfanti et al., 1998). We were particularly interested to know if procollagen occurs in GPCs en route to the ECM. Of particular relevance to this question is that proteolytic cleavage of the N- and C-propeptides results in spontaneous collagen fibril formation. N-propeptide removal is catalyzed by the procollagen N-proteinases, which include a disintegrin and metalloprotease (reprolysin type) with thrombospondin motifs (ADAMTS)-2, -3, and -14 (Colige et al., 1997, 2002; Fernandes et al., 2001), whereas C-proteinase activity is possessed by all members of the tolloid family of zinc metalloproteinases including bone morphogenetic protein-1 (BMP-1; Scott et al., 1999). Both BMP-1 and ADAMTS-2 are activated by a furin-like proprotein convertase, and in the case of pro-BMP-1, activation has been shown to occur in the TGN (Leighton and Kadler, 2003; Wang et al., 2003). Furin itself undergoes autocatalytic activation and is thought to cycle between the TGN, the cell surface, and the endosomal system (Molloy et al., 1999; Thomas, 2002).

Seminal studies in the early 1980s by Birk, Trelstad, and coworkers (Trelstad and Hayashi, 1979; Birk and Trelstad, 1984, 1986) suggested that collagen fibrils occur in deep PM recesses and that the recesses increase in diameter at distances from the cell to accommodate fibril bundles. Evidence from EM autoradiography indicated that newly synthesized collagen molecules pass through these recesses en route to the ECM. However, recent findings that pro–BMP-1 is converted to BMP-1 in the TGN, as well as studies of GPCs in cultured cells, prompted us to relate these new observations to the description of fibril formation in embryonic tendon described by Trelstad and colleagues (see references above). Here, we show that GPCs are indeed present in embryonic tendon fibroblasts and that some GPCs contain 28-nm-diam collagen fibrils (GPCs+cf). Moreover, GCPs+cf are targeted to novel PM protrusions, which we have termed “fibripositors” (fibril depositors). In addition, we show that procollagen can be converted to collagen within the confines of the cell membrane, which is consistent with the observation of collagen fibrils in some GPCs and the known intracellular activation of BMP-1. A novel observation was that fibripositors are always oriented along the tendon axis, which establishes a link between intracellular transport and the organization of the ECM. Interestingly, fibripositor formation is not a constitutive process in procollagen-secreting cells but occurs only during a narrow window of embryonic development when tissue architecture is being established.

Results

GPCs containing collagen fibrils occur in chick embryo tendon fibroblasts in vivo

Transverse (i.e., orthogonal to the tendon axis) sections of metatarsal tendons from chick embryos show membrane encapsulated collagen fibrils within the cytoplasm and bundles of fibrils in the ECM (not depicted), whereas longitudinal sections (i.e., parallel to the tendon long axis) showed GPCs containing cross-banded collagen fibrils (GPCs+cf; Fig. 1 A). Three-dimensional (3-D) reconstructions were performed on six separate serial longitudinal sections from chick embryo leg tendons. The reconstructions showed the presence of GPCs+cf, which varied in length up to ~2 μm, and associated small pleiomorphic membrane enclosures (Fig. 1 B). These GPCs+cf often were completely enclosed within the cell and the 67-nm banding pattern provided unequivocal identification of collagen fibrils. However, it was also shown that the GPCs+cf were reactive to a collagen I (triple helical region) antibody (Fig. 1 C).

The N- and C-propeptides of procollagen are cleaved in post-Golgi compartments

Pulse-chase experiments were performed on tendons from 13-d chick embryos. At this stage, the tendon fibroblasts synthesize mainly collagen I (Graham et al., 2000), which simplifies biochemical studies. To examine procollagen processing, pulse-chase experiments were performed on whole tendons using a 10-min pulse and 10–180-min chase. Using a differential extraction procedure (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200312071/DC1) we analyzed the ECM-resident proteins in a neutral high salt buffer and then the intracellular proteins in the same buffer supplemented with NP-40. The extracts were analyzed separately by SDS-PAGE and autoradiography. Trimeric type I procollagen (comprising two proα1(I) and one proα2(I) chain) can be processed by sequential removal of the N- and C-propeptides, and vice versa, producing eight different α-chains. In decreasing size order these are: proα1(I), pCα1(I), pNα1(I), proα2(I), pCα2(I), α1(I), pNα2(I), and α2(I). All eight bands can be seen in the 20-min chase NP-40 extract (Fig. 2 A). The migration of each chain was confirmed by comparison with radiolabeled procollagen digested with recombinant BMP-1, ADAMTS-2, or trypsin (unpublished data). The collagen chains were differentially distributed between the
NaCl (S1) and NP-40 (N) extracts (Fig. 2A). Most of the 14C-label was initially present in the NP-40 extract as procollagen. At later chase times, the NP-40 extract contained (a) procollagen; (b) a naturally occurring intermediate in the cleavage of procollagen to collagen that contains the N-propeptides and not the C-propeptides (pNcollagen); (c) a naturally occurring intermediate in the cleavage of procollagen to collagen that contains the C-propeptides and not the N-propeptides (pCcollagen); and (d) collagen, and the amount of label in the NP-40 extracts progressively decreased over the course of the experiment. In contrast, little 14C-collagen was present in the NaCl extract at the start of the experiment. Procollagen, pCcollagen, and collagen occurred in the NaCl extract in the 20-min chase sample. The amount of procollagen and pCcollagen in the NaCl extract decreased during the chase such that after ~3 h all of the labeled protein was fully processed collagen I in the NaCl extract.

To gain further confidence that procollagen intermediates (e.g., pNcollagen and pCcollagen) occurred within the cell, we performed a pulse-chase experiment to produce a mixture of processed procollagen chains in both the NaCl and NP-40 extracts, and then digested the tendons with trypsin at 20°C for 30 min. At this temperature cellular protein export is prevented but trypsin can still degrade nontriple helical protein molecules. The tendons were then sequentially extracted. Analysis of the first NaCl extract is shown. S1, the first salt extract. N, the NP-40 extract. In a repeat experiment, the 30-min chase samples were incubated with trypsin and the labeled proteins displayed by autoradiography. Control, untreated; trypsin, trypsin treated; S, the first salt extract; N, the NP-40 solubilized proteins. The migration positions of the α-chains of procollagen, pCcollagen, pNcollagen, and collagen are indicated. (C) Tendons were subjected to a mock pulse chase (pulse 10 min, chase 60 min) in which no radiolabeled proline was added. They were then subjected to two salt (S1 and S2) extractions and a final NP-40 (N) extraction to which exogenous purified 14C-labeled procollagen was added. The extracts were prepared for SDS-PAGE and the proteins analyzed by autoradiography. Procollagen remained uncleaved in the S and N samples.

Figure 2.  **Pulse-chase analysis of procollagen conversion by chick tendon fibroblasts in situ.** (A) Chick metatarsal tendons were incubated in medium containing [14C]proline for 10 min and then in unlabeled medium for the times indicated. The tendons were successively extracted four times in buffer containing NaCl and then in a buffer containing NP-40. Autoradiography was used to display the labeled proteins in the chase samples. For brevity, only the first NaCl extract is shown. S1, the first salt extract. N, the NP-40 extract. (B) In a repeat experiment, the 30-min chase samples were incubated with trypsin and the labeled proteins displayed by autoradiography. Control, untreated; trypsin, trypsin treated; S, the first salt extract; N, the NP-40 solubilized proteins. The migration positions of the α-chains of procollagen, pCcollagen, pNcollagen, and collagen are indicated. (C) Tendons were subjected to a mock pulse chase (pulse 10 min, chase 60 min) in which no radiolabeled proline was added. They were then subjected to two salt (S1 and S2) extractions and a final NP-40 (N) extraction to which exogenous purified 14C-labeled procollagen was added. The extracts were prepared for SDS-PAGE and the proteins analyzed by autoradiography. Procollagen remained uncleaved in the S and N samples.
proα1(I) and proα2(I) chains occurred (Fig. 2 C), indicating that the labeled procollagen in the pulse-chase experiment must have been processed before extraction. To further confirm that intracellular collagen molecules could only be removed by extraction with buffer containing NP-40, pulse-chase experiments were performed in the presence of α,α-dipyridyl and brefeldin A. These treatments are both known to result in the accumulation of procollagen within the ER. Two successive NaCl extractions (S1 and S2) were performed before the NP-40 extraction (N), which was found to contain only unprocessed procollagen (unpublished data). These results are consistent with cleavage of procollagen to collagen occurring in post-Golgi compartments and the results of EM showing collagen fibrils in GPCs.

Appearance of PM protrusions coincides with onset of post-Golgi collagen fibril polymerization in 14.5-d mouse tail tendon

Transverse sections through the presumptive tail tendon of 13.5 dpc (days post coital) mouse embryos showed closely packed and apparently undifferentiated cells, which lacked evidence of GPCs or a fibrillar ECM (Fig. 3 A). However, cells in the proximal region of tails from 14.5 dpc embryos were loosely packed and had large extracellular spaces that contained numerous collagen fibrils. Cellular projections (Fig. 3 B, open arrows) were an obvious feature at this stage of development. Some of the projections contained tubular carriers in which collagen fibrils were clearly visible (Fig. 3 B, closed arrows). Analysis of the distal region of the same tails showed spaces between cells and a conspicuous absence of collagen fibrils and cellular projections, indicating that the development of mouse tail tendon proceeds from the proximal to the distal end. Therefore, the occurrence of parallel collagen fibrils in the ECM coincided with the appearance of cellular projections having GPCs.

Figure 3. Onset of fibripositor formation in mouse tail at 14.5 dpc. (A) EM of tail tendon from a 13.5-d embryonic mouse showing predifferentiated fibroblasts with no obvious Golgi apparatus. Note the absence of collagen fibrils in the intercellular spaces. Bar, 500 nm. (B) EM of the proximal region of the tail from a 14.5-d embryonic mouse. Spaces between cells contain collagen fibrils in PM protrusions (open arrows), some of which contain collagen fibrils contained within lumens (closed arrows). Bar, 500 nm.

Well-developed Golgi, parallel GPCs and parallel fibripositors in 15.5-d embryonic mouse tail tendon fibroblasts

By 15.5 dpc the mouse tail tendon contains an elaborate ECM containing parallel bundles of narrow collagen fibrils that are interconnected by filamentous strands (Fig. 4, A and B). Image analysis of collagen fiber bundles showed that the fibrils had a uniform diameter of 28 nm and were hexagonally packed with a mean center to center spacing of 58 nm; n = 380. The packing arrangement, which has not previously been reported, is highly indicative of a cell-mediated mechanism of fibril deposition. The cells contained a well-defined Golgi complex and a highly vacuolar TGN (Fig. 4 A). There was an abundance of membrane encapsulated collagen fibrils within the cytoplasm of the same diameter as the extracellular fibrils (Fig. 4 A, arrow).
Five separate transverse serial section series were reconstructed (Video 1, available at http://www.jcb.org/cgi/content/full/jcb.200312071/DC1). These showed that the membrane-encapsulated collagen fibrils were aligned parallel to the tendon axis and that these carriers could either be enclosed within the cell (GPCs^{+d}) or open to the extracellular space at one end (see Fig. 8 A). Closed GPCs^{+d} could either be located within membrane protrusions, as shown, or close to the Golgi complex (see Fig. 10 H). It was possible to trace the paths of individual fibrils through a transverse series from bundles in the ECM through the lumen of PM protrusions and into the main body of the cell, although most collagen fibrils exceeded the z-dimension of the reconstructions (Fig. 5). For descriptive purposes we have designated the PM protrusions “fibripositors”. Occasionally it was possible to capture a fibrpositor and its associated collagen fibrils in longitudinal section (Fig. 6, A–D). The fibrpositors contained a lumen that extends several microns into the body of the cell. There were several common features of fibrpositors: (a) in 85 fibrpositors examined, 75 had a single fibril exiting the distal tip, five had two fibrils exiting, and five were closed at the distal end (i.e., contained a GPC containing one or more 67-nm periodic collagen fibrils [GPC^{+d}] that was totally enclosed within the PM); (b) there was an S-shaped kink at the site where the fibrpositor protruded from the main body of the cell; (c) the lumen was not uniform in diameter along the length of the fibrpositor but often constricted to the diameter of a single fibril; (d) several fibrils could be located within the central lumen of the fibrpositor,
i.e., within the main body of the cell; (e) the fibrils were often hexagonally packed within the lumen and had the same diameter as fibrils in the ECM; and (f) numerous short and very narrow fibrils occurred at the base of the fibripositor (Fig. 5 B, section 10).

Collagen fibrils are deposited by fibripositors into extracellular channels formed by adjacent fibroblasts

As cultured fibroblasts do not deposit parallel collagen fibrils, nor do they exhibit apparent GPCs/H11001 cfS or fibripositors it was not possible to use correlative microscopy techniques (including the use of GFP-tagged procollagen) to study the formation of fibripositors or the secretion of collagen fibrils. Therefore, to determine whether fibripositors deposit rather than remove fibrils from the ECM the serial sections were reexamined in further detail. In all examples in which a fibril could be seen exiting a fibripositor there was clear space around the fibril (a gallery of examples is shown in Fig. 5 C). In 3-D, the fibril was at the center (on axis) of a cylindrical space, which was identical in size and shape to the tip of the fibripositor. It is difficult to imagine how such a space could be generated by an encroaching fibripositor; therefore, it appears that the spaces are generated by retreating fibripositors. In addition, the fibrils deep within the fibripositor lumen were uniformly 28 nm in diameter, as were ECM fibrils, providing no evidence for fibril disassembly inside the fibripositors.

3-D reconstruction showed that tendon fibroblasts are roughly cylindrical in shape with their long axis parallel to the axis of the tendon. Moreover, the PM adopts a novel conformation to generate cylindrical channels that are parallel to the tendon-long axis. The fibripositors were also parallel to the long axis of the tendon and to the collagen fibril bundles (Figs. 6 and 7). Fibripositors projected into channels that were formed by close contacts between the PMs of adjacent cells (Fig. 7 B, open circles). The longitudinal axes of channels, which had smooth concave surfaces, were always parallel to the tendon-long axis (Fig. 7, A and B). Channels presumably provide a confined environment for the supramolecular organization of collagen fibrils into parallel bundles. Fibrils were observed exiting fibripositors in either direction along the tendon axis and branched fibripositors and branched lumen were also observed (Fig. 7 D).

The lumen of the fibripositor is accessible to HRP

To determine whether exogenous molecules such as nondegergent buffers and trypsin are able to penetrate into the lumen of the fibripositors in pulse-chase experiments, tendons from 13-d chick embryos were incubated in medium containing HRP. The presence of HRP was then detected using DAB, and transverse sections examined using EM. As shown in Fig. 8 B, electron dense material occurred in some fibripositors (closed arrowheads) whereas others remained clear (open arrowheads).

GPCs/H11001 cfS are absent during postnatal development despite active procollagen synthesis

Fibroblasts from the tails of 6-wk-old mice were stellate in cross section, surrounded by a dense matrix of collagen fibrils and lacked fibripositors or intracellular collagen fibrils (unpublished data). The fibrils had a diameter of 151 nm (±69; n = 521) and the distribution was broad (Fig. 9 C).
No hexagonal packing arrangement was observed presumably because of the heterogeneity in fibril diameters. The cells contained distinct populations of “large” and “small” compartments that had a mean diameter of 361 nm (±22 SEM; n = 210) and 66 nm (±7 SEM; n = 379), respectively (Fig. 9, A and B). The larger compartments were immunoreactive to an anticornagen antibody, and the small compartments were abundant beneath the PM. Analysis of the immunoEM images showed that the large compartments labeled with 35.4 (±2.5 SEM) gold particles per μm².
whereas the small compartments labeled with 2.0 (±1.1 SEM) gold particles per µm². The background labeling within the cell, which included areas of the ER, was 2.3 (±0.4 SEM). Therefore, there was a 17-fold increase in labeling/µm² of the large compartments compared with the small compartments. Pulse-chase analysis showed that procollagen and pCollagen occurred in NP-40–sensitive compartments although pNcollagen and collagen appeared transiently around 40 min of chase. In contrast, the salt extract contained pCollagen and collagen (Fig. 9 D).

Discussion

Here, we show that in embryonic tendon fibroblasts, (a) procollagen is converted to collagen in the post-Golgi secretory pathway; (b) this collagen assembles into narrow-diameter fibrils within elongated GPCs; (c) the collagen-fibril–containing GPCs (GPCs<sup>+</sup>) are targeted to novel PM protrusions, termed fibripositors, that are parallel to the tendon axis; and (d) the lumen of fibripositors open into channels formed between cells to deposit the fibrils into hexagonally packed bundles. Furthermore, fibripositors occur in tendon only during embryonic development when seeding of the ECM occurs; fibripositors are absent during postnatal development despite active procollagen synthesis. Thus, we have identified a novel PM organelle for the ordered assembly of tissues and we have demonstrated a link between the secretory pathway and the synthesis of an organized ECM.

This work has shown that processing of procollagen to collagen begins in Golgi to PM compartments that are refractory to extraction with high salt buffer but which can be solubilized using NP-40 detergent, suggesting that they are within the cell. In addition, we have shown that GPCs<sup>+</sup> are tubular in shape and are completely enclosed within the fibroblast PM. A question of interest is, once GPCs<sup>+</sup> have fused to the PM to form a fibripositor, is the lumen of the fibripositor accessible to salt extraction buffer or does it require NP-40 for solubilization? Incubation of tendons with HRP indicates that diffusion of exogenous molecules (such as trypsin, HRP, and salt ions) into the lumen does occur (Fig. 8 B). Some compartments did not contain DAB-reactive HRP, which is consistent with the presence of GPCs<sup>+</sup> that are enclosed within the cell. It is unlikely that fibripositor lumen close during the extraction process and then become refractory to salt extraction because the compartments that are accessible to salt extraction are also accessible to trypsin (Fig. 2 B). Trypsin digestion was performed directly after labeling and before the salt extractions when tissue shrinkage might be expected to occur. Therefore, it is likely that the material in the NP-40 extract is derived from ER, Golgi, GPCs<sup>+</sup>, and potentially from material at the very base of the fibripositor lumen where short early fibrils are found (Fig. 5 B). pNcollagen was absent from the salt extract indicating that the N-propeptides are removed before secretion into the lumen of the fibripositor or ECM and that pCollagen is the major intermediate used for the extension (embryonic) or broadening (6 wk) of pre-existing fibrils.

Two lines of evidence indicate that collagen fibril polymerization during embryogenesis begins in the TGN or in TGN exit sites, although future studies are needed to identify the origin of collagen-fibril-containing vesicles and precursors of the GPCs<sup>+</sup>. First, GPCs<sup>+</sup> near to the Golgi stacks contained cross-rafted collagen fibrils. Second, processing of procollagen to collagen was completely prevented in the presence of brefeldin A. The idea that intracellular procollagen processing could be mediated by N- and C-proteinases, which are concomitantly synthesized and trafficked with procollagen is supported by recent work in our laboratory showing intracellular activation of BMP1 in the TGN (Leighton and Kadler, 2003). Cleavage of procollagen would require neutral pH and a concentration of free calcium ions between 2 and 5 mM (Hojima et al., 1985). Alternatively, the procollagen proteinases could be targeted to the ECM and/or the base of the fibripositors and a cycling mechanism, similar to that used by furin (Molloy et al., 1999), could be used to localize the enzymes to the transface of the TGN. Fusion of procollagen containing GPCs with vesicles containing the processing enzymes would then trigger fibrillogenesis and the formation of new GPCs<sup>+</sup>.

At 6 wk of development very little cleavage of procollagen to collagen occurs within the cell: the NP-40–soluble compartments contain procollagen and pCollagen, whereas the NaCl-soluble compartments contain pCollagen and collagen. No GPCs<sup>+</sup> are observed despite immunolocalization of triple helical collagen to GPCs. Unfortunately, it is not possible to determine which intermediates are present within the GPCs because antibodies that are directed to the collagen triple helix, terminal propeptides, or cleaved neoepitopes will inevitably recognize at least two procollagen intermediates. Further studies are needed to explain the observed difference of N- and C-proteinase activity in embryonic and older tendon fibroblasts. The low abundance and high sequence homology between the various gene products has so far complicated the use of specific antibodies for immunolocalization studies.

Seed and feed mechanism of ECM assembly

Evidence from in vitro studies indicates that collagen fibrillogenesis is a nucleation-propagation process in which the formation of a thermodynamically unstable nucleus occurs slowly but once formed, the nucleus propagates rapidly in size by accretion of collagen molecules (Wood and Keech, 1960; Holmes and Chapman, 1979; Kadler et al., 1987, 1990; Silver et al., 1992). This assembly mechanism predicts the formation of a nucleus having a high fidelity structure because it contains the structural blueprint for the final fibril. The data from embryonic tendon showing the formation of early fibrils at the base of fibripositors, and the fact that the fibrils exhibit the same diameter as the fibrils in the ECM, are strongly suggestive that a nucleation-propagation assembly mechanism occurs in vivo. An important observation was that fibrils exceeding 10 µm in length could be traced from the center of a fiber bundle within the ECM to the lumen of a fibripositor deep within the cell. There is no evidence that collagen fibrils of this length can be synthesized de novo and deposited whole. Thus, we propose that the nucleation step occurs in GPCs and at the base of the fibripositors, at least in embryonic fibroblasts in situ. Further studies are needed to identify the site of fibril propagation, although the pulse-chase observations of pCollagen
being cleaved to collagen in a NaCl-extractable compart-
ment are consistent with propagation occurring in the ECM
or in open fibripositors. The increase in fibril diameter be-
tween embryonic and 6-wk stages of development and the
subsequent absence of GPCs\textsuperscript{+cf} is consistent with switching
off of the nucleation step of fibrillogenesis at postnatal stages
(Fig. 10, schematic). The results of immunoEM at 6 wk
unequivocally showed the presence of procollagen and/or
pCollagen in \textasciitilde350-nm-diam compartments, which pre-
sumably are responsible for delivering procollagen and pC-
collagen to the ECM.

**Biogenesis of GPCs\textsuperscript{+cf}, PM channels, and fibripositors**

It is a novel observation that procollagen processing can be
initiated in the late secretory pathway in vivo. Cultured
chick embryo tendon or human fibroblasts secrete procollagen
into the cell medium (Jimenez et al., 1971) and pro-
collagen peptidase activity is detected in the medium but not
in the cell layer of cultured fibroblasts (Kerwar et al., 1973;
Hayashi, 1973). In addition, recesses containing collagen fibrils have not been observed in cultured fibro-
blasts (Birk and Trelstad, 1986), and we have similarly
found no evidence of GPCs\textsuperscript{+cf} or fibripositor formation (un-
published data). Cells in culture therefore appear unable to
orchestrate the formation of extracellular channels between
cells into which parallel collagen fibrils are deposited. This
suggests that the parallelism of the tendon is the culmina-
tion of the stacking of fibroblasts along the long axis of the ten-
don, the 3-D organization of the post-Golgi secretory path-
way (presumably involving alignment of the cytoskeletal
components of adjacent cells) and the extrusion of PM pro-
cesses into which GPCs\textsuperscript{+cf} are targeted. The intercellular
channels appeared to be stabilized by specific points of con-
tact between adjacent cells (Fig. 7 B). The molecular com-
ponents of these cell–cell contacts remain unknown. Fur-
thermore, the positioning of fibripositors specifically into
the intercellular channels presumably involves altered distri-
bution of cell-matrix adhesion molecules, and specific adap-
tor molecules to ensure fusion of the GPCs\textsuperscript{+cf} only with the
distal tip of the fibripositor. Mechanical stimuli might also
be expected to influence fibripositor formation, the coal-
ignment of the fibripositors with the ECM, and the directional
deposition of collagen fibrils into the ECM. The current
work has established a functional link between the post-
Golgi secretory pathway and the organization of the ECM
and generates a platform for future studies of the cell and
molecular basis of tissue assembly.

**Perspectives**

The 3-D serial section reconstruction studies by Trelstad
and colleagues (Trelstad and Hayashi, 1979; Birk and Trel-
stad, 1984, 1986) in the 1970s and ‘80s used high voltage
transmission electron microscopes to image relatively thick
sections. These approaches were so far beyond the technical
expertise of most collagen biologists at the time, and the data
were so compelling, that few people contemplated extending
these studies. However, recent development of state-of-the-
art software such as IMOD has made it possible to revisit
this approach using larger numbers of thinner sections im-
aged on a conventional transmission electron microscope.
The results of our work and those obtained by Trelstad, Birk, and colleagues differ in several respects (see references above). First, it was speculated that only uncleaved procollagen was transported to the PM recesses. Our results show that procollagen can be converted to collagen within the cell and that fibril formation can occur in closed intracellular carriers. Furthermore, the recesses were envisioned to broaden at increasing distances from the body of the cell, and it was in these wider zones that fibrils formed into bundles. Our results show that the recesses are long channels in the PM that do not protrude into the cell but run down the surface of the cell. We also show that fibroblasts exhibit fibripositors that are finger-like protrusions from the PM.

Although the study of the cellular aspects of tissue assembly has been simplified by improvements in image analysis software such as IMOD, there remains a major technical hurdle concerning the cells. Fibroblasts flatten when plated out in culture and consequently the PM channels and fibripositors disappear. It is possible to culture whole tendons for up to 3 h and still observe normal procollagen processing but beyond this time the cells lose their embryonic phenotype. Therefore, dissection of the molecular mechanisms involved in PM channel formation and fibripositor biogenesis will rely on the development of novel cell or organ culture methods that preserve the 3-D shape of the cell and provide additional signals needed to specify continued cell differentiation.

Materials and methods

Pulse-chase analysis of procollagen processing in chick embryo tendons

Metatarsal tendons were obtained from day 13 chick embryos. Pulse-chase experiments were performed at 37°C in DME/F12 containing 1% (vol/vol) PS, 2 mM l-glutamine, 200 μM ascorbate, 400 μM β-APN, and supplemented with 2.5 μCi/ml of [14C]proline, 1 mM α,α-dipyridyl or 3.5 mM brefeldin A, as required. Pulse chase was stopped by transferring the tendons to 25 mM EDTA, and 50 mM Tris-HCl, pH 7.5, at 4°C. Trypsin digestion was performed in trypsin-EDTA in HBSS buffer for 30 min at 20°C and control digestions in HBSS buffer alone. For the detection of open fibripositors, tendons were incubated in DME/F12 supplemented with HRP (Chemicon, Temecula, CA) at 37°C, fixed with 2.5% glutaraldehyde, and treated with fast DAB (Sigma-Aldrich) before EM analysis. Tendons subjected to pulse-chase analysis in 1 ml aliquots of supplemented medium were extracted in 100 μl aliquots of salt extraction buffer (1 M NaCl, 25 mM EDTA, 50 mM Tris-HCl, pH 7.4) containing protease inhibitors and supplemented as required with 1% NP-40 detergent. Tendons were usually extracted in four changes of salt extraction buffer; overnight (S1), 6 h (S2), overnight (S3), 6 h (S4), and overnight in NaCl extraction buffer containing NP-40 (N). Extracts were analyzed on 4% precast SDS polyacrylamide gels (Invitrogen) under reducing conditions. The gels were fixed in 10% methanol, 10% acetic acid, dried under vacuum, and exposed to a phosphorimaging plate (Fuji BAS-III or BAS-M). After overnight exposure the phosphorimaging plates were processed using a phosphorimagery (Fuji BAS 2000 or 1800).

EM

Freshly dissected chick metatarsal tendons were cut into 3-mm lengths and frozen to –196°C using an EM PACT high pressure freezer (Leica). Freeze substitution for ultrastructure was performed using an AFS system (Leica), starting at –90°C in 2% wet/vol osmium tetroxide in aceton, going through pure aceton at –50°C and ending in several changes of Spurr’s resin (Spurr, 1969). At 20°C. Polymerization in fresh resin was then performed at 60°C for 24 h. Freeze substitution for immunolabeling was performed using an AFS system (Leica) using pure aceton at –90°C, pure ethanol at –50°C in ethanol, and ending in several changes of HM20 Lowicryl resin at –50°C. UV polymerization in fresh resin was then performed at –50°C for 48 h and continued at 20°C for 48 h. Embryonic mouse tails were fixed in 2% glutaraldehyde in 100 mM phosphate buffer, pH 7.0, for 30 min at RT. The tails were then diced and fixed for 2 h at 4°C in fresh fixative. After washing in 200 mM phosphate buffer they were fixed after 1% glutaraldehyde and 1% Oso4 in 50 mM phosphate buffer, pH 6.2, for 40 min at 4°C. After a rinse in distilled water they were in bloc stained with 1% aqueous uranyl acetate for 16 h at 4°C, dehydrated and embedded in Spur’s resin.

Ultra-thin sections for normal transmission electron microscopy were collected on uncoated copper 200 grids, serial sections for 3-D reconstruction on formvar-coated copper 1,000 μm slot grids (stabilized with carbon film) and ultra-thin sections (~60 nm) for immunolabeling on formvar-coated nickel 400 grids. A postembedding labeling technique was used to detect type I collagen using a rabbit anti–chicken collagen-I antibody (Biodesign International) at a dilution of 1:500 followed by a gold-conjugated goat anti-rabbit antibody (British Biocell International) at a dilution of 1:200. All sections were subsequently stained with uranyl acetate and lead citrate, and examined using either a JEOL 1200EX, Philips EM 400, or Philips BioTwin transmission electron microscope. Images were recorded on 4489 film (Kodak) and scanned using an Imacor Flexight 848 scanner (Precision Camera & Video). Images from EM serial sections were aligned and reconstructed in IMOD for Linux (Kremer et al., 1996) and visualized using OpenSynu for Linux (Hessler et al., 1992).

Online supplemental material

Experimental procedures are available online concerning the differential extraction of secretory pathway proteins and ECM proteins. Fig. S1 depicts the differential extraction of ECM proteins and intracellular proteins. Methods were developed that facilitated the differential extraction of ECM proteins and proteins enclosed within membrane-bound compartments. Proteins in the ECM were solubilized in neutral buffers at 4°C, whereas those in membrane compartments were subsequently extracted in buffers containing NP-40. EM was used to examine the ultrastructure of the cells after each extraction. Western blot analysis was used to examine the protein composition of each extract. Video 1 depicts a 3-D reconstruction of part of a tendon fiber that runs down the mouse tail showing cells and associated fibripositors. Cells are color rendered. Some fibripositors are shown in yellow. The video shows that the cells are cylindrical in shape. Cylindrical channels occur between cells. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200312071/DC1.

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