Active Negative Control of Collagen Fibrillogenesis in Vivo

INTRACELLULAR CLEAVAGE OF THE TYPE I PROCOLLAGEN PROPEPTIDES IN TENDON FIBROBLASTS WITHOUT INTRACELLULAR FIBRILS

Received for publication, October 2, 2007, and in revised form, January 14, 2008. Published, JBC Papers in Press, February 19, 2008, DOI 10.1074/jbc.M708198200

Sally M. Humphries, Yinhui Lu, Elizabeth G. Canty, and Karl E. Kadler

From the Wellcome Trust Centre for Cell-Matrix Research, University of Manchester, Faculty of Life Sciences, Michael Smith Building, Oxford Road, Manchester M13 9PT, United Kingdom

It is established fact that type I collagen spontaneously self-assembles in vitro in the absence of cells or other macromolecules. Whether or not this is the situation in vivo was unknown. Recent evidence shows that intracellular cleavage of procollagen (the soluble precursor of collagen) to collagen can occur in embryonic tendon cells in vivo, and when this occurs, intracellular collagen fibrils are observed. A cause-and-effect relationship between intracellular collagen and intracellular fibrils was not established. Here we show that intracellular cleavage of procollagen to collagen occurs in postnatal murine tendon cells in situ. Pulse-chase analyses showed cleavage of procollagen to collagen via its two propeptide–retained intermediates. Furthermore, immunoelectron microscopy, using an antibody that recognizes the triple helical domain of collagen, shows collagen molecules in large-diameter transport compartments close to the plasma membrane. However, neither intracellular fibrils nor fibripositors (collagen fibril-containing plasma membrane protrusions) were observed. The results show that intracellular collagen occurs in murine tendon in the absence of intracellular fibrillogenesis and fibripositor formation. Furthermore, the results show that murine postnatal tendon cells have a high capacity to prevent intracellular collagen fibrillogenesis.

The functional integrity of connective tissues is dependent upon the production of an extensive and highly organized network of extracellular collagen fibrils. Curiously, collagen fibrils occur in a wide spectrum of tissues in which diverse properties are elicited from the tissue-specific organization and size of the fibrils. For example, orthogonal layers of narrow-diameter collagen fibrils account for the toughness and optical transparency of the cornea, whereas the parallel arrangement of the fibrils in tendon produces a tough, tensile tissue. An organized extracellular matrix is established in these tissues during embryonic development, but the molecular mechanisms are poorly understood.

Tendons have a highly ordered, yet relatively simple extracellular matrix (ECM) composed of elongated collagen fibrils in parallel alignment with the tendon long axis. At embryonic stages of development when the architecture of the ECM is being established, the tendon precursor cells are cylindrical in shape with the long axis of the cells parallel to that of the tendon long axis. Convolutions of the plasma membranes of tendon fibroblasts form intercellular and intracellular channels in which the collagen bundles form (1). These bundles are contiguous from one cell to another along the tendon long axis and are stabilized by adherens cell-cell junctions (2). Fibril bundles and their associated cells are arranged into tendon fascicles with several fascicles composing the tendon proper. As tendon development proceeds, the fibril bundles associate laterally to form the large fascicles of mature tendons (3). With increasing age, bundle size increases, and the tendon fibroblasts reduce in number and become more widely dispersed (4). This organization of collagen fibrils, taking into account fibril diameter, packing density, bundle size, and the parallel orientation of the fibrils, defines the mechanical qualities demonstrated by tendon (3).

The motivation for the work presented here came from evidence that the late secretory pathway is involved in orchestrating the parallel organization of collagen fibrils in tendon (5). Using a combination of three-dimensional electron microscopy (serial section three-dimensional reconstruction) and biochemical approaches, it was shown that during embryonic development, collagen processing begins within the cell in post-Golgi transport carriers. Actin-stabilized plasma membrane processes (fibripositors) protrude into the ECM to organize the newly assembled collagen fibrils in parallel alignment with the existing matrix (5, 6). The intracellular cleavage of procollagen and the concomitant appearance of fibripositors pointed to a link between the intracellular cleavage of procollagen to collagen and the formation of intracellular fibrils. Here we show using pulse-chase analysis of procollagen processing that the intracellular conversion of procollagen to collagen can occur within the cell during postnatal stages of tendon development in the absence of intracellular collagen fibrils and fibripositors. Therefore, cells can prevent intracellular formation of collagen fibrils.

* This work was supported by the Wellcome Trust (to K. E. K.) and the Biotechnology and Biological Sciences Research Council (as an equipment grant to K. E. K. and a postgraduate studentship to S. M. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 To whom correspondence should be addressed. Tel.: 44-0-161-275-5086; Fax: 44-0-161-275-1505; E-mail: karl.kadler@manchester.ac.uk.

2 The abbreviations used are: ECM, extracellular matrix; pCcollagen, procollagen lacking the C-propeptides; pNcollagen, procollagen lacking the N-propeptides but retaining the N-propeptides; *collagen, a collective term for all procollagen, pCcollagen, pNcollagen, and collagen molecules or polypeptide chains of these molecules.
**Negative Control of Collagen Fibrillogenesis**

**EXPERIMENTAL PROCEDURES**

_Pulse-Chase Analysis of Procollagen Processing and Secretion_—Reagents were obtained from Invitrogen or Sigma unless stated otherwise. Mice were obtained from the University of Manchester Biological Supply facility and killed using Schedule 1 procedures prior to dissection of the tail tendons. Pulse-chase experiments were performed at 37 °C in Dulbecco’s modified Eagle’s medium/nutrient mixture F-12 containing penicillin (100 units/ml), streptomycin (100 µg/ml), l-glutamine (2 mM), l-ascorbic acid 2-phosphate (200 µM), and β-amminopropionitrile (400 µM). Pulse-chase experiments were performed at 37 °C; tendons were pre-equilibrated for 30 min, labeled for 10 min with 2.5 µCi/ml [14C]proline (GE Healthcare), and then transferred to unlabeled medium for 15 min to 3 h. Pulse-chase was stopped by transferring the tendons to 25 mM EDTA and 50 mM Tris-HCl, pH 7.5, at 4 °C. Tendons subjected to pulse-chase analysis in 1-ml aliquots of supplemented medium were then extracted in 100-µl aliquots of salt extraction buffer (1 M NaCl, 25 mM EDTA, and 50 mM Tris-HCl, pH 7.5) containing protease inhibitors and supplemented as required with Nonidet P-40 detergent (1%, v/v). A concentrated stock solution of protease inhibitors was made using EDTA-free protease inhibitor mixture tablets (Roche Applied Science). Tendons were extracted in four changes of salt extraction buffer (overnight (S1), 6 h (S2), overnight (S3), and 6 h (S4)) and overnight in salt extraction buffer containing Nonidet P-40. S1 extracts are shown as _S_ lanes in Figs. 1, 2, and 3. Extracts were analyzed using 4% precast SDS-polyacrylamide gels (Novex) under reducing conditions. The gels were fixed for 40 min using two changes of methanol (10%, v/v) and acetic acid (10%, v/v) and dried under vacuum. The gels were exposed to a BAS-MS phosphorimaging plate, which was processed using an FLA3000 phosphoimager.

_Tryptsin Test of Membrane-enclosed Procollagen Molecules—_To verify that the procollagen intermediates in the Nonidet P-40 extracts originated from membrane-enclosed compartments, we used the fact that the procollagen propeptides are susceptible to cleavage by trypsin unless the procollagen and trypsin are separated by a membrane (as shown previously in Ref. 5). Tail tendons were incubated at 37 °C in 1-ml aliquots of culture medium supplemented with [14C]proline for 10 min and chased in unlabeled medium for 15 min. Tendon samples were then incubated at 20 °C for 30 min with trypsin/EDTA (trypsin/Versene, final concentration of 0.25%; BioWhittaker) in Dulbecco’s modified Eagle’s medium/nutrient mixture F-12 buffer. Soybean trypsin inhibitor was added to 0.25% to inhibit subsequent trypsinization. Proteins were sequentially extracted at 4 °C in 100-µl aliquots of four changes of neutral salt extraction buffer (overnight, 6 h, overnight, and 6 h) and finally in buffer containing Nonidet P-40 overnight. Only the first salt extracts and Nonidet P-40 extracts are shown under “Results.” Samples were analyzed using 4% precast Novex SDS-polyacrylamide gels under reducing conditions. The gels were fixed for 40 min using two changes of methanol (10%, v/v) and acetic acid (10%, v/v) and dried under vacuum. The gels were exposed to a BAS-MS phosphorimaging plate, which was processed using an FLA3000 phosphoimager.

_Electron Microscopy—_Tendons were prepared for electron microscopy as described previously (5). Ultrathin (60–70 nm) sections were collected on uncoated copper 200-mesh grids and stained with lead citrate (0.3% in 0.1 M sodium hydroxide) for 1 min. Immunelectron microscopy samples were prepared by high pressure freezing (Leica EM PACT) followed by freeze substitution (APS, Leica) and embedding in LR White. A post-embedding labeling technique was used to detect type I collagen using a rabbit anti-mouse collagen type I primary polyclonal antibody (Biosign Ecuador International) at a dilution of 1:50 for 1 h at room temperature, followed by a 10-nm gold-conjugated goat anti-rabbit antibody (British Biocell International) at a dilution of 1:50 for 1 h at room temperature. Post-treated sections were stained with 1% aqueous uranyl acetate. Sections were examined on an FEI Tecnai 12 Biotwin transmission electron microscope, and images were recorded on Kodak 4498 film and digitized using an Imacron Flexlight 848 scanner (Precision Camera and Video).

_Collagen Fibril Diameter Measurements—_Electron micrographs of near-transverse sections of fibril bundles were recorded. Magnification calibration was performed using a diffraction grating replica (2160 lines/mm; Agar Scientific, Stanstead, UK). Subregions of micrographs were digitized using a Bosch T101 91D TV camera and A/D converter. Diameters were measured from the digital images using the image analysis software Semper5 (Synoptics, Cambridge, UK).

**RESULTS**

During our studies of the occurrence and structure of fibri-positors, we noted that tail tendons of postnatal mice lacked fibripositors and the associated fibricarriers that contain intra-cellular collagen fibrils. These observations prompted a study of procollagen processing in postnatal murine tendons in combination with ultrastructural observations of the cells and collagen fibrils. We used our sequential protein extraction procedure to examine separately procollagen cleavage intermediates inside and outside the cell (5). In brief, ECM proteins are extracted into buffers containing sodium chloride, and intracellular proteins are extracted into buffers further supplemented with Nonidet P-40.

_Procollagen Processing during Postnatal Development of C57BL/6 Mouse Tail Tendon Occurs Predominantly in the ECM—_Tail tendons were removed from 4- and 6-week-old C57BL/6 strain mice, and sequential pulse-chase analyses of procollagen cleavage were performed as described (5). As shown in Fig. 1, the appearance of procollagen processing intermediates mirrored that described previously for postnatal C57BL/6 mouse tail tendon (5), with a differential distribution of procollagen processing intermediates in the salt and Nonidet P-40 extracts. At both ages, the [14C]procollagen chains were predominately present in the Nonidet P-40 extract as proα1(I) and proα2(I) after a 15-min chase time (Fig. 1, _A_ and _B_, _lanes_ 1). Procollagen α-chains could be seen in the Nonidet P-40 extracts at subsequent chase times up to 60 min (Fig. 1, _A_ and _B_, _lanes_ 3, 5, and 7) along with low levels of pCcollagen. Few or no labeled procollagen α-chains could be seen at 120- or 180-min chase times (Fig. 1, _A_ and _B_, _lanes_ 9 and 11). Conversely, no labeled collagen could be seen in the 15-min salt...
extracts (Fig. 1, A and B, lanes 2). After longer chase times, some partially processed α-chains (cleaved of the N-terminal propeptide) and fully processed α-chains could be seen in the first salt extracts. At 120- and 180-min chase times (Fig. 1, A and B, lanes 10 and 12), all detectable 14C-proteins were present in the salt extract as collagen and pCcollagen. These observations indicate that procollagen processing occurs predominately in the ECM in postnatal C57BL/6 mice and that processing is via pCcollagen.

C57BL/6 and CD1 Mice Exhibit Differences in Postnatal Procollagen Processing—To determine whether the intracellular/extracellular pattern of postnatal procollagen processing was the same in another strain of mouse, pulse-chase experiments were performed on postnatal CD1 mouse tail tendons. Surprisingly, procollagen, pNcollagen, pCcollagen, and fully processed collagen were present in intracellular (Nonidet P-40) fractions (Fig. 2A). This was particularly apparent with chase times of 60 min or less (Fig. 2A, lanes 1, 3, 5, and 7). The amount of labeled collagen in the Nonidet P-40 extract decreased progressively as chase time increased, consistent with the secretion of collagen to the ECM. Interestingly, procollagen was observed in the Nonidet P-40 extract after a 180-min chase, whereas collagen chains had been seen almost entirely in the salt extract of C57BL/6 mice at chase times longer than 60 min (compare Figs. 1 and 2). Another surprising result was the amount of labeled collagen present in the salt extract after a 15-min chase time.

Analysis of postnatal processing in C57BL/6 mice revealed no difference in procollagen processing in CD1 and CD1 tail tendons. Extracellular protein extracts were otherwise similar to those observed for C57BL/6. These data show that intracellular processing of procollagen can occur via pCcollagen or pNcollagen in postnatal CD1 mouse tendon but primarily via the pCcollagen intermediate in the ECM. These results also demonstrate that there are murine strain-dependent differences in procollagen processing.

Intracellular Procollagen Processing Persists in Adult CD1 Tail Tendons—Examination of procollagen cleavage in 3-month-old CD1 mouse tendons demonstrated rapid intracellular conversion of procollagen to collagen (Fig. 2B). The results showed intracellular cleavage of procollagen to collagen within a short 15-min time frame followed by secretion of fully processed collagen within 30 min (Fig. 2B, lane 2). It was noteworthy that the rate of secretion of collagen was apparently faster in the 3-month-old CD1 mice than in the 4-week-old mice.

The Sequential Isolation of Extracellular and Then Intracellular Proteins Is Effective in Postnatal Murine Tendon—Although Canty et al. (5) had demonstrated that the sequential sodium chloride/Nonidet P-40 extraction procedure successfully separated intracellular from extracellular proteins in embryonic chick, we considered the possibility that the procedure might not be optimized for postnatal murine tendon, especially in light of the results using the CD1 mouse tendons. We therefore validated the procedure on CD1 mouse tendons using the trypsin sensitivity of the procollagen propeptides, based on the method described by Bruckner and Prockop (7) and used by Canty et al. (5). In brief, the propeptides are sensitive to trypsin digestion but are protected from cleavage when enclosed within the cell or within a membrane-bound compartment. Fig. 3 shows that proteins extracted in the salt-containing buffer were accessible to trypsin, whereas proteins in pre-Nonidet P-40 extracts of the cells were protected from trypsin cleavage.
Intracellular procollagen is resistant to trypsin digestion prior to Nonidet P-40 removal of membranes. CD1 tail tendons were incubated in medium supplemented with \[^{14}C\]proline for 10 min and chased in unlabeled medium for 15 min. Samples were incubated with trypsin, and then proteins were sequentially extracted in four changes of neutral salt extraction buffer and finally in buffer containing Nonidet P-40. Only the first salt extracts (S) and Nonidet P-40 extracts (N) are shown. Samples were analyzed by SDS-PAGE and autoradiography. R, a reference sample containing α-chains from *collagen.

Other Strains of Mice Exhibit Intracellular Procollagen Cleavage—Procollagen cleavage was examined at 3 weeks postbirth in the tail tendons of four additional mouse strains: BALB/c, C3H, FVB (MDR1A knock-out), and MF1-nude. For each of the strains, intracellular processing via pCcollagen and pNcollagen intermediates occurred (data not shown). These results imply that cleavage of procollagen to collagen within the confines of the cell is a feature of collagen biosynthesis during postnatal murine tendon development in many strains of mice, with C57BL/6 being a notable exception.

Intracellular Collagen Does Not Directly Initiate Fibripositor Formation in Postnatal Mouse Tendon Cells—Transverse sections through tail tendon bundles of 3-week-old C57BL/6, CD1, and C3H strain mice were examined by transmission electron microscopy for evidence of fibripositors and fibricarriers. Furthermore, immunoelectron microscopy using an anti-triple helix type I collagen antibody was performed on tail tendons of 6-week-old CD1 and C3H mice to identify transport compartments containing procollagen, pNcollagen, pCcollagen, and collagen. Unfortunately it is not possible to determine which intermediates are present within the transport compartments because antibodies that are directed to the collagen triple helix, terminal propeptides, or cleaved neoepitopes will inevitably recognize at least two procollagen intermediates. In the interest of brevity, we use *collagen to describe collectively procollagen, pNcollagen, pCcollagen, and collagen. As shown in Fig. 4, the tendon cells appeared similar for each of the strains. At 3 weeks of age, the plasma membranes of the tendon fibroblasts were bordered closely by their surrounding ECM, which was dominated by closely packed parallel collagen fibrils heterogeneous in diameter (see collagen fibrils (CFs) in Fig. 4, A1, B1, and C1, of conventionally embedded and stained samples and in the collagen immunolocalization images in A2, B2, and C2). No evidence of fibricarriers, intracellularly assembled collagen fibrils, or fibripositors was observed in any of the samples. Furthermore, large-diameter *collagen-containing compartments observed by Canty et al. (5) in the stellate projections of tendon fibroblasts in 6-week-old C57BL/6 strain mice were also observed in CD1 and C3H cells (labeled with black arrows in Fig. 4, A2, B2, and C2). The stellate projections are at 90° to the tendon long axis and should not be confused with fibripositors, which are parallel to the tendon long axis. In each of the strains, the stellate projections were closely bordered by parallel type I collagen-containing fibrils and an abundance of small compartments in close association with the plasma membrane that did not label for type I collagen. Again, there was no evidence of intracellular fibril assembly or fibripositors. The results indicate that the intracellular conversion of procollagen to collagen is not sufficient to initiate their assembly into collagen fibrils or to activate the formation of fibripositors.

Strain Variations in Collagen Processing Do Not Affect Collagen Fibril Diameter—Observations of procollagen processing in several strains of mice indicate that C57BL/6 strain mice can only effectively cleave the procollagen C-propeptide following the secretion of collagen into the ECM. This is in contrast to the other strains examined, which showed active C-proteinase activity in both intracellular and extracellular locations. To examine whether the lack of C-terminal procollagen processing...
in C57BL/6 strain mice had an impact on fibril diameter, measurements were taken from the tail tendons of C57BL/6, CD1, and C3H strain mice at 3 weeks of postnatal development (Fig. 5). Each of the strains had a broad distribution of fibril diameters, although CD1 fibrils had a slightly higher mean diameter of 144.6 ± 1.6 nm (S.E.) (Fig. 5A) compared with 139.6 ± 1.6 nm (S.E.) for C57BL/6 (Fig. 5C) and 129.6 ± 1.4 nm (S.E.) for C3H (Fig. 5B). The data were analyzed by one-way analysis of variance and Tukey post-hoc analysis, and CD1 fibril diameters were significantly larger than C3H and C57BL/6 fibril diameters ($p < 0.01$). Fibril diameter differences between C3H and C57BL/6 strains were not significant ($p = 0.579$). These data show that differences in intracellular processing of type I procollagen in C57BL/6 tendons do not appear to affect collagen fibril diameter.

DISCUSSION

It was in the 1970s that type I collagen was shown to be synthesized as a soluble precursor procollagen containing N- and C-terminal propeptides (8). Since that time, it has been shown that cleavage of the C-terminal propeptides by procollagen C-proteinase (which, it is noteworthy, is a neutral metalloproteinase) is essential for the assembly of collagen fibrils (9) and that, following cleavage, spontaneous fibrillogenesis occurs (see Refs. 10 and 11 and references therein). Here we show that cleavage of the procollagen propeptides can occur in intracellular compartments in postnatal mouse tendon cells in the absence of intracellular fibrillogenesis. Thus, mechanisms must be in place to prevent intracellular collagen fibril formation. The mechanisms in postnatal mouse tendon cells are unknown. However, from extensive studies of collagen fibrillogenesis in vitro, we can speculate that factors such as low pH, high pH, restricted size of transport compartments, the absence of nucleating molecules (e.g. type V collagen), and the presence of inhibitors or a combination of these factors might be at play in postnatal tendon cells. We expand on these possibilities below.

We had first examined postnatal C57BL/6 tendon and showed that procollagen processing was predominately extracellular (5). In subsequent studies, we were surprised to find that in tail tendons of 3.5-week-old CD1 mice (an outbred mouse strain), marked intracellular procollagen processing occurred. The relative amounts of procollagen, pNcollagen, pCcollagen, and collagen $\alpha$-chains were similar to those observed in embryonic day 13 chick tendons, with processing via both pNcollagen and pCcollagen in intracellular extracts and processing via the pCcollagen intermediate in extracellular extracts (5). Interestingly, intracellular fibrils are also seen in the embryonic chick tendon cells at the same time that intracellular cleavage of procollagen occurs.

The discovery (described here) that complete procollagen processing can occur within intracellular compartments of postnatal tendons prompted us to examine whether intracellular fibril formation and fibripositor formation also occurs in these tissues. However, electron microscopy revealed no evidence of intracellularly assembled fibrils or fibrispotors. In addition, immunoelectron microscopy localized type I collagen chains to large-diameter compartments in the stellate projections of the cells. We noticed considerable differences in intracellular procollagen cleavage between C57BL/6 and CD1 mice. We therefore investigated whether these differences might manifest themselves in differences in collagen fibril diameter. However, although diameter differences were observed between CD1 and C57BL/6, there was no significant difference between C3H and C57BL/6 despite differences in intracellular procollagen cleavage. We concluded that intracellular cleavage of procollagen per se does not determine the diameter of collagen fibrils. An explanation eludes us for the remarkable similarity of fibril diameters across strains of mice despite differences in procollagen processing. Different strains of mice might be expected to have minor differences in ECM components, presumably caused by genetic variation. It is there-
before tempting to speculate that the diameter of fibrils is determined by a structure-based fibril assembly mechanism, perhaps coupled with the ability of collagen-binding proteins (such as proteoglycans, thrombospondins, and minor collagens) to modulate fibril assembly in specific tissues.

The intracellular processing of procollagen to collagen is paradoxical given that the procollagen N- and C-proteases are neutral metalloproteinases (for review, see Ref. 12) and that the late secretory pathway is increasingly acidic during anterograde protein transport; the pH of the trans-Golgi network is ~6 (13), becoming more acidic in post-trans-Golgi network transport carriers and secretory vacuoles. The pH optimum for activity of both the procollagen C- and N-proteases is pH 8.0–8.5, and negligible activity is observed at pH 6 or lower (9, 14). Therefore, how procollagen is cleaved within intracellular compartments is puzzling. Cleavage of pro-BMP-1 to BMP-1 occurs at the trans-Golgi network (15), and therefore, if BMP-1 is active in post-trans-Golgi network compartments, these compartments would require neutralization by the release of H+ ions. This might require the formation of specialized compartments containing ECM proteins and the neutral procollagen proteinases. In fact, the existence of specialized transport compartments for procollagen-containing compartments has recently been suggested; it was shown that different transport machinery is required for the trafficking of small (vesicular stomatitis virus G) and large (procollagen I) cargo (16), supporting the hypothesis that constitutive cargo may be segregated into numerous classes based on the protein properties prior to their secretion.

The intracellular processing of procollagen in the apparent absence of intracellular fibril formation is a further enigma. Cleavage of the N- and C-terminal propeptides is sufficient for spontaneous fibrillogenesis in vitro (10, 17); thus, a purely self-assembly-based mechanism of fibril formation would promote the presence of intracellular fibrils. Thus, the absence of fibrils in compartments containing collagen implies negative control of collagen fibrillogenesis. How cells in postnatal tendon prevent intracellular collagen fibril formation is unknown. In one scenario, type I collagen binds at least 50 different molecules (for review, see Ref. 18), and therefore, fibrillogenesis could be inhibited by sequestering the molecule, thereby sterically blocking binding sites that would otherwise promote fibril assembly. In other ways, fibril assembly could be prevented by rapidly secreting the fully processed collagen molecules before they have time to form early fibrils. This would be a simple explanation for the absence of intracellular fibrils in postnatal CD1 mouse tendon cells, in which procollagen cleavage and secretion of collagen occur within 15 min (see Fig. 2B). As another alternative, the secretory compartments could be mildly acidified, which would efficiently prevent collagen fibril formation (for review, see Ref. 11 and references therein). Elucidating which, if any, of these scenarios explains the absence of intracellular fibril formation will require new methods to determine the concentration of collagen in secretory compartments, the pH of collagen-containing compartments, and the identification of collagen-containing macromolecular complexes in secretory compartments in vivo.

Conversely, the absence of fibrils in compartments containing collagen molecules might suggest the need for molecules that promote effective fibril assembly. It is possible that such a regulatory molecule might associate with processed collagen molecules to accelerate fibrillogenesis or prevent errant fibrillogenesis in embryonic tendon. A recent study has shown that the cartilage oligomeric matrix protein, which occurs in tendon, accelerates collagen fibrillogenesis (19). Alternatively, the induction of intracellular fibrillogenesis may require the presence of essential nucleators; initiation of type I collagen fibril assembly is dependent on the presence of type V collagen in vivo (20). Thus, independent trafficking of these collagens might be expected to prevent fibrillogenesis. In addition, type I/III collagen fibrillogenesis in cell culture is reliant upon a preformed fibronectin matrix and is facilitated by collagen-binding integrins (21, 22). Fibronectin assembly occurs at the cell surface and is mediated by interactions with fibronectin-binding integrin α5β1 and the actin cytoskeleton (23); therefore, intracellular fibrillogenesis may require the internalization of fibronectin and/or integrins.

On a final note, the absence of fibripositors despite intracellular procollagen processing suggests that fibripositor formation is dependent on appropriate presentation of collagen fibrils to cellular membranes. The occurrence of fibripositors coincides with the deposition or alignment of collagen fibrils during tendon embryogenesis. However, it has not escaped our attention that the formation of fibripositors might require presentation of preformed collagen fibrils in transport compartments or at the plasma membrane and not just the presence of non-fibrillar (i.e. non-aggregated) collagen molecules per se.

Acknowledgments—We thank Drs. David Holmes and Tobias Starborg for technical advice and critical reading of the manuscript.

REFERENCES
1. Trelstad, R. I., and Hayashi, K. (1979) Dev. Biol. 71, 228–242
2. Richardson, S. H., Starborg, T., Lu, Y., Humphries, S. M., Meadows, R. S., and Kadler, K. E. (2007) Mol. Cell. Biol. 27, 6218–6228
3. Birk, D. E., and Trelstad, R. L. (1986) J. Cell Biol. 103, 231–240
4. Nakagawa, Y., Majima, T., and Nagashima, K. (1994) Acta Physiol. Scand. 152, 307–313
5. Canty, E. G., Lu, Y., Meadows, R. S., Shaw, M. K., Holmes, D. F., and Kadler, K. E. (2004) J. Cell Biol. 165, 553–563
6. Canty, E. G., Starborg, T., Lu, Y., Humphries, S. M., Holmes, D. F., Meadows, R. S., Huffman, A., O’Toole, E. T., and Kadler, K. E. (2006) J. Biol. Chem. 281, 38592–38598
7. Breuckner, P., and Prockop, D. J. (1981) Anal. Biochem. 110, 360–368
8. Bellamy, G., and Bornstein, P. (1971) Proc. Natl. Acad. Sci. U. S. A. 68, 1138–1142
9. Hojima, Y., van der Rest, M., and Prockop, D. J. (1985) J. Biol. Chem. 260, 15996–16003
10. Kadler, K. E., Hojima, Y., and Prockop, D. J. (1987) J. Biol. Chem. 262, 15696–15701
11. Kadler, K. E., Holmes, D. F., Trotter, J. A., and Chapman, J. A. (1996) Biochem. J. 316, 1–11
12. Greenspan, D. S. (2005) Top. Curr. Chem. 247, 149–183
13. Demaurex, N., Furuya, W., D’Souza, S., Bonifacino, J. S., and Grinstein, S. (1998) J. Biol. Chem. 273, 2044–2051
14. Hojima, Y., Morgelin, M. M., Engel, J., Boutillon, M. M., van der Rest, M., McKenzie, J., Chen, G. C., Rafi, N., Romanic, A. M., and Prockop, D. J.
15. Leighton, M., and Kadler, K. E. (2003) 
   *J. Biol. Chem.* 278, 18478–18484

16. Starkuviene, V., and Pepperkok, R. (2007) 
   *Traffic* 8, 1035–1051

17. Fessler, J. H., and Fessler, L. I. (1978) 
   *Annu. Rev. Biochem.* 47, 129–162

18. Di Lullo, G. A., Sweeney, S. M., Korkko, J., Ala-Kokko, L., and San Antonio, J. D. (2002) 
   *J. Biol. Chem.* 277, 4223–4231

19. Halasz, K., Kassner, A., Morgelin, M., and Heinegard, D. (2007) 
   *J. Biol. Chem.* 282, 31166–31173

20. Wenstrup, R. J., Florer, J. B., Brunskill, E. W., Bell, S. M., Chervoneva, I., and Birk, D. E. (2004) 
   *J. Biol. Chem.* 279, 53331–53337

21. Li, S., Van Den Diepstraten, C., D’Souza, S. J., Chan, B. M. C., and Pickerling, J. G. (2003) 
   *Am. J. Pathol.* 163, 1045–1056

22. Velling, T., Risteli, J., Wennerberg, K., Mosher, D. F., and Johansson, S. (2002) 
   *J. Biol. Chem.* 277, 37377–37381

23. Pankov, R., Cukierman, E., Katz, B. Z., Matsumoto, K., Lin, D. C., Lin, S., Hahn, C., and Yamada, K. M. (2000) 
   *J. Cell Biol.* 148, 1075–1090