Procollagen trafficking, processing and fibrillogenesis

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Summary

Collagen fibrils in the extracellular matrix allow connective tissues such as tendon, skin and bone to withstand tensile forces. The fibrils are indeterminate in length, insoluble and form elaborate three-dimensional arrays that extend over numerous cell lengths. Studies of the molecular basis of collagen fibrillogenesis have provided insight into the trafficking of procollagen (the precursor of collagen) through the cellular secretory pathway, the conversion of procollagen to collagen by the procollagen metalloproteinases, and the directional deposition of fibrils involving the plasma membrane and late secretory pathway. Fibril-associated molecules are targeted to the surface of collagen fibrils, and these molecules play an important role in regulating the diameter and interactions between the fibrils.

Key words: Procollagen, Collagen, Fibrillogenesis, ECM

Introduction

Collagen fibrils are the principal source of tensile strength in animal tissues and define the shape and form of tissues in which they occur. They have a distinctive 67 nm axial periodicity, are millimetres in length and range in diameter from a few nanometres to ~500 nm (depending on the tissue and stage of development). Fibrils are arranged in elaborate three-dimensional (3D) arrays, such as parallel bundles (e.g. in tendons and ligaments), orthogonal lattices (e.g. in the cornea) and concentric weaves (e.g. in bone). The fibrils are synthesized and secreted by fibroblasts but how this process is orchestrated and controlled, particularly during embryonic development, regeneration and tissue repair, is poorly understood. Collagen molecules secreted by cells might self-assemble in the extracellular matrix (ECM) but accumulating evidence suggests that fibril assembly can begin in the secretory pathway and at the plasma membrane (PM).

Here, we focus on the synthesis, trafficking, post-translational processing and assembly of collagen into fibrils, as well as some potentially important interactions between collagen and other macromolecules. There are comprehensive reviews of mutations in collagen and related genes (Myllyharju and Kivirikko, 2004), fibril structure (Hulmes, 2002; Ottani et al., 2001) and the polarity of collagen molecules in the fibrils (Kadler et al., 1996) and therefore these topics are not described in detail here.

Collagen is an abundant component of the ECM

Collagens are trimeric molecules in which each chain comprises a repeating Gly-X-Y triplet, in which X and Y can be any residue but are usually proline and hydroxyproline, respectively (van der Rest and Garrone, 1991). This triplet motif results in a left-handed helix that can intertwine with two other helices to form a right-handed triple-helical structure, which can be homotrimeric or heterotrimeric, depending on the collagen type. To date, 27 different collagen types have been identified (Table 1). We focus here on the fibrillar collagens (types I, II, III, V and XI) (Boot-Handford and Tuckwell, 2003; Vuorio and de Crombrugghe, 1990), which are characterized by a triple-helical domain, 300 nm in length, flanked by terminal globular domains known as the N- and C-propeptides that do not exhibit the Gly-X-Y repeat structure. Proteolytic removal of the propeptides results in triple-helical collagen molecules that have short telopeptides at either end and can assemble into highly ordered, string-like aggregates known as fibrils. In tendon, bone and skin, type I collagen is the major component of these fibrils; by contrast, in cartilage, fibrils mainly comprise type II collagen. Fibrils are usually heterotypic in that they can contain more than one type of fibrillar collagen. For example, the fibrils in skin are made up of both type I and type III collagen, and those in cartilage contain types II and XI. Fibrils in tendons vary in diameter from 30 nm to 300 nm, depending on the stage of development (Fleischmajer et al., 1988), and in mature tissues the fibrils are indeterminate in length and far longer than the fibroblasts that synthesize the constituent collagen molecules (Craig et al., 1989). Fibrils in tissues are most often assembled into higher-order structures. However, although fibroblasts in culture are able to produce collagen fibrils that have the distinctive 67 nm banding pattern characteristic of correctly assembled fibrils, the fibrils display no higher-order organization and are randomly distributed in the cell culture dish (Fig. 1).

Collagen biosynthesis

Procollagen is cotranslationally translocated into the lumen of the endoplasmic reticulum (ER), in which a number of molecular chaperones and enzymes assist its folding and trimerization (Hendershot and Bulleid, 2000; Lamande and Bateman, 1999). The C-propeptides play an important role in procollagen folding because they ensure association between

monomeric procollagen chains (Bulleid et al., 1997) and determine chain selectivity (Lees et al., 1997) (Fig. 2). Association between procollagen chains is preceeded by folding and disulphide bond formation within the individual C-propeptides (Doege and Fessler, 1986). Protein disulphide isomerase (PDI) has also been implicated in the subsequent formation of inter-chain disulphide bonds (Koivu, 1987), but this step is not required for the directional folding of the collagen triple helix, which occurs in the C-to-N direction (Bulleid et al., 1996). However, propogation of the collagen

Table 1. The collagen family of proteins

Collagen type	Genes	Supramolecular organization in tissues (where known)	References
I	*COL1A1* *COL1A2*	Fibrils in tendon, bone, skin, cornea and blood vessel walls	Chu et al., 1982 Myers et al., 1981
II	*COL2A1*	Fibrils in cartilage	Miller and Matukas, 1969
III	*COL3A1*	Forms heterotypic fibrils with type I collagen	Cameron et al., 2002
IV	*COL4A1* *COL4A2* *COL4A3* *COL4A4* *COL4A5* *COL4A6*	Network in basement membrane	Timpl and Brown, 1996; Timpl et al., 1981
V	*COL5A1* *COL5A2* *COL5A3*	Forms heterotypic fibrils with type I	Birk, 2001
VI	*COL6A1* *COL6A2* *COL6A3*	Fine microfibrils with ubiquitous distribution (distinct from fibrillin-containing microfibrils	Kielty et al., 1992
VII	*COL7A1*	Forms anchoring fibrils in skin at the dermal/epidermal junction (basement membrane)	Keene et al., 1987
VIII	*COL8A1* *COL8A2*	3D hexagonal lattice in Descemet's membrane in the eye	Kapoor et al., 1986; Kapoor et al., 1988; Stephan et al., 2004
IX	*COL9A1* *COL9A2*	Associated with type II collagen fibrils	Olsen, 1997; Shimokomaki et al., 1990
X	*COL10A1*	Mat-like structure/hexagonal lattice in the hypertrophic zone of the growth plate	Kwan et al., 1991
XI	*COL11A1* *COL11A2* *COL2A1*	Forms heterotypic fibrils with type II	Mendler et al., 1989
XII	*COL12A1*	Associated with type I fibrils	Keene et al., 1991; Nishiyama et al., 1994; Zhang et al., 2003
XIII	*COL13A1*	Transmembrane and possibly involved in cell adhesion	Latvanlehto et al., 2003
XIV	*COL14A1*	Associated with type I fibrils	Young et al., 2000b; Young et al., 2002
XV	*COL15A1*	Specialized basement membranes, cleaved to produce antiangiogenic fragment (restin)	Myers et al., 1996; Ramchandran et al., 1999
XVI	*COL16A1*	Component of specialized fibrillin-rich microfibrils in skin and type II collagen fibrils in cartilage	Kassner et al., 2003
XVII	*COL17A1*	Transmembrane component of hemidesmosomes (cell-cell junctions), which attach epidermis to basement membrane in skin	Hopkinson et al., 1998
XVIII	*COL18A1*	Cleaved to produce antiangiogenic fragment (endostatin)	Sasaki et al., 1998
XIX	*COL19A1*	Radially distributed aggregates formed by association at one end in vitro	Myers et al., 2003
XX	*COL20A1*	May be associated with type I collagen fibrils	Koch et al., 2001
XXI	*COL21A1*	May be fibril associated, widespread expression pattern	Fitzgerald and Bateman, 2001
XXII	*COL22A1*	Located in specific tissue junctions and may be associated with microfibrils	Koch et al., 2004
XXIII	*COL23A1*	Transmembrane collagen identified in cell culture	Banyard et al., 2003
XXIV	*COL24A1*	Expressed in tissues containing type I collagen	Koch et al., 2003
XXV	*COL25A1*	Transmembrane collagen, cleaved form present in Alzheimer's amyloid plaques in neurons	Hashimoto et al., 2002
XXVI	*COL26A1*	Expressed in testis and ovary of adult tissues	Sato et al., 2002
XXVII	*COL27A1*	Widespread expression particularly in cartilage	Boot-Handford et al., 2003; Pace et al., 2003

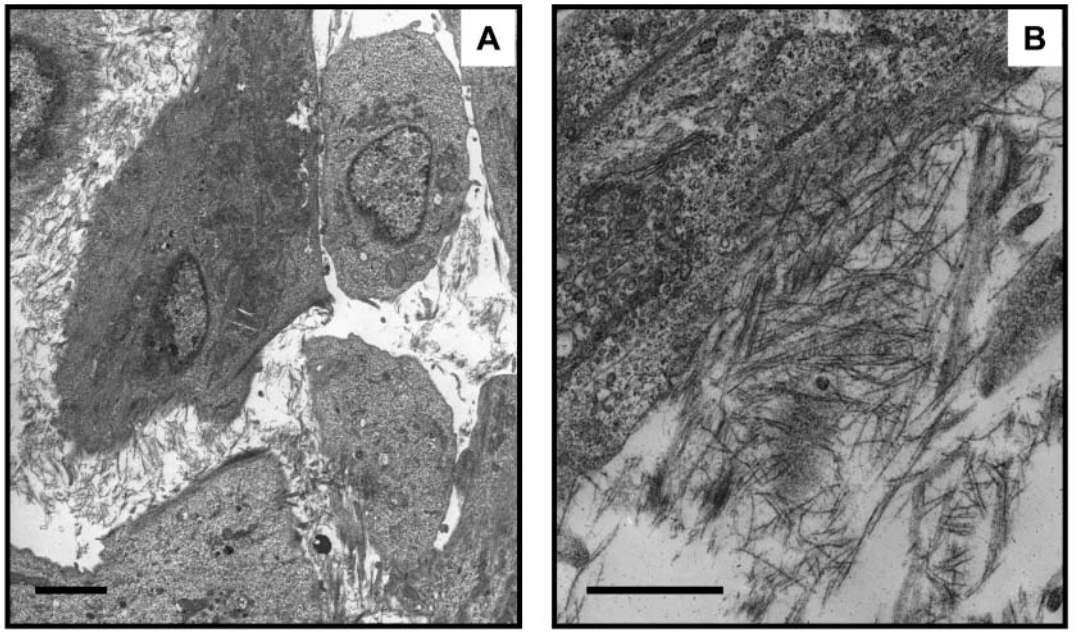

Fig. 1. Transmission electron microscopy of cultured embryonic tendon fibroblasts shows randomly orientated extracellular collagen fibrils. A: bar, 2 μm; B: bar, 1 μm.

triple helix does require further modifications within the polypeptide chain: peptidylproline cis-trans isomerase (PPI) activity (Galat, 1995) is required to convert the proline residues to the trans form (Bachinger, 1987; Davis et al., 1989) and prolyl-4-hydroxylase (P4H) is required to convert proline residues to hydroxyproline residues (Kivirikko and Myllyharju, 1998; Myllyharju, 2003). PDI itself makes up the β subunit of the P4H $\alpha_2\beta_2$ tetramer (Koivu et al., 1987; Pihlajaniemi et al., 1987) and functions to prevent aggregation of the catalytically active α subunits (John et al., 1993).

If hydroxylation is prevented, unfolded procollagen remains bound to P4H and is retained within the ER (Juva et al., 1966; Kao et al., 1979; Walmsley et al., 1999). Hydroxylation stabilizes the structure of the collagen triple helix and increases its denaturation temperature (Berg and Prockop, 1973; Jimenez et al., 1973; Rosenbloom et al., 1973). There is also a positive correlation between the melting temperature of the triple helix and the extent of hydroxylation of proline residues (Burjanadze, 1979; Burjanadze, 2000; Burjanadze and Veis, 1997), and with the physiological temperature of an organism (Privalov, 1982). Interestingly, a recent report indicates that the melting temperature of collagen might lie slightly below, rather than above, body temperature and be further modulated in vivo by interactions with surrounding proteins (Leikina et al., 2002; Persikov and Brodsky, 2002).

Hydroxyproline coordinates an extensive network of water molecules within the triple helix of collagen such that water bridges occur within and between the collagen chains (Bella et al., 1995; Bella et al., 1994; Privalov, 1982). The presence of hydroxyproline in the Y position also appears to favour a specific conformation of the imino acid necessary for the packing of the collagen triple helix (Berisio et al., 2004; Vitagliano et al., 2001). It may even favour the trans conformation of the hydroxyprolyl peptide bond (Holmgren et al., 1998). The importance of hydroxylation is exemplified by the consequences of long-term dietary deficiency in vitamin C. This results in scurvy, which is a disease characterized by inadequate connective tissue renewal. Ascorbic acid (vitamin C) is an essential cofactor for P4H; in the absence of P4H, new collagen fibrils cannot be synthesized, because most of the procollagen is unable to leave the ER. Interestingly, only humans, primates and guinea pigs are unable to synthesize ascorbic acid in the liver as they lack a functional gene for gulonolactone oxidase (Nishikimi et al., 1994; Nishikimi et al., 1992; Nishikimi et al., 1988; Nishikimi and Udenfriend, 1976; Ohta and Nishikimi, 1999).

Prolyl-3-hydroxylase is much less well characterized but has similarly been shown to act on unfolded collagen (Vranka et al., 2004). Lysine residues are also hydroxylated by a family of lysyl hydroxylases (Kellokumpu et al., 1994; Mercer et al., 2003; Passoja et al., 1998; Ruotsalainen et al., 1999; Valtavaara et al., 1998; Wang et al., 2002) and can be subsequently further modified by specific enzymes (galactosyl transferase and galactosylhydroxylysyl-glucosyl transferase) that add galactose and glucose moieties to hydroxylysine residues in the ER (Harwood et al., 1974; Harwood et al., 1975). Hydroxylysyl residues are also further modified to form extracellular collagen crosslinks (see below).

The collagen-specific chaperone HSP47 (Nagata, 1998; Nagata, 2003) is also required for the folding of procollagen but its function is unknown. Interestingly, HSP47 binds to both folded and unfolded procollagen chains (Satoh et al., 1996; Sauk et al., 1994). The importance of HSP47 in collagen maturation is demonstrated by the embryonic lethality associated with mice lacking a functional *HSP47* gene (Nagai et al., 2000). HSP47 could have both chaperone and anti-chaperone properties in that it could both facilitate and control the level of aggregation of procollagen at different stages of biosynthesis (Smith et al., 1995; Tasab et al., 2002). HSP47

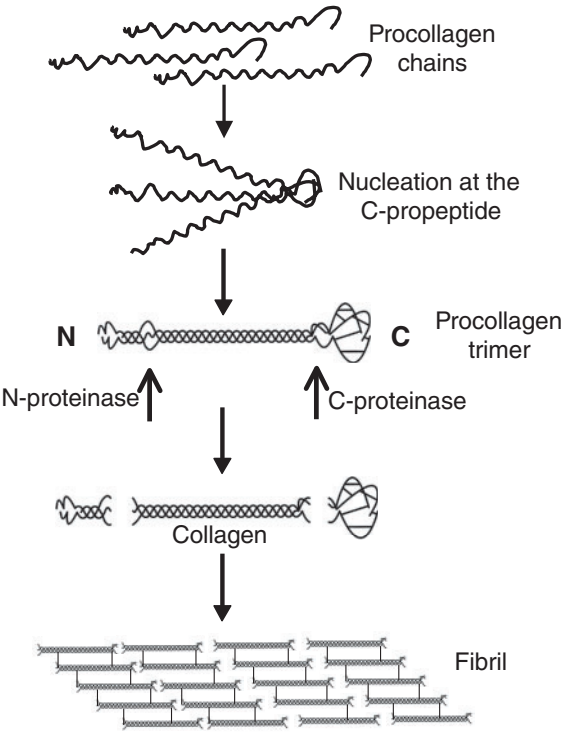

Fig. 2. Overview of the steps involved in the production of collagen fibrils by fibroblasts. Procollagen chains are synthesized in the endoplasmic reticulum (ER), are brought together by interactions between the C-propeptides and fold to form a rod-like triple-helical domain flanked by globular N- and C-propeptides. The large number of post-translational modifications that occur in the ER are not depicted. Removal of the N- and C-propeptides from fully folded procollagen only occurs after transport of procollagen across the Golgi stacks and results in collagen molecules that are then able to assemble into fibrils. Covalent crosslinks occur within and between triple-helical collagen molecules in fibrils.

travels from the ER to the cis-Golgi with procollagen, at which point it dissociates and is recycled (Satoh et al., 1996). It is possible that concentration of procollagen at ER exit sites is accomplished by the anti-chaperone activity of HSP47 and other ER-resident proteins (Smith et al., 1995).

Collagen trafficking

The mechanism by which molecules destined for secretion from the cell are transported from their site of synthesis in the ER through the Golgi to the PM has long been of interest to cell biologists. Vesicles are abundant in the vicinity of the Golgi complex, and a wealth of evidence favours a model in which cargo is transported through the secretory pathway using spherical transport vesicles, the molecular details of which are well described (Bonifacino and Glick, 2004). Incorporation of cargo into vesicles and budding of vesicles from the donor membrane is thought to be mediated by two transient protein complexes – COPI and COPII – which can coat the outer surface of vesicles. Targeting of vesicles to acceptor membranes, after vesicle uncoating, is mediated by specific proteins known as v- and t-SNARES. However, these vesicles are typically only 60-80 nm in diameter whereas the triple-helical domains of the fibril-forming collagens are about 300 nm long. Furthermore, procollagen molecules have a tendency to aggregate (Hulmes et al., 1983). Nevertheless, procollagen is effectively and efficiently secreted by fibroblasts and other cell types in vivo. Studies on the mechanism of its secretion have fuelled a controversy as to whether the vesicular transport model is generally applicable to protein secretion or a new model needs to be invoked for the transport of some or all secreted proteins (Glick and Malhotra, 1998; Pelham, 2001; Stephens and Pepperkok, 2001).

ER-to-Golgi transport

Proteins cannot exit the ER until they have achieved their correctly folded conformation (Hammond and Helenius, 1995). Their transport from the ER to the Golgi requires the sequential action of COPII and COPI; COPII is involved in the export of cargo from the ER, whereas COPI might mediate the retrograde transport of ER-resident proteins and the later stages of ER-to-Golgi transport (Duden, 2003; Murshid and Presley, 2004; Scales et al., 1997). The use of green fluorescent protein (GFP)-tagged vesicular stomatitis virus G-protein (VSVG), which is much smaller than procollagen, to study ER-to-Golgi transport by light microscopy has shown that ER-to-Golgi carriers are larger than individual coated vesicles, are pleiomorphic in shape and move along microtubules towards the Golgi (Presley et al., 1997; Scales et al., 1997). Furthermore, freeze-fracture replication of semi-intact cells has shown that ER export complexes are composed of arrays of budding vesicles and cytoplasmic vesicular tubular clusters (VTCs) (Bannykh et al., 1996).

Procollagen molecules en route from the ER to the cis-Golgi are found in tubular-saccular VTCs surrounded by small vesicles (Bonfanti et al., 1998) and transport of GFP-tagged procollagen requires the action of both COPI and COPII (Stephens and Pepperkok, 2002). Detailed investigations using correlative light or video-electron microscopy, serial-section 3D reconstruction and electron tomography have shown that both VSVG and procollagen are transported in saccular structures formed directly from protruding portions of the ER membrane (Mironov et al., 2003). In this study, the morphologies of four different types of carrier at different stages of maturity were characterized. COPII was found to be closely associated with VSVG but not with procollagen during ER exit and, at later stages, carriers containing the two cargos were associated with COPI. COPII might generate and maintain ER export domains rather than function in the formation of spherical transport vesicles (Palmer and Stephens, 2004). Interestingly, procollagen and VSVG reside in different regions of the ER prior to export and, although not transported separately, segregate to different domains of the same carrier. This raises the possibility that proteins destined for incorporation into the ECM are segregated from one another at this early stage in secretion. In fact, in cartilage cells (chondrocytes), the distribution of aggrecan, a large secreted ECM proteoglycan that undergoes post-translational xylosylation in the early secretory pathway, has a distribution distinct from that of type II procollagen in the ER (Vertel et al., 1989). Such segregation could prevent premature interactions between matrix molecules and/or ensure protein-specific post-translational modifications.

Table 2. Location of enzymes involved in the modification of N-linked oligosaccharides within the Golgi stacks*

Cisternal location	Enzyme
Cis	α mannosidase I
Medial	N-acetylglucosaminyl transferase I
Medial	α mannosidase II
Medial	N-acetylglucosaminyl transferase II
Medial	Fucosyltransferase
Trans	Galactosyltransferase
Trans	Sialyltransferase

*The number of cisternae in the Golgi stacks is variable and each cisternal location might comprise 2-3 individual cisternae.

Traversing the Golgi apparatus

The Golgi apparatus consists of polarized stacks of cisternae that mediate the sequential post-translational modification of a wide range of secreted proteins. The processing of N-linked oligosaccharides in the Golgi is particularly well characterized, and the enzymes responsible differentially localize to specific Golgi cisternae (Table 2) (Kornfeld and Kornfeld, 1985). Numerous COPI-coated vesicles are associated with the Golgi stacks, and the COPI coat is essential for both the structure and function of the Golgi apparatus (Guo et al., 1994). However, studies of the secretion of procollagen and other proteins have challenged the view that COPI-coated vesicles are involved in the anterograde (forward) trafficking of secreted proteins.

Transport of procollagen through the secretory pathway can result in distensions in the cisternae of the Golgi apparatus and it has been suggested that the morphology of these distensions and the supramolecular organization of the procollagen aggregates change during transport (Leblond, 1989; Marchi and Leblond, 1984; Trelstad and Hayashi, 1979; Weinstock and Leblond, 1974). Procollagen moves across the Golgi stacks without ever leaving the lumen of the Golgi cisternea (Bonfanti et al., 1998). This supports a model for intra-Golgi transport in which cisternae mature in the cis-to-trans direction and there is a retrograde transport of Golgi-resident enzymes in COPI-coated vesicles but no requirement for the anterograde transport of cargo in vesicles. However, if this is the case, Golgi enzymes (such as α mannosidase I and sialyltransferase) should be enriched in COPI-coated vesicles between the relevant Golgi stacks. In fact, there are conflicting reports regarding this issue: Golgi enzymes are enriched in vesicles isolated by subcellular fractionation techniques (Lanoix et al., 1999; Love et al., 1998; Sonnichsen et al., 1996) whereas, in single sections from intact cells, enrichment may (Martinez-Menarguez et al., 2001) or may not (Cosson et al., 2002; Orci et al., 2000a) appear to occur. Recent 3D analysis using serial-sectioning and electron tomography has found no enrichment of Golgi enzymes in vesicles, and retrograde transport of Golgi enzymes was proposed to occur through cargo-dependent intercisternal tubular connections between cisternae (Kweon et al., 2004; Trucco et al., 2004).

Whether some or the majority of cargos are transported by small COPI-coated vesicles is also controversial, i.e. non-default cisternal-maturation-like pathways might operate only in circumstances in which the cargo protein to be transported is unable to fit into small vesicles. Subcellular fractionation and single sections through cultured cells have been used to demonstrate that anterograde cargo that can fit into COP1-coated vesicles might be transported by a vesicular mechanism (Malsam et al., 1999; Nickel et al., 1998; Orci et al., 1986; Orci et al., 2000b; Orci et al., 1997; Ostermann et al., 1993). However, other reports indicate that small cargo is not found in isolated vesicles (Lanoix et al., 1999; Love et al., 1998; Sonnichsen et al., 1996), and a large cargo protein has even been shown to be transported in megavesicles (Volchuk et al., 2000). Thus, a working model for cis-to-trans Golgi transport that is consistent with all the observations remains elusive at this stage. It is unclear what the essential function of COPI-coated vesicles is, if they are not required for the transport of cargo such as procollagen or the retrograde transport of Golgi enzymes. One possibility is that they play a role in the preservation of Golgi structure by opposing any net flux of membrane in the retrograde or anterograde direction as cargo passes through the stack.

Golgi-to-PM transport

Sorting of proteins to their final destination occurs as they exit the trans-Golgi network (TGN; Griffiths and Simons, 1986; Keller and Simons, 1997; Orci et al., 1987), resulting in numerous diverse routes from the TGN to the PM within individual cells (Ponnambalam and Baldwin, 2003). Insights into the morphology of Golgi to PM transport carriers have been provided by confocal and internal reflection microscopy using fluorescently tagged proteins (Hirschberg et al., 1998; Schmoranzer et al., 2000; Toomre et al., 1999; Toomre et al., 2000). These studies have indicated that carriers are heterogeneous in size and morphology, with either a tubular or vesicular appearance, and that their transport is microtubule dependent.

Electron microscopy (EM) of fibroblasts in situ has indicated that bundles of procollagen are released from the trans face of the Golgi apparatus to form secretory vacuoles (Leblond, 1989; Marchi and Leblond, 1984; Trelstad, 1971; Trelstad and Coulombre, 1971; Trelstad and Hayashi, 1979; Weinstock and Leblond, 1974). These vacuoles, which are seen as electron-dense containers around 500 nm in length by transmission electron microscopy, appear to be associated with microtubules and are found in a variety of connective tissues. The transport of labelled procollagen through these structures has also been verified by EM autoradiography (Marchi and Leblond, 1984; Weinstock and Leblond, 1974).

More recently, fluorescent microscopy has been combined with electron microscopy in studies of the ultrastructural morphology of post-Golgi carriers in cultured cells (Polishchuk et al., 2000; Polishchuk et al., 2003). Post-Golgi carriers containing VSVG protein are generally tubular-saccular but are heterogeneous in shape. These carriers are 300-1700 nm long and appear to be formed by the detachment of large regions of the TGN. Procollagen is transported in the same type of carrier as VSVG, and detachment of procollagen-containing Golgi-to-PM carriers (GPCs) from the TGN has been visualized in human fibroblasts by antibodies directed against the C-propeptide of type I collagen.

Extrusion of collagen fibrils

The PM of fibroblasts in situ during the early stages of connective tissue deposition is highly convoluted. Cross-

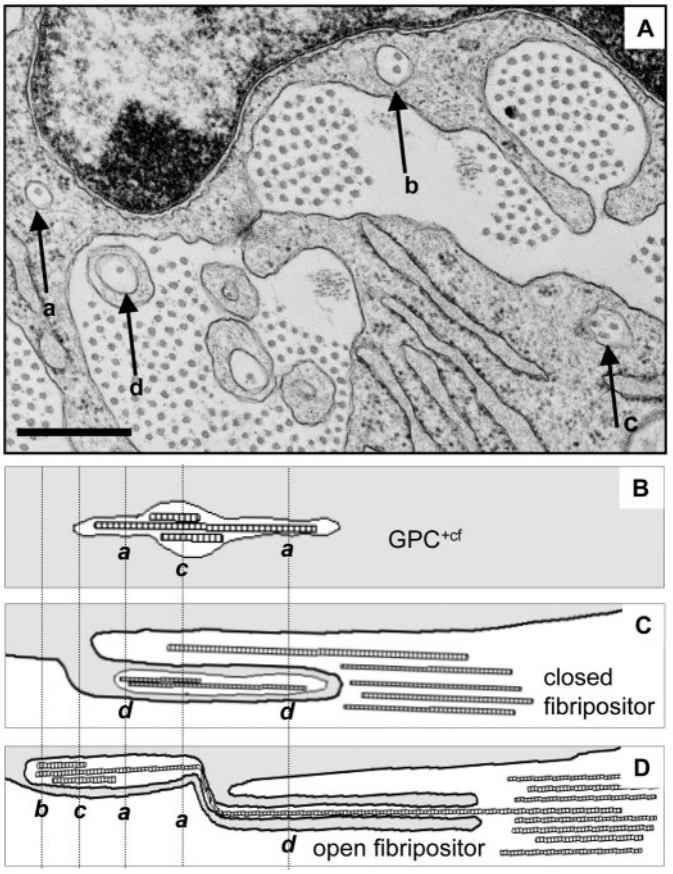

Fig. 3. The fibripositor secretory pathway. (A) Transmission electron microscopy of transverse sections through embryonic mouse tail tendon shows bundles of extracellular collagen fibrils between adjacent cells. One (a), two (b), three (c) or more (not shown) membrane-bounded collagen fibrils are also frequently observed within the cytoplasm. Membrane-bounded collagen fibrils are also observed in plasma membrane (PM) extensions called fibripositors (d). Bar, 500 nm. (B-D) Schematics showing longitudinal representations of collagen fibrils in (B) a Golgi-to-PM transport compartment (GPC^{+cf}) and within both (C) closed and (D) open fibripositors. The topology of membrane-bounded collagen fibrils observed in cross-section and the PM was determined by serial-section 3D reconstruction. Selected potential planes of section are represented by dotted lines. (a-c) Cross-section through one (a), two (b) and three (c) fibrils located within the cytoplasm; (d) cross-section through a single fibril located within a fibripositor.

sections through tendon, cornea and skin appear to show collagen fibrils embedded in the cytoplasm surrounded by cell membrane. Observation of numerous transverse and longitudinal sections reveals that collagen fibrils are present within narrow cellular recesses and that collagen is secreted through these structures (Birk and Trelstad, 1984; Birk and Trelstad, 1986; Ploetz et al., 1991; Trelstad and Hayashi, 1979; Yang and Birk, 1986).

On the basis of these observations, a model for connective tissue growth was proposed in which single collagen fibrils are produced in cellular recesses, they are brought together to form collagen fibril bundles and the bundles are assembled into higher-order structures such as tendon fascicles or corneal lamella. Subsequent advances in computer-aided reconstruction have enabled the acquisition of 3D images of cellular structures (Bonfanti et al., 1998; Hessler et al., 1992; Kremer et al., 1996; Ladinsky et al., 1999; McDonald et al., 1992; O'Toole et al., 2003). 3D reconstruction from serial-sections of embryonic tendon has revealed important features relevant to collagen assembly that are difficult to identify or impossible to verify in single sections (Canty et al., 2004). Notably, short collagen fibrils embedded in the cytoplasm can be completely enclosed within the cell by intracellular membranes. These may be the same structures identified earlier as secretory vacuoles; although, because they contain cross-banded collagen fibrils, they are probably more mature than vacuoles or represent carriers on an independent route of procollagen secretion from the cell. In addition, long collagen fibrils can be traced from locations deep within the cell, where they may coexist with numerous shorter fibrils, through a distinctive fibripositor (fibril-depositor) structure. This is located at the side of the cell, aligns along the long axis of the tendon and protrudes into the spaces between cells to extracellular collagen fibril bundles (Fig. 3). In a revision of the original model for collagen fibril assembly at early stages of development, collagen fibrillogenesis is initiated in GPCs. These carriers may then push out of the cell and eventually fuse with the PM to form a new fibripositor, or fuse with the base of existing fibripositors. Collagen fibril growth may then occur at the base of the fibril in the fibripositor by the addition of individual collagen molecules to the ends of the fibrils (Holmes et al., 1992; Holmes et al., 1998) or by end-to-end fusion (Graham et al., 2000; Kadler et al., 2000) with nascent short fibrils.

Procollagen processing

A key step in collagen fibril formation is the removal of the globular N- and C- propeptides from procollagen by the procollagen N- and C-proteinases (Fig. 2) (Leung et al., 1979). Of particular importance is the removal of the C-propeptides, which markedly decreases the critical concentration required for fibril assembly and thereby triggers the self-assembly of collagen into fibrils (Hulmes et al., 1989; Kadler et al., 1987). This process can be reconstituted in vitro and results in the spontaneous aggregation of triple-helical collagen molecules into fibrils (Kadler et al., 1987; Miyahara et al., 1982; Miyahara et al., 1984). C-proteinase activity (Hojima et al., 1985) is possessed by members of the tolloid family of zinc metalloproteinases [bone morphogenetic protein 1 (BMP-1), mammalian tolloid (mTLD) and tolloid like 1 (TLL-1); (Hartigan et al., 2003; Kessler et al., 1996; Li et al., 1996; Scott et al., 1999)] and N-proteinase activity is provided by members of the ADAMTS (for 'a disintegrin and metalloproteinase with thrombospondin motifs') family: ADAMTS-2, ADAMTS-3 and ADAMTS-14 (Colige et al., 1997; Colige et al., 2002; Fernandes et al., 2001). Type V collagen is unusual because it undergoes chain-specific processing in which the N-propeptides can be removed by tolloid family members and the C-propeptides can be removed by either BMP-1-like enzymes or furin-like proprotein convertases (Gopalakrishnan et al., 2004; Unsold et al., 2002). The ubiquitous furin-like proprotein convertases, which are responsible for the maturation of a wide variety of substrates (Nakayama, 1997), have also been implicated in the conversion of latent BMP-1 and ADAMTS2 to their enzymatically active forms (Fig. 4) (Leighton and

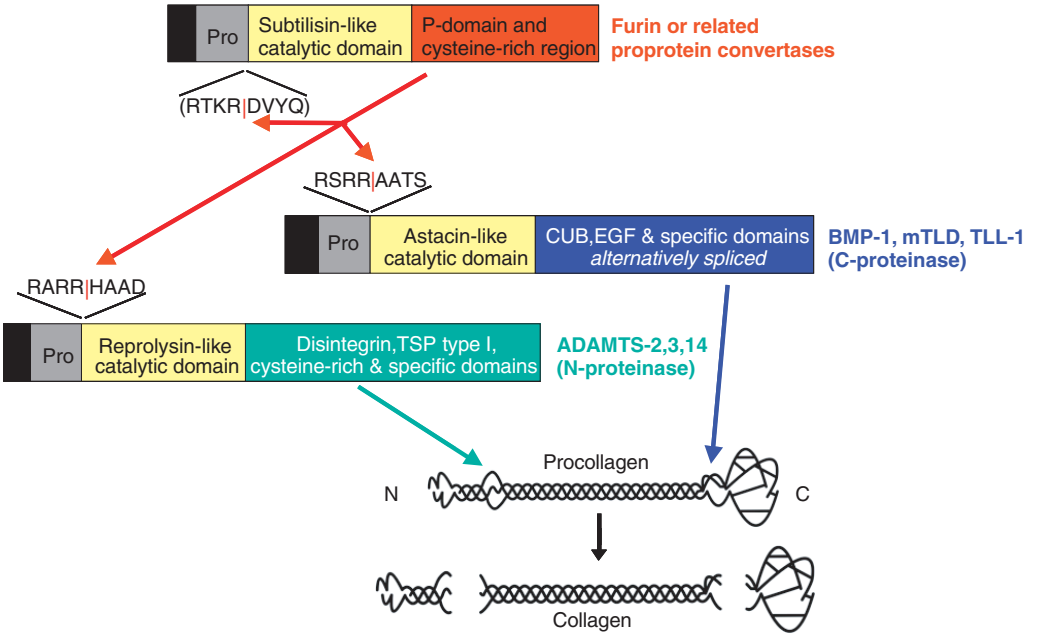

Fig. 4. Schematic of the activation pathway for the procollagen N- and C-proteinases. This enzyme cascade is initiated by autocatalytic activation of furin or furin-like proprotein convertases in the trans-Golgi network and is responsible for the processing of procollagen to collagen by ADAMTS (N-proteinase) and tolloid (C-proteinase) family enzymes. Amino acid sequences cleaved by furin are shown between the pro- and catalytic domains of the enzymes. The signal sequence for secretory targeting is represented as a black rectangle. Abbreviations: ADAMTS, a disintegrin and metalloproteinase with thrombospondin motifs; BMP-1, bone morphogenetic protein 1; CUB domains, so-called owing to their occurrence in complement components, a sea urchin protein and BMP-1; EGF, epidermal growth factor; mTLD, mammalian tolloid; P-domain, region required for processing activity; Pro, prodomain, removal of which is required for enzyme activation; TLL-1, tolloid like 1; TSP, thrombospondin.

Kadler, 2003; Wang et al., 2003). Activation of pro-BMP-1 has been shown to occur in the TGN (Leighton and Kadler, 2003).

Studies on cells in culture have shown that aligned aggregates of procollagen, known as segment long spacing (SLS) crystallites, which can also be seen in sections through fibroblasts in situ, are secreted from cells and are present in the cell culture medium (Bruns et al., 1979; Goldberg, 1974; Hulmes et al., 1983). In addition, enzymatic activity capable of converting procollagen to collagen is found in the medium of cultured cells (Kerwar et al., 1973; Layman and Ross, 1973). Furthermore, comparison of SDS extracts of tendon and isolated cells that have been subjected to pulse-chase analysis has shown that procollagen processing in tendon (which retains the collagenous ECM), occurs much more efficiently than in cell culture (Jimenez et al., 1971). Thus, processing of procollagen to collagen in tissue has previously appeared to be an extracellular event, occurring within infoldings of the PM. By studying the developmental changes in procollagen processing in chick embryo cornea, Mellor et al. used a high-salt buffer to extract processing intermediates from the tissue and noted that preferential extraction of extracellular proteins may have occurred (Mellor et al., 1991). A differential extraction procedure for extracellular and intracellular proteins based on these methods has revealed that processing of procollagen can begin within the confines of the PM in chick embryo tendon (Canty et al., 2004). This is consistent with the observation of short intracellular collagen fibrils in GPCs. However, further work is required to determine whether cleavage of procollagen to collagen in a transport container is sufficient to initiate fibrillogenesis and whether intracellular processing is a specific feature of tendon development.

The role of cell-surface proteins in collagen fibrillogenesis

Collagen fibril assembly can be reconstituted in vitro by neutralizing and warming solutions of collagen obtained by tissue extraction with acetic acid (Kadler et al., 1996; Trelstad and Hayashi, 1979; and references therein) and by cleavage of procollagen to collagen (Miyahara et al., 1982). Procollagen secreted by fibroblasts in culture is processed to collagen (Goldberg and Sherr, 1973) and collagen fibrils are found associated with the cell layer (Goldberg and Green, 1964). However, collagen fibril formation in cell culture is dependent on the assembly of fibronectin into fibrils. Fibronectin is a large extracellular protein that plays a crucial role in embryogenesis (George et al., 1993) and is composed of multiple tandem domains, which are responsible for interaction with cell-surface and matrix molecules (Wierzbicka-Patynowski and Schwarzbauer, 2003). An antibody directed against a 60 kDa collagen-binding fragment of fibronectin inhibits assembly of both fibronectin and collagen produced in cell culture (McDonald et al., 1982). Furthermore, assembly of collagen produced from fibronectin-null fibroblasts requires the addition of exogenous fibronectin to the cell culture medium, and transfection with collagen-binding integrins potentiates the effect of fibronectin addition (Velling et al., 2002). Assembly of fluorescently labelled exogenous collagen into fibrils has

also been described in cultures containing smooth muscle cells (Li et al., 2003). Collagen fibrillogenesis occurs on the cell surface, and assembly of both collagen and fibronectin is prevented by an antibody against the fibronectin-binding $\alpha_5\beta_1$ integrin. Inhibition of the collagen-binding $\alpha_2\beta_1$ integrin affects collagen but not fibronectin assembly. These results point to the possibility that, in cell culture at least, collagen fibrillogenesis is downstream of fibronectin assembly and is mediated by interactions with cell-surface integrins. Rho-mediated cytoskeletal changes, which can be induced by the serum component lysophosphatidic acid (LPA), have also been shown to induce fibronectin assembly by exposing a cryptic site in fibronectin (Zhong et al., 1998). Collagen fibril assembly is also stimulated by LPA (Li et al., 2003), presumably through upstream effects on fibronectin assembly.

In cultured fibroblasts, some procollagen processing could also occur in solution, resulting in solvated collagen molecules that associate together to form fibrils. However, because there appears to be a distinct reduction in the amount of fibrillar collagen (at the light microscopy level) when fibronectin assembly is inhibited, most, if not all, of the collagen assembly in cultured cells is probably mediated by cell-surface molecules. The role of fibronectin in collagen fibril assembly in vivo has yet to be investigated. However, processing of procollagen to collagen is probably not sufficient for the formation of collagen fibrils, and interactions of collagen molecules with fibronectin and specific integrins either at the cell surface or within post-Golgi carriers might also be required. Inducible tissue-specific knockouts of these molecules in mice could help to determine their role in collagen matrix assembly.

Extracellular collagen fibril growth

The initial stages of ECM deposition result in arrays of narrow fibrils of uniform diameter. In cornea, these fibrils are prevented from increasing further in diameter (Birk et al., 1990; Holmes and Kadler, 2005) but in other tissues their diameter can increase by ~tenfold. Collagen fibrils also increase dramatically in length as development proceeds. The isolation of intact fibrils from tissue is only possible at the earliest stages of development (Birk et al., 1995) and the length of collagen fibrils in mature tissues has been estimated on the basis of the number of fibril ends observed in cross-section (Craig et al., 1989). Collagen fibril growth is thought to occur by accretion as well as by lateral and end-to-end fusion of collagen fibrils in the ECM (Birk et al., 1995; Graham et al., 2000). The extracellular collagenous matrix is strengthened not just by an increase in the size of the collagen fibrils but also by the formation of intra- and inter-molecular crosslinks within fibrils as a result of the action of lysyl oxidase (Eyre et al., 1984) (Fig. 2). Lysyl oxidase catalyses the crosslinking reaction by activating lysine and hydroxylysine residues and, to date, five different lysyl oxidase enzymes have been identified (Maki et al., 2001).

Trafficking and assembly of fibril-associated molecules

Although collagen fibrils are the major component of many connective tissues, other molecules can bind to collagen and modulate fibril diameter. Small leucine-rich repeat proteoglycans (SLRPs) such as decorin, fibromodulin and lumican affect collagen fibrillogenesis in vitro (Hedbom and Heinegard, 1989; Oldberg et al., 1989; Rada et al., 1993; Vogel et al., 1984); they are located at the surface of collagen fibrils (Hedlund et al., 1994; Pringle and Dodd, 1990; Svensson et al., 2000) and mice lacking these molecules have abnormal collagen fibril diameters (reviewed by Ameye and Young, 2002). Decorin binds to procollagen (Keene et al., 2000) and the distribution of surface-bound proteoglycans at the ends of isolated collagen fibrils indicates that it might bind to collagen prior to fibril assembly (Graham et al., 2000). However, at what point, within the secretory pathway or the ECM, SLRPs interact with collagen is unknown.

The FACIT (for 'fibril-associated collagen with interrupted triple helices') family of collagens, of which types IX, XII and XIV are the best characterized, consist of multiple collagenous domains separated by non-collagenous regions. Type IX collagen is associated with the surface of type II collagen fibrils and interacts with cartilage oligomeric matrix protein, matrillin-3 and heparin (Briggs and Chapman, 2002; Holden et al., 2001; Pihlajamaa et al., 2004; Thur et al., 2001). Similarly, type XII and type XIV collagens are associated with the surface of type I collagen fibrils (Keene et al., 1991; Young et al., 2000a). Type XII collagen interacts with decorin and fibromodulin (Font et al., 1996), whereas type XIV collagen interacts with decorin, perlecan and procollagen N-proteinase (Brown et al., 1993; Colige et al., 1995; Font et al., 1993; Giry-Lozinguez et al., 1998). The presence of type XII and XIV collagen at the fibril surface appears to decrease interactions between fibrils, enabling them to slide past each other (Nishiyama et al., 1994). Together, these finding indicate that FACIT collagens might play a role in tissue integrity and plasticity. Whether these molecules are co-trafficked with fibrillar collagen has yet to be investigated.

Conclusions and future goals

Research during the past few years has brought the focus of collagen research back to the cell and, in particular, the membrane trafficking events in the late secretory pathway that appear to exercise exquisite control over collagen fibril self-assembly. It is becoming increasingly clear that the late secretory pathway and the PM are vital components of the toolkit for tissue assembly within cells. Future work will be directed at understanding the segregation and co-trafficking of fibrillar collagens, collagen-associated molecules (including FACIT collagens, some integrins and some proteoglycans), fibrillins and other ECM macromolecules, as well as the procollagen-processing enzymes. Cells in culture do not always faithfully repeat the synthesis of ECM macromolecules that is observed in situ. Therefore, future studies should include, where possible, cells in tissue.

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