Type XXVI Collagen, a New Member of the Collagen Family, Is Specifically Expressed in the Testis and Ovary*

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Keiji Sato‡, Kentaro Yomogida§, Takayuki Wada‡, Tetuya Yorihuzi‡, Yoshitake Nishimune§, Nobuko Hosokawa‡, and Kazuhiro Nagata‡¶

From the ‡Department of Molecular and Cellular Biology, Institute for Frontier Medical Sciences, Kyoto University, Sakyo-ku, Kyoto, 606-8397 and the §Department of Science for Laboratory Animal Experimentation, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamada-oka, Suita City, Osaka 565, Japan

HSP47 is a collagen-specific molecular chaperone that specifically recognizes and binds to the triple helical domain of various types of collagens. Here we report the cloning of the entire coding region of a novel collagen-like protein by yeast two-hybrid screening of a 17.5-day whole mouse embryo cDNA library using HSP47 as a bait. The cDNA of this protein and its deduced amino acid sequence are 2,690 bp and 438 amino acids long, respectively. The protein contains two clusters of Gly-X-Y collagenous repeats and three noncollagenous domains. Northern blot analysis showed that its mRNA is specifically expressed in the testis and ovary in adult tissues and that expression in these tissues is highest in the neonate. Biochemical characterization of this protein revealed that its proline residues are hydroxylated, it undergoes N-linked glycosylation, it forms trimers, and it is secreted in vitro. Immunohistochemical studies showed that the myoid cells and the pre-theca cells synthesized it in the testis and ovary, respectively, resulting in the accumulation of this protein in the extracellular spaces of these organs. These observations suggest that this protein is a new member of the collagen protein family. We thus designated this protein as type XXVI collagen.

Collagen is the most abundant protein in vertebrates. It is a major component of the extracellular matrix, which consists of specialized fibrils and networks around cells and in the interstitial spaces between the cells. Twenty-five types of collagen have now been identified (1–5). During the development of mammals, these collagens are expressed in various spatiotemporal patterns. The fibril-forming collagens are the most abundant of the collagens and include types I, II, and III (6). Other collagens known as the FACIT collagens (Fibril-associated Collagens and Osteopontin) form interrupted triple helices, which include types IX, XII, XIV, XVI, and XIX, associate with the surface of the collagen fibrils and modify their interactive properties (7–9). In addition, nonfibrillar collagens, which include types XIII, XVII, and XXV, are reported to have transmembrane domains and appear to localize at the cell surface (5, 10, 11).

Despite the differences among the collagens, all share in common a triple helical structure composed of three polypeptides consisting of Gly-X-Y repeats, where X is any amino acid, and Y is frequently proline or hydroxyproline. Each chain is a left-handed helix, and the three chains wind around each other in a right-handed superhelix (1, 6).

The procollagens are synthesized in and cotranslationally transported into the endoplasmic reticulum (ER). After translation is completed, the procollagens form a trimer at the C terminus. Only properly folded triple helical forms of procollagens are secreted by the cells. During collagen biosynthesis, the nascent procollagen chain is modified by unique enzymes that include procollagen prolyl-4 hydroxylase, which stabilize the triple helical structure. After being secreted by the cell, the N- and C-propeptides are cleaved by specific proteases, thereafter forming collagen bundles or basement membranes (1, 6, 9, 12–15).

HSP47 is an ER resident stress protein that functions as a collagen-specific molecular chaperone. This protein associates with procollagen during its folding and/or post-translational modification in the ER. Recent studies have revealed that HSP47 plays a critical role in collagen biosynthesis (16). Type I collagen secreted by cells from hsp47-null mice is abnormal in that its N- and C-propeptides are improperly cleaved, and the protease-sensitivity in its triple helix domain is altered (17). The tissues of hsp47-null mice lack collagen fibrils and basement membranes, and the mice died before E11.5. HSP47 has been reported to bind to various types of collagen (at least types I–V) in vitro (18–20). Furthermore, synthetic model peptides representing the collagenous triple helix domain and yeast two-hybrid screening revealed that HSP47 specifically binds to the helix-forming portions of procollagen (21–24). Thus, HSP47 appears to play a critical role in collagen biosynthesis.

To investigate the role of HSP47 further, we searched for proteins that interact with HSP47 by performing yeast two-hybrid screening using HSP47 as a bait. This experiment identified a gene encoding a novel collagen-like protein that contains two collagenous domains consisting of Gly-X-Y repeats and three noncollagenous domains. We demonstrate here that this protein is secreted into the extracellular matrix with modifications typically seen in other types of collagens, namely, N-linked glycosylation and hydroxylation of proline and/or lysine residues. This protein also forms a trimer and accumulates in the extracellular matrix of mouse tissues. These collagen-like proteins that interact with HSP47 by performing yeast two-hybrid screening using HSP47 as a bait. This experiment identified a gene encoding a novel collagen-like protein that contains two collagenous domains consisting of Gly-X-Y repeats and three noncollagenous domains. We demonstrate here that this protein is secreted into the extracellular matrix with modifications typically seen in other types of collagens, namely, N-linked glycosylation and hydroxylation of proline and/or lysine residues. This protein also forms a trimer and accumulates in the extracellular matrix of mouse tissues. These collagen-like
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features led us to designate this protein as type XXVI collagen. This protein is produced in the testis and ovary of adult mice but is also present at higher levels in the reproductive tissues of neonates, suggesting that it plays an important role in the development of the reproductive tissues.

EXPERIMENTAL PROCEDURES

General Reagents and Animals—Restriction enzymes and other DNA-modifying enzymes were purchased from Takara Shuzo Co. Ltd. (Tokyo, Japan). DNA fragments were synthesized by Hokkaido System Science, (Sapporo, Japan). Chemicals and reagents were purchased from either Nakalai Tesque (Kyoto, Japan) or Wako Pure Chemical Co. (Osaka, Japan). DNA sequencing was carried out on an ABI PRISM 377A sequencer (PerkinElmer Life Sciences, Forester City, CA). ICR mice were purchased from Japan SLC, Inc. (Shizuoka, Japan).

Yeast Two-hybrid Screening and Cloning of Type XXVI Collagen cDNA—The ER retention sequence of mouse hsp47 cDNA was deleted, and the remaining cDNA was used as bait in yeast two-hybrid screening. The cDNA was amplified by PCR and inserted into pAS2-1 (Clontech, Palo Alto, CA). Following the methods recommended by the manufacturer, a 1.8-kb cDNA fragment of type XXVI collagen was cloned from the cDNA library of 17.5-day whole mouse embryo (Clontech). A 2.7-kb type XXVI collagen cDNA was also cloned from a 15.5-day mouse embryo cDNA (Invitrogen) using a specific probe, based on the sequence data of the 1.8-kb cloned fragment. Marathon-ReadyTM cDNA from mouse testis (Clontech) was used as a template for 5'-RACE and resulted in the acquisition of 79 bp of upstream sequence. The program BLAST was used to predict the putative signal sequence (25). Northern Blot Analysis—Freshly removed organs of mice of various ages were homogenized in TRIZOL Reagent (Invitrogen). Total RNA was extracted with TRIZOL according to the manufacturer’s instructions. RNA samples were fractionated on an agarose gel, MOPS, EDTA, 1% agarose gel and then transferred to nylon membranes and cross-linked by baking at 80 °C for 2 h. The filters were subsequently incubated with several radiolabeled probes. These included a 696-bp fragment amplified by PCR from type XXVI collagen cDNA, a 1.5-kb EcoRI-HindIII fragment of hsp47 cDNA (26), and a 1.3-kb PstI fragment of chick GAPDH cDNA (27). Prehybridization and hybridization were performed using Perfect Hyb (Toyobo, Tokyo) according to the manufacturer’s instructions.

Plasmid Constructs and Production of Antibodies against the Type XXVI Collagen—Type XXVI collagen cDNA generated by PCR was cloned into the expression vector pCMV-Tag4 (Stratagene, La Jolla, CA) to construct type XXVI collagen tagged with FLAG at its C terminus. The same expression vector was also used to create the protein without the tag. Polyclonal antibodies were raised in rabbits against the cDNA clone encoding type XXVI collagen tagged with FLAG at its C terminus. The same expression vector was also used to create the protein without the tag. Polyclonal antibodies were raised in rabbits against the cDNA clone encoding type XXVI collagen tagged with FLAG at its C terminus.

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RESULTS

Isolation of cDNA Encoding Type XXVI Collagen—In search of proteins interacting with HSP47, a collagen-specific molecular chaperone, we performed yeast two-hybrid screening using as bait HSP47 lacking its ER retention sequence, RDEL. We obtained a clone containing a 1,825-bp fragment that encoded a protein with two collagenous domains. Because this clone was thought to contain only part of the full-length cDNA, further cloning was performed on a 15.5-day whole mouse embryo cDNA library encompassing 5 × 106 clones. The probe used was a digoxigenin-labeled oligonucleotide probe prepared from the sequence data of the previously cloned fragment. A clone containing the 2,690-bp full-length cDNA sequence was obtained (Fig. 1A). 5'-RACE was also performed using a specific probe prepared from the cDNA sequence data to obtain the 79-bp upstream region of the cDNA (Fig. 1A). The full-length cDNA clone encodes a 438-amino acid protein (Fig. 1A) that consists of two collagenous domains (COL1 and COL2) and three noncollagenous domains (NC1–NC3) (Fig. 1B). The protein also contains a putative ER-targeting signal sequence at its N terminus and two Asn-X-Ser/Thr sequences that are consensus motifs for Asn-linked glycosylation. The homology search using BLAST revealed that this protein is almost identical to the mouse emu2 protein and the putative human emu2 protein with the exception of 2 amino acids. Only the sequences of these two proteins have been reported previously, but studies to characterize them have, to our knowledge, not been performed. Apart from its two collagenous regions, this protein has no obvious sequenceology to any other collagen. However, as described below, this protein is also collagen-like in its secretion from the cells, the localization in the extracellular spaces, and the post-translational modifications it undergoes. We have thus designated this novel protein as type XXVI collagen.

The 13 cysteine residues of the protein are found only in the NC1 domain, including in the putative signal sequence (Fig.
A, shown by circles), which is a unique feature of this collagen-like protein relative to the other types of collagens. There is also a possible furin protease cleavage site (RRRR, Fig. 1A, broken line) just before the COL1 domain, which is commonly observed in the transmembrane collagens such as types XIII and XXV. These latter transmembrane collagens are cleaved just before the COL1 domain. However, we did not observe that type XXVI collagen undergoes this cleavage because the fragment that would result from this cleavage was not detected in the murine tissues that express type XXVI collagen (data not shown). Furthermore, cells transfected with cDNA encoding type XXVI collagen did not release the cleaved fragment into the medium (data not shown). Type XXVI collagen also does not have transmembrane domains.
The Type XXVI Collagen Gene Is Specifically Expressed in the Testis and Ovary in Adult Tissues—We next examined the regional expression of this novel collagen-like gene in adult murine tissues. Northern blotting revealed that type XXVI collagen gene mRNA is markedly expressed in the testis and ovary (Fig. 2A) and weakly in the kidney. We next examined its expression level during the postnatal development of the testis and ovary (Fig. 2B). In the testis, the type XXVI collagen gene is expressed at its highest levels 1 day after birth, after which the mRNA levels gradually decrease during germ cell development. In the ovary, the type XXVI collagen gene is highly expressed in the first 2 weeks after birth, after which expression also decreases. These expression patterns of the type XXVI collagen gene are very similar to that of hsp47 expression in both the testis and ovary (Fig. 2B). That the expression of hsp47 correlates spatiotemporally with the expression of various collagen genes has previously been reported by our group (16–19).

Type XXVI Collagen Protein Is Highly Expressed during Testis and Ovary Development—We raised two antibodies against type XXVI collagen in rabbits using GST-NC2 and GST-NC3 fusion proteins as antigens (see Fig. 1B). These antibodies were tested for specificity with an extract of cells transfected with an expression plasmid containing type XXVI collagen cDNA. Both antibodies recognized a single 60 kDa band (data not shown). Western blot analysis under reducing conditions showed that the anti-NC3 antiserum (Fig. 3) and the anti-NC2 antiserum (data not shown) recognize an ~60 kDa band in extracts of neonatal mouse testes and ovaries (Fig. 3). The levels of this 60 kDa band gradually decreased after birth as the testis and ovary matured (Fig. 3). These expression patterns are consistent with those revealed by Northern blot analysis. In adult mice (8 weeks old), low levels of type XXVI collagen protein were observed in both the testis and ovary, but no protein was detected in the somatic tissues such as the brain, heart, intestine, and kidney (data not shown). The antibodies also faintly recognized a 70-kDa protein in both the testis and ovary (shown by * in Fig. 3). This band is thought to be an unspecific signal rather than a modified form of type XXVI collagen because its expression pattern did not coincide with that of the mRNA in Northern blotting, nor did immunoprecipitation with anti-type XXVI collagen antibody precipitate a 70-kDa protein (data not shown).

Intracellular Localization of Type XXVI Collagen—COS-7 cells were transiently transfected with cDNA encoding type XXVI collagen tagged at its C terminus with the FLAG tag. The cells were then fixed, permeabilized, and double stained with mouse antibody specific for FLAG and rabbit anti-calnexin antibody. Visualization was achieved with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG for calnexin and rhodamine-conjugated goat anti-mouse IgG for type XXVI collagen. Images of the type XXVI collagen and calnexin stainings were obtained separately with the Axioplan 2 microscope and then overlapped.
ble staining with anti-FLAG monoclonal antibody and anticalnexin polyclonal antibody revealed that type XXVI collagen colocalizes with calnexin, an ER marker, indicating that type XXVI collagen is targeted to the ER (Fig. 4).

**Secretion of Type XXVI Collagen**—To determine whether type XXVI collagen is secreted, HEK-293 cells were transiently transfected with a plasmid encoding type XXVI collagen, and a pulse-chase experiment was performed in the presence of ascorbate, a cofactor of prolyl-4-hydroxylase, prolyl-3-hydroxylase, and lysyl hydroxylase (Fig. 5). Cell extract and the medium were immunoprecipitated with the NC3 antibody, and the precipitates were run on a SDS-polyacrylamide gel under reducing and nonreducing conditions. The transfected HEK-293 cells release type XXVI collagen that appears as a ~60-kDa protein under reducing conditions (Fig. 5A, lane 2). Under nonreducing conditions, two higher molecular size species of ~120 and ~200 kDa were observed in the medium (Fig. 5B, lane 2), suggesting that type XXVI collagen forms dimer and/or trimer structures like the other collagens. However, type XXVI collagen is secreted into the culture medium more slowly than other collagen molecules (Fig. 5A) (29). Notably, in the medium, the trimer form of type XXVI collagen predominates (Fig. 5B, lanes 7 and 8), whereas the type XXVI collagen in the cell extract is present as a mix of monomers, dimers, and trimers (Fig. 5B, lanes 2–4). The type XXVI collagen proteins secreted into the medium have a slightly higher molecular size than the proteins within the cells (Fig. 5A, compare lanes 2–4 and lanes 7 and 8), suggesting the protein is glycosylated just before it is secreted (see Figs. 6 and 7).

To assess whether collagen XXVI is subjected to prolyl and lysyl hydroxylations, the cells were labeled and treated with α,α’-dipyridyl, an iron-chelating agent that prevents prolyl and lysyl hydroxylations in the presence of ascorbate. The type XXVI collagen originating from α,α’-dipyridyl-treated cells migrated slightly faster under reducing conditions than the collagen from untreated cells (Fig. 6A, compare lanes 2 and 3). In the absence of ascorbate, treatment with α,α’-dipyridyl did not affect the migration of type XXVI collagen derived from the cell (Fig. 6A, lanes 5 and 6). Furthermore, in the absence of ascorbate, the secretion of type XXVI collagen was negligible (Fig. 6A, lane 4). Cells treated with α,α’-dipyridyl in the presence of ascorbate dramatically reduced their secretion of type XXVI collagen (Fig. 6A, lane 7). Thus, type XXVI collagen is hydroxylated in the ER like other collagens, and only hydroxylated type XXVI collagen is secreted into the medium.

When the cells were labeled in the presence of tunicamycin, an inhibitor of N-linked glycosylation, a 51-kDa form of type XXVI collagen was detected in the cell extract (Fig. 6A, lane 4). This suggests that the two putative N-linked glycosylation sites in type XXVI collagen are indeed both glycosylated. The secretion of type XXVI collagen was also prevented by the tunicamycin treatment (Fig. 6A, compare lanes 6 and 8).

As shown in Figs. 5 and 6, secreted type XXVI collagen migrates slower than the cell-derived protein under reducing conditions (Fig. 5A, lanes 3 and 7, and Fig. 6A, lanes 2 and 6). To assess why the migration rate differs, we treated the cells with PNGase F and endoglycosidase H. Both enzymes released the N-glycans from the cell-derived type XXVI collagen (Fig. 7A, lanes 7 and 5) but the N-glycans from the secreted type XXVI collagen were released by treatment with PNGase F only (Fig. 7A, lanes 9 and 5), not endoglycosidase H (Fig. 7A, lane 11). Thus, the N-linked glycans on type XXVI collagen are processed to the complex type when the protein is in the Golgi apparatus awaiting secretion.

**Localization of Type XXVI Collagen in Mouse Testis and Ovary**—To determine the localization of type XXVI collagen in the testis and ovary, we performed immunohistochemical analysis using the NC2 antibody. In the 5-day-old testis, only myoid cells were stained (Fig. 8D). Other somatic cells such as Leydig or Sertoli cells were not stained nor were germ cells such as spermatogonia. In the 7-day-old ovary, although the primary follicles were not stained, pre-theca cells surrounding primary follicles and the extracellular matrix region of medulla and cortex were recognized by the antibody (Fig. 8D). This observation confirms that type XXVI collagen is secreted in vivo. In both the testis and ovary, no signal was detected after staining...
with preimmune serum (Fig. 8, A and B). Thus, like other collagen molecules, type XXVI collagen accumulates in the extracellular matrix. This suggests that this novel collagen may be involved in the early development of testis and ovary as an extracellular matrix component.

**DISCUSSION**

We performed yeast two-hybrid screening using HSP47 as a bait to identify proteins that interact with HSP47. This resulted in the cloning and characterization of a novel protein. This protein has a signal sequence at its N terminus, two collagenous domains, and three noncollagenous domains. Collagen-like features of this protein are indicated by several observations: first, it possesses two clusters of Gly-X-Y repeats; second, it forms a trimer and is secreted into the culture medium; third, it is hydroxylated and N-linked glycosylated; and fourth, immunohistochemical analysis of the testis and ovary indicates its presence in the extracellular matrix region. These features indicate that this protein can be considered as a new member of collagen. Thus, we designated this protein as type XXVI collagen because 25 types of collagens have been reported to date (1–5). In adult mice, type XXVI collagen mRNA is found mainly in the testis and ovary and slightly in the kidney. During early testis and ovary development, type XXVI collagen is highly expressed in myoid cells and pre-theca cells, respectively.

That HSP47 is indispensable in collagen biosynthesis has been demonstrated by the analysis of hsp47−/− mice (17). The null mutation of hsp47 is lethal during embryonic development, and it has been shown that the embryos lack collagen fibrils and basement membranes in their tissues. This suggests that in these mice, the molecular maturation of collagen is

**FIG. 6.** Effects of the addition of ascorbate, α,α′-dipyridyl, and tunicamycin on the hydroxylation, glycosylation, and secretion of type XXVI collagen. HEK-293 cells were transiently transfected with cDNA encoding type XXVI collagen and then cultured for 24 h in the presence or absence of 136 μg/ml ascorbate phosphate. The cells were then preincubated with either 0.3 mM α,α′-dipyridyl for 30 min or with 5 μg/ml tunicamycin for 12 h, after which the cells were labeled by culturing them with 0.1 mCi/ml [35S]methionine in medium lacking methionine for 3 h. The cell extract and the medium were then immunoprecipitated with anti-type XXVI collagen NC3 antibody. Immunoprecipitated samples were resolved by 8% SDS-PAGE under reducing (A) or nonreducing (B) conditions.

**FIG. 7.** Characterization of N-linked glycosylation of secreted type XXVI collagen. HEK-293 cells transiently transfected with type XXVI collagen cDNA were cultured for 24 h in medium containing 136 μg/ml ascorbate phosphate. The cells were labeled by culturing them with 0.1 mCi/ml [35S]methionine in medium lacking methionine for 3 h. The cellular and medium fractions were immunoprecipitated with anti-type XXVI collagen antibody NC3. Each immunocomplex was then divided into equal aliquots and treated with either PNGase F or endoglycosidase H (Endo H). The samples were resolved by 8% SDS-PAGE under reducing conditions.

**FIG. 8.** Immunohistochemical staining of type XXVI collagen in the neonatal mouse testis and ovary. Sections of neonatal testis and ovary (5 μm) were fixed and immunostained with preimmune rabbit serum (A and C) or anti-type XXVI collagen antibody NC2 (B and D). Signals were detected with the Tyramide Signal Amplification plus dinitrophenyl horseradish peroxidase system. After detection of the signals, sections were counterstained with 1% methyl green. Arrowheads in B indicate the myoid cells. In D, arrowheads indicate the extracellular matrix of cortex. Bars = 50 μm (A and B), 100 μm (C and D), and 25 μm (insets).
impaired. This notion is supported by the improper cleavage of the type I collagen N- and C-propeptides and the enhanced sensitivity of secreted type I collagen to protease. Recently, it was shown that Gly-X-Arg triplets in the collagen triple helix are dominant binding sites for HSPG47 (24). Type XXVI collagen also possesses two Gly-X-Y clusters, both of which contain Gly-X-Arg triplets. Thus, HSPG47 may also interact with this novel collagen through these motifs.

Further supporting the importance in development of interactions between HSPG47 and collagen is that although collagen XXVI is expressed in the adult testis and ovary, this expression is higher in the neonatal tissues. Furthermore, we also detected high levels of type XXVI collagen expression during embryogenesis. Thus, type XXVI collagen together with HSPG47 may be important not only during testis and ovary development but also in embryogenesis. This is similar to type XIX collagen, which was reported to be ubiquitously expressed during embryogenesis, whereas in adulthood its expression was restricted to just a few tissues (30). The roles that type XXVI collagen and HSPG47 play during embryogenesis are currently being investigated.

In the case of type I collagen, three α-chains are associated in the C-propeptide region and form intermolecular disulfide bonds (1, 6). In contrast, type XXVI collagen bears its 13 cysteine residues only in the N-terminal portion of its first non-collagenous region (NC1), including in its signal sequences. The electrophoresis of cell-derived and secreted XXVI collagen revealed that this protein forms multimers by establishing intermolecular disulfide bonds. Notably, the trimer form was the predominantly secreted form. Thus, trimers of type XXVI collagen must arise from intermolecular disulfide bonds between the NC1 regions. This is a unique feature of this protein relative to the other collagens. When extracts of the neonatal testis or of cells transfected with type XXVI collagen cDNA were immunoprecipitated with anti-type XXVI collagen antibody, only one band was detected. This suggests that the type XXVI collagen trimer probably consists of three identical α-chains (COL26A1). However, it remains possible that the trimer may actually be a heterotrimer consisting of different α-chains with identical molecular sizes. The studies to discriminate between these two possibilities are now in progress.

HEK-293 cells transfected with type XXVI collagen cDNA release a protein that is ~60 kDa in reducing SDS-PAGE. This is a slightly higher molecular size than is predicted from the actual amino acid length (438 amino acids). The additional mass appears to come from post-translational modifications of the protein that include glycosylation and hydroxylation of proline residues within the collagenous domain. Pulse-chase experiments revealed that both modifications are required for the secretion of the protein. Furthermore, the N-glycans that are added are further modified to more complex forms in the Golgi apparatus before the protein is secreted.

Type XXVI collagen possesses RRRR sequences just before the first collagenous (COL1) region. These sequences are also found in types XIII and XXV collagens and were cleaved by furin convertase (5, 11). However, Western blotting of testis and ovary did not detect a short peptide fragment of type XXVI collagen resulting from cleavage by furin convertase (data not shown). The role this site plays in type XXVI collagen function is thus unclear at present. It also appears that type XXVI collagen is not a FACIT collagen. The FACIT collagens reported to date all have a thrombospondin N terminus-like domain and various numbers of von Willebrand factor-like A-domains (31). Type XXVI collagen does not bear such domains.

In summary, we have identified a new collagen that interacts with HSPG47 and is secreted into the extracellular matrix. It may be important in the adult reproductive organs as well as during the development of these organs. It bears many collagen-like features, including the trimeric form it assumes in its secreted state and its post-translational modifications. It also has some unusual features, including the fact that the disulfide bonds that form the trimer are made in an N-terminal non-collagenous domain. Further studies to investigate the functions of this novel collagen in development and adulthood are currently being conducted.

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