A NOVEL 3-HYDROXYPROLINE (3HYP)-RICH MOTIF MARKS THE TRIPLE-HELICAL C-TERMINUS OF TENDON TYPE I COLLAGEN

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Running head: A novel 3-hydroxyproline motif in tendon collagen

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Because of its unique physical and chemical properties, rat tail tendon collagen has long been favored for crystallographic and biochemical studies of fibril structure. In studying the distribution of 3-hydroxyproline in type I collagen of rat bone, skin and tail tendon by mass spectrometry, the repeating sequences of GPP triplets at the C-terminus of \( \alpha_1(I) \) and \( \alpha_2(I) \) chains were shown to be heavily 3-hydroxylated in tendon but not in skin and bone. By isolating the tryptic peptides and subjecting them to Edman sequence analysis, the presence of repeating 3-hydroxyprolines in consecutive GPP triplets adjacent to 4-hydroxyproline was confirmed as a unique feature of the tendon collagen. A 1960s study by Piez and colleagues in which they compared the amino acid compositions of rat skin and tail tendon type I collagen chains indeed showed 3-4 residues of 3Hyp in tendon \( \alpha_1(I) \) and \( \alpha_2(I) \) chains but only one 3Hyp residue in skin \( \alpha_1(I) \) and none in \( \alpha_2(I) \). The present work therefore confirms this difference and localizes the additional 3Hyp to the GPP repeat at the C-terminus of the triple-helix. We speculate on the significance in terms of a potential function in contributing to the unique assembly mechanism and molecular packing in tendon collagen fibrils and on mechanisms that could regulate 3-hydroxylation at this novel substrate site in a tissue-specific manner.

Prolyl 3-hydroxylation, a long-recognized quantitatively minor post-translational modification of collagen (1), has received much attention in the last few years after gene mutations affecting its formation were found to cause recessive forms of osteogenesis imperfecta (OI, 2-5). A single primary site of 3-hydroxyproline (3Hyp) is present in normal collagen \( \alpha_1(I) \) and \( \alpha_1(II) \) chains at P986 of the triple-helix (6,7), but is not hydroxylated in the tissues of mice and humans with recessive OI caused by mutations in CRTAP or LEPRE1 (2-5). The LEPRE1 gene encodes P3H1, which is one of three prolyl 3-hydroxylases (P3H1, P3H2, and P3H3) in the mammalian genome. CRTAP encodes a protein that is homologous to the amino-terminal half of P3H1, which it associates with together with CypB to form the functional enzyme complex required for P986 3-hydroxylation of unfolded collagen chains in the ER (8).

We recently identified several other sites of prolyl 3-hydroxylation in fibrillar collagen chains including P707 in \( \alpha_2(I) \) and \( \alpha_2(V) \), P944 in \( \alpha_1(II) \) and \( \alpha_2(V) \), P470 in \( \alpha_2(V) \) and \( \alpha_3(IV) \), P665 and P692 in \( \alpha_1(VI) \), \( \alpha_1(XI) \) and \( \alpha_2(XI) \) but a lack of any 3-hydroxyproline in the mammalian \( \alpha_1(III) \) chain (7). The D-periodic spacing between three of these additional sites in clade A collagen chains (\( \alpha_1(I) \), \( \alpha_2(I) \), \( \alpha_1(II) \) and \( \alpha_2(V) \)) and between two in clade B (\( \alpha_1(V) \), \( \alpha_1(XI) \) and \( \alpha_2(XI) \)) suggested a role in fibril formation. In pursuing this concept further, we investigated the potential for differences in 3-hydroxylation pattern between tissues known to have very different fibril architectures (9,10) though based on the same primary structural collagen, type I.

Using ion-trap tandem mass spectrometry, we interrogated all candidate GPP triplets in collagen \( \alpha_1(I) \) and \( \alpha_2(I) \) chains from rat skin, bone and tail tendons for the presence of additional hydroxyls (+16 dalton mass). The results revealed that the tendon type I collagen chains were distinguished by having up to four 3Hyp residues in series in the (GPP)_n motif at the extreme C-terminus of the triple-helical domain, whereas bone and skin type I collagen had none. The findings may be fundamentally important for understanding the mechanisms by which the unique features of cellular assembly, deposition and structure of the parallel arrays of collagen fibrils secreted by tendon cells (11,12) are controlled.

**Experimental Procedures**

**Tissue collagen preparation** - Skin, bone and tail tendons were dissected from the carcasses of adult normal Sprague-Dawley laboratory rats obtained as a by-product from approved and completed animal studies. Bovine Achilles tendon, dissected from adult steer (18 month), was obtained from a local abattoir. All tissues were stored at -20°C prior to analysis. Tendons were pulled from proximal tail ends using the usual two-hemostat repetitive method (13). Skin, bone and bovine tendon were scraped clean and defatted in chloroform/methanol (3:1 v/v); bone was
demineralized for several days in 0.5M EDTA according to established methods, all at 4°C (13).

Collagen was solubilized from each tissue matrix by heat denaturation for 2-3 min at 100°C directly in SDS-PAGE sample buffer. Acid-extracted collagen (3% acetic acid v/v, 4°C) was also prepared and digested with trypsin for chromatographic resolution of individual peptides for N-terminal sequence analysis.

**SDS-polyacrylamide electrophoresis and in-gel trypsin digestion** - The method of Laemmli (15) was used with 6% gels for denaturant extracts of tissue collagen. Collagen α-chains were cut from SDS-PAGE gels and subjected to in-gel trypsin digestion (7,16).

**Tandem mass spectrometry of tryptic peptides** - Electrospray MS was performed on the tryptic peptides using an LCQ Deca XP ion-trap mass spectrometer equipped with in-line liquid chromatography (LC) (ThermoFinnigan) using a C8 capillary column (300µm x 150mm; Grace Vydac 208MS5.315) eluted at 4.5µl min. The LC mobile phase consisted of buffer A (0.1% formic acid in MilliQ water) and buffer B (0.1% formic acid in 3:1 acetonitrile:n-propanol v/v). The LC sample stream was introduced into the mass spectrometer by electrospray ionization (ESI) with a spray voltage of 3kV. The machine is normally run in automatic triple play mode cycling through a full scan, zoom scan and ms/ms every few milliseconds. The machine can also be made to target specific low abundance ions by narrowing the selecting mass range. Sequest search software (ThermoFinnigan) was used for peptide identification using the NCBI protein database. Large collagenous peptides not found by Sequest were identified manually by calculating the possible MS/MS ions and matching these to the actual MS/MS spectrum (17).

**Reverse-phase HPLC of C-terminal (GPP)₅-containing peptides** - Tryptic peptides prepared from acid-extracted rat tail tendon were separated by reverse-phase HPLC on a C8 column (Brownlee Aquapore RP-300, 4.6mm x 25cm) with a linear gradient of acetonitrile:n-propanol (3:1, v/v) in aqueous 0.1% (v/v) trifluoroacetic acid. (18).

**Edman N-terminal sequence analysis** - N-terminal sequence analysis of the α1(I) GPP repeat containing tryptic peptide was carried out by Edman chemistry on a Porton 2090E machine equipped with on-line HPLC analysis of phenylthiohydantoin-derivatives as reported (7).

**RESULTS**

Tandem mass spectrometry revealed a repeating 16-dalton difference across a ladder of molecular ions given by a tryptic peptide from the C-terminus of the α1(I) chain of tendon type I collagen but not from its equivalent peptide from skin or bone (Fig. 1). The MSMS fragmentation pattern established that it consisted of the C-terminus of the triple-helix running into the C-telopeptide and ending at the cross-linking lysine. Peptides that originated from chains in which the lysine had been converted to lysine aldehyde by lysyl oxidase or remained as lysine were both identified in the LCMS profiles, with similar 16-dalton ladder distinction from tendon. Results are shown for the unmodified lysine (and for bone, lysine and hydroxylysine) containing-peptides. The MSMS fragmentation profiles established that the source of the 16 dalton ladder was the (GPP)₅ repeat, specifically the X-position prolines next to Y-position 4-hydroxyprolines. This strongly suggested that prolyl 3-hydroxylation was responsible. The relative quantities of the individual sequences making up the ion ladder indicated an average of 2.8 3Hyp per collagen α1(I) chain in its (GPP)₅ C-terminal sequence. The MSMS data showed the highest occupancy on the N-terminal GPP triplet tailing off C-terminally with the maximum number detected in any one peptide and hence α1(I)-chain of four 3Hyp residues in a repeating series.

In contrast, no extra hydroxyls (and hence potential 3Hyp residues) were detected in the equivalent peptides from rat skin or bone (Fig. 1). The latter differed in their MS profile because from bone the α1(I) C-telopeptide lysine is partially hydroxylated, whereas it is not from skin and tail tendon. The MSMS fragmentation spectra clearly reveal this, since the y-ion fragments from the C-terminus of the peptide show only a lysine mass from skin and tendon, but the presence of both hydroxylysine-containing and lysine-containing y-ions from bone. This explains why from bone α1(I) C-terminal tryptic peptide the +16 dalton parent ion (1860²) is prominent but not from skin.

The equivalent peptide purified from a
trypsin digest of acid-soluble rat tail tendon collagen by reverse-phase HPLC (not shown) was subjected to N-terminal Edman sequencing. The results confirmed that the additional +16 daltons on the X-position prolines on the (GPP)₅ repeat were due to 3-hydroxyproline, based on its distinctive PTH-amino acid signature, compared with 4Hyp or Pro as we previously reported for a secondary 3Hyp site at P944 in the α1(II) chain of bovine type II collagen (7).

We similarly interrogated the tryptic peptide LCMS profiles given by the α2(I) chains of tendon, skin and bone for a peptide with a +16 dalton ladder derived from the (GPP)₅-repeat that forms the C-terminus of its triple-helical domain. Again the results show a high occupancy with an average of 2.8 3Hyp residues per (GPP)₅ in tendon α2(I) but none from skin or bone α2(I) (Fig. 2a). The mass spectral parent ion results clearly show this but the α2(I) peptide MSMS fragmentation profiles (Fig. 2) lack a strong C-terminal y-ion contribution since no lysine (or arginine) is present in the tissue collagen C-telopeptide sequence to produce a charged C-terminus on trypsin cleavage.

The known lone site of prolyl 3-hydroxylation in α1(I) at P986 was also hydroxylated in tendon α1(I) (91% 3Hyp) as it was from bone (94%) and skin (84%) (not shown). Similarly, the single site in α2(I) P707 (7) was partially 3-hydroxylated in tendon α2(I) (85% 3Hyp) and much less so in bone (18%) and skin (32%) (not shown).

Though rodent tail tendon type I collagen appears to be especially highly 3-hydroxylated at its C-terminus, the phenomenon extends to tendon in general. For example, results from a bovine tendon α2(I) chain preparation are shown in Fig. 2b. The α1(I) chain from the same collagen sample showed a similar +16 dalton 3Hyp ladder for the tryptic peptide from its C-terminus (not shown, but the results presented illustrate the phenomenon). Human Achilles tendon type I collagen also showed an average of one 3Hyp per C-terminal (GPP)₅ domain in α1(I) and α2(I) (not shown). Precise 3Hyp percentages at each of the GPP triplets of the (GPP)₅ patch cannot simply be derived from the MS data, but it was clear for both α1(I) and α2(I) from tendon of all species examined that the highest occupancy was on the N-terminal GPP with falling percentages C-terminally. Tendons in general, therefore, seem to express this post-translational modification as a marker of their phenotype, though other collagens, notably type II collagen, have a GPP repeat at their triple-helix C-terminus and so are potential substrates.

Fig. 3 compares the sequences of all human clade A and clade B fibril-forming collagen chains. Collagen type II has (GPP)₅ compared with (GPP)₄ and (GPP)₃ for α1(I) and α2(I) chains. Screening of type II collagen from various tissues indicates that some 3Hyp may be present, the level depending on the tissue (data not shown). Prolyl 3-hydroxylation of the (GPP)₅ repeat motif may therefore not be restricted completely to tendon type I collagen, though the latter, particularly in rodents, is uniquely hyper-hydroxylated.

**DISCUSSION**

The findings establish a previously unrecognized site and sequence motif for 3-hydroxyproline formation that appears to be a phenotypic characteristic of tendon collagen. In rat tail tendon α1(I) about 3 residues of 3Hyp are present on average per C-terminal (GPP)₅ repeat together with 1 residue at P986. But in skin and bone α1(I) no 3Hyp is present in the C-terminal (GPP)₅ repeat, just the 1 residue at P986. The α2(I) chain showed a similar tissue-dependent difference in the GPP-repeat hydroxylation. This is consistent with results in a classic study by Piez and colleagues in which they reported the amino acid compositions of rat skin and tail tendon α1(I) and α2(I) chains (19). Their data show four residues of 3Hyp in each α1(I) and α2(I) chain from tendon but only one in α1(I) and none in α2(I) from skin. The additional 3Hyp they observed from tendon all appears, therefore to be in the C-terminal triple-helical (GPP)₅ repeat.

This newly discovered site of prolyl 3-hydroxylation, the extreme C-terminal (GPP)₅ repeat of the α1(I) and α2(I) chains, can be defined as a third class of substrate motif for prolyl 3-hydroxylation in fibril-forming collagens. We recently reported finding several new sites of full and partial prolyl 3-hydroxylation in clade A (α1(I), α2(I), α1(II), α2(V)) and clade B (α1(V), α1(XI) and α2(XI)) collagen chains (7), in addition to the long recognized P986 site in α1(I) and α1(II). For example in α1(II), P944 was
variably 3-hydroxylated dependent on the tissue of origin, ranging from >80% hydroxylated in vitreous, 40% in nucleus pulposus to <20% in mature articular cartilage type II collagen. The α2 chain had 3Hyp only at P707 whereas α2(V) was hydroxylated at P986, P944, P707 and P470. Notably the latter three sites, P944, P707 and P470, are spaced apart within three residues of the D-period (234 residues) the distance by which collagen molecules are axially staggered from each other along a fibril. The collagen type V/XI clade B gene products α1(V), α1(XI) and α2(XI) also revealed a D-spacing between two of their uniquely placed 3Hyp sites (7).

Based on their sequence motifs the three classes of 3Hyp substrate site are: class 1, the unique P986 motif; class 2 includes all the other sites (which tend to be preceded N-terminally by a phenylalanine residue (7)), and class 3, the new (GPP)_n site at the C-terminus of the triple-helix of tendon collagen. This apparent class distinction may be significant as three P3H enzymes are present in the mammalian genome (20). P3H1 is known to be required for P986 3-hydroxylation in types I and II collagens (4) as part of a complex with CRTAP protein and CypB (8). Recombinant P3H2 was shown using synthetic peptide substrates to actively 3-hydroxylate (Gly-Pro-P3H) by guest on March 24, 2020http://www.jbc.org/Downloaded from

specificities among the above defined three classes of substrate site in fibril-forming collagens.

A key question remains, however. What is the function of 3Hyp residues in collagen? It is clearly an ancient post-translational modification, found in the most primitive invertebrates including sponge (Porifera) fibrillar collagens (22) and prominent in basement membrane type IV collagens in which about one in ten hydroxyprolines are 3Hyp (23). This implies biological benefits through a fundamental contribution to the properties of collagen structure itself. We recently suggested based on the observed D-periodic spacing (7) and externally directed 3(S)OH (24), that inter-triple-helical hydrogen bonding may be involved, for example in helping fine-tune the polymeric assembly of fibrils and basement membrane networks. With this concept in mind, the possible consequences of a 3Hyp repeat at the junction of the triple helix and the C-terminal telopeptide/propeptide are worth considering.

The triple helix folds from the C-terminus after the propeptides have formed a complex in the ER and the (GPP)_n repeat is important for its nucleation (25). The chain order in the type I collagen heterotrimer has not been defined (α1α1α2, α1α2α1 or α2α1α1) for any tissue (26). Possibly the tertiary structure of the triple-helix initiated when 3Hyp residues are present in the GPP repeat could direct a particular chain order in tendon that results in an assembled molecule, polymer and subsequent cross-linking interactions that differ from those of skin and bone collagen type I fibrils. Tendon cells secrete collagen fibrils in a linear direction through what appear to be cellular invaginations and what ultrastructurally Kadler and colleagues have referred to as fibripositors (27,28). The unique post-translational phenotype of tendon type I collagen may have evolved in conjunction with this cellular machinery to assemble fibrils better suited to tendon growth and function. Indeed, the cross-linking chemistry of tendon collagen differs from that of skin, despite both using exclusively the lysine aldehyde pathway (29). The placement of intermolecular cross-links also appears to differ between tendon and skin type I collagen specifically for those formed from the α1(I) C-telopeptide lysine aldehyde. We have evidence for an intramolecular aldol cross-link at the C-terminus of collagen type I in skin but not tendon. An effect of this is apparent in Fig. 1 with a slow form of β11 dimer from skin but not tendon collagen. This component appears to be a β11 dimer in which the α1(I) chains are linked at both ends by allysine aldol (Eyre,D.R. and Weis,M. unpublished). We suspect that an altered triple-helix chain order or another effect of the neighboring 3Hyp C-terminal repeat is responsible for the altered cross-linking properties.
between hole and overlap domains on the surface of collagen fibrils. Such molecules are thought to influence fibril size and collagenase susceptibility (30-32). Fig. 3 illustrates the site of the (GPP)n 3Hyp motif in the collagen molecule and assembled fibril.

Lastly, the mechanisms that regulate (GPP)n 3Hyp formation in tendon cells and prevent it in dermal fibroblasts and osteoblasts will be important to define. Conceivably, pathological conditions may exist in which misregulation results in lack of C-terminal 3Hyp and defective tendon properties or presence of C-terminal 3Hyp in non-tendon tissues with negative consequences. It will be important therefore to determine which of the three P3H enzymes is primarily responsible for (GPP)n prolyl 3-hydroxylation and whether other proteins are required in a complex for the selective activity.

REFERENCES

1. Ogle, J. D., Arlinghaus, R. B., and Logan, M. A. (1962) J. Biol. Chem. 237, 3667-3673
2. Morello, R., Bertin, T. K., Chen, Y., Hicks, J., Tonachini, L., Monticone, M., Castagnola, P., Rauch, F., Glorieux, F. H., Vranka, J. Bächinger, H. P., Pace, J. M., Schwarze, Y., Byers, P. H., Weis, M., Fernandes, R. J., Eyre, D. R., Yao, Z., Boyce, B. F., and Lee, B. CRTAP is required for prolyl 3-hydroxylation and mutations cause recessive osteogenesis imperfecta. (2006) Cell 127, 291-304
3. Barnes, A. M., Chang, W., Morello, R., Cabral, W. A., Weis, M., Eyre, D. R., Leikin, S., Makareeva, E., Kuznetsova, N., Uveges, T. B., Ashok, A., Flor, A. W., Mulvihill, J. J., Wilson, P. L., Sundaram, U. T., Lee, B., and Marini, J. C. (2006) N. Engl. J. Med. 355, 2757-2764
4. Cabral, W. A., Chang, W., Barnes, A. M., Weis, M., Scott, M. A., Leikin, S., Makareeva, E., Kuznetsova, N., Rosenbaum, K. N., Tiffet, C. J., Bulas, D. I., Kozma, C., Smith, P. A., Eyre, D. R., and Marini, J. A. (2007) Nat. Genet. 39, 359-365
5. Baldridge, D., Schwarze, U., Morello, R., Lennington, J., Bertin, T. K., Pace, J. M., Pepin, M. G., Weis, M. A., Eyre, D. R., Walsh, J., Lambert, D., Green, A., Robinson, H., Michelson, M., Houge, G., Lindman, C., Martin, J., Ward, J., Lemyre, E., Mitchell, J. J., Krakow, D., Rimoin, D. L., Cohn, D. H., Byers, P. H., and Lee, B. (2008) Hum. Mutat. 29, 1435-1442
6. Fietzek, P. P., Rexrodt, F. W., Wendt, P., Stark, M., and Kühn, K. (1972) Eur. J. Biochem. 30, 163-168
7. Weis, M. A., Hudson, D. M., Kim, L., Scott, M., Wu, J-J., and Eyre, D R. (2010) J. Biol. Chem. 285, 2580-2590
8. Ishikawa, Y., Wirz, J., Vranka, J. A., Nagata, K., and Bächinger, H. P. (2009) J. Biol. Chem. 284, 17641-17647
9. Brodsky, B., Eikenberry, E. F., and Cassidy, K. (1980) Biochim. Biophys. Acta. 621,162-166
10. Ottani, V., Martini, D., Franchi, M., Rugareri, A., and Raspanti, M. (2002) Micron 33, 587-596
11. Zhang, G., Young, B. B., Ezura, Y., Favata, M., Soslowsky, L. J., Chakravarti, S., and Birk, D. E. (2005) J. Musculoskelet. Neuronal Interact. 5, 5-21
12. Bayer, M. L., Yeung, C. Y., Kadler, K. E., Qvortrup, B., Baar, K., Svensson, R. B., Magnusson, S. P., Krogsaard, M., Koch, M., and Kjaer, M. (2010) Biomaterials 31, 4889, 4897
13. Kessler, A., Rosen, H., and Levenson, S. M. (1960) J. Biol. Chem. 235, 989-994
14. Hanson, D. A., and Eyre, D. R. (1996) J. Biol. Chem. 271, 26508-26516
15. Lämmli, U. K. (1970) Nature 227, 680-685
16. Hanna, S. L., Sherman, N. E., Kinter, M.T., and Goldberg, J. B. (2000) Microbiology 146, 2495-2508
17. Eyre, D. R., Weis, M. A., and Wu, J. J. (2008) Methods 45, 65-74
18. Wu, J. J., Woods, P. E., and Eyre, D. R. (1992) J. Biol. Chem. 267, 23007-23014
19. Piez, K. A., Eigner, E. A., and Lewis, M. S. (1963) Biochemistry 2, 58-66
20. Vranka, J.A., Sakai,L.Y. and Bächinger, H.P. (2004) J Biol Chem 279, 23615-23621
21. Tiainen, P., Pasanen, A., Sormunen, R., and Myllyharju, J. (2008) J. Biol. Chem. 283, 19432-
22. Garrone, R. (1985) In Bairati, A., and Garrone, R. (eds.), NATO ASI Series A. 93, 157-175, Plenum, NY
23. Kefalides, N. A. (1973) Int Rev Connect Tissue Res 6, 63-104
24. Schumacher, M. A., Mizuno, K., and Bächinger, H. P. (2006) J. Biol. Chem. 281, 27566-27574
25. Bulleid, N. J., Dalley, J. A., Lees, J. F. (1997) EMBO J. 16, 6694-6701
26. Okuyama, K., Bächinger, H. P., Mizuno, ., Boudko, S., Engel, J., Berisio, R., and Vitagliano, L. (2009) Acta. Cryst. D65, 1007-1008
27. 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
28. Kapacee, A., Richardson, S. H., Lu Y., Starborg, T., Holmes, D. F., Baar, K., and Kadler, K. E. (2008) Matrix Biol. 27, 371-375
29. Eyre, D.R. (1987) Methods Enzymol 144, 115-139
30. Matsushima, N., Ohyanagi, T., Tanaka T., and Kretsinger, R. H. (2000) Proteins 38, 210-225
31. Zhang, G., Ezura, Y., Chervoneva, I., Robinson, P. S., Beason, D. P., Carine, E. T. Soslowsky, L. J., Iozzo, R. V., and Birk, D. E. (2006) J. Cell. Biochem. 98, 1436-1449
32. Geng, Y., McQuillan, D., Roughley, P. J. (2006) Matrix Biol. 25, 484-491

FOOTNOTES

Key Words: collagen, 3-hydroxyproline, tendon, rat, mass spectrometry

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The abbreviations used are: CRTAP, the gene encoding cartilage-associated protein; LEPRE1, the gene encoding P3H1; CypB, cyclophilin B; ER, endoplasmic reticulum; 4Hyp, 4-hydroxyproline; 3Hyp, 3-hydroxyproline; P3H, prolyl 3-hydroxylase; OI, osteogenesis imperfecta; HPLC, high performance liquid chromatography; MS, mass spectrometry; MS/MS, tandem MS, LC, liquid chromatography

FIGURE LEGENDS

**Fig. 1.** Tandem mass spectrometry of tryptic peptides from the α(I) chain reveals a 3Hyp repeat in the C-terminal sequence from the triple-helical domain of tendon but not skin or bone type I collagen

The three collagen preparations were run on SDS-PAGE for in-gel trypsin digestion of the excised α1(I) chains as shown in a). Also identified in a) are the principal β dimer components common to all three tissues and a slower B11 band prominent in extracts of skin collagen (see Discussion). The parent ion ladders given by the C-terminal tryptic peptide from each tissue’s α-chain are shown in b). These full scan mass spectra are taken from each α-chain’s tryptic-peptide LCMS profile scrolling across an elution window that combines all post-translational variants of this peptide. In c) the MSMS fragmentation spectrum of the parent ion (1876.32+) of the tendon peptide with three additional hydroxyls on X position prolines is shown. The sequence is shown with b and y ion breakages that establish the proline (P)
residues bearing the additional 16-dalton masses. P#, 3Hyp; P*, 4Hyp.

**Fig. 2.** Tandem mass spectrometry of tryptic peptides from the α2(I) chain reveals a similar 3Hyp ladder to that in α1(I) for tendon collagen but not skin or bone

The α2(I) chains were resolved as shown in Fig 1, cut out and subjected to in-gel trypsin digestion and LCMS analysis. In a) the parent ion ladder from the C-terminal tryptic peptide of rat tail tendon α2-chain is shown and below it the MSMS fragmentation profile from the ion bearing three additional 16-dalton masses on Y position prolines. The position of predicted 3Hyp residues was determined from the b and y ion masses as indicated for this variant and also for the other variant parent ions in the ladder. Sequence determined matches that in the Ensembl rat genomic database (ENSRNOT00000016423). In b) MS results from the α2(I) chain of mature bovine Achilles tendon show a similar +16 dalton series of molecular ions for this peptide. The bovine Achilles α1(I) chain gave a similar ladder (data not shown). P#, 3Hyp P*, 4Hyp.

**Fig. 3.** Molecular location of the novel 3Hyp repeat at the C-terminus of the triple-helix in relation to known 3Hyp sites

The axial positioning of the three classes of 3Hyp that can be defined by their sequence context is shown in the upper procollagen molecule. How these are positioned relative to each other in a fibril of D-staggered molecules is shown below. Sequences of the C-terminal GPP repeat domain for all clade A and clade B collagen α-chains from the human genome (Ensembl and NCBI genomic databases) are aligned for comparison and to put the rat sequences studied here in a broader context.
a) SDS-PAGE

b) α1(I) MS profiles

TGDSGPAGPPPGPPGPPGPPGPPGPPSGGYDFSFLPQPPQEK

Rat Tendon α1(I) four 4Hyp 1852.02+ all with four 4Hyp and Lys (K)

1884.02+ four 3Hyp 1860.02+ four 4Hyp + Lys

Rat Skin α1(I) three 4Hyp 1843.92+ four 4Hyp + Lys

1860.62+ one 3Hyp 1868.02+ two 3Hyp

Rat Bone α1(I) three 4Hyp 1851.52+ four 4Hyp + Hyl

1851.72+ four 4Hyp + Lys

1860.62+ four 4Hyp + Hyl

Hyl

c) Tendon MSMS

TGDSGPAGPPPGPPGPPGPPGPPGPPSGGYDFSFLPQPPQEK

Figure 1
a) Rat Tail Tendon α2(I)

b) Bovine Tendon α2(I)

Figure 2
C-terminal sequence

rat

GRTGDSGPAGPPGPAGPPGPAGPPGPPSAGYDFS–FLPQPPQEK \( \alpha_1(I) \)
GSQGSQGPAGPPGPAGPPGPAGPPGVSGGGYDFEGDFY \( \alpha_2(I) \)

human

GRTGDSGPVGPAGPPGPAGPPGPAGPPSAGYDFS–FLPQPPQEK \( \alpha_1(I) \)
GPOHQGPAGPPGPAGPPGPAGPPGVSGGGYDFEGDFY \( \alpha_2(I) \)
GRSTGEPAGPPGPAGPPGPAGPPGPAGPDMSAFAGLPREK \( \alpha_1(II) \)
GERGDWSSPSGPAGPPGPAGPPGPAGPDMSAFAGLPREK \( \alpha_1(III) \)
GSVGEAGPEGPPGPAGPPGPAGPPGPAGPDMSAFAGLPREK \( \alpha_2(V) \)
GSSGPTGPKEGAGHPGPAGPPGPAGPEVI \( \alpha_1(V) \)
GSTGPGPQKGDSGLGPAGPPGPAGPEVI \( \alpha_1(XI) \)
GATGPGPKEKVGQPPGPAGPPGPAGPEVI \( \alpha_2(XI) \)

Figure 3
A novel 3-hydroxyproline (3HYP)-rich motif marks the triple-helical C-terminus of tendon type I collagen
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