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Developmental Stage-dependent Regulation of Prolyl 3-Hydroxylation in Tendon Type I Collagen*

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Background: The physiological role of 3-hydroxyproline (3-Hyp) rarely found in collagen is unclear.
Results: Significant increases in 3-Hyp were observed at specific sites in tendon type I collagen at early ages.
Conclusion: The tendon-specific alterations in the 3-Hyp level were correlated with tissue development, rather than aging.
Significance: Prolyl 3-hydroxylation is suggested to contribute to fibril diameter regulation in tendon collagen.

3-Hydroxyproline (3-Hyp), which is unique to collagen, is a fairly rare post-translational modification. Recent studies have suggested a function of prolyl 3-hydroxylation in fibril assembly and its relationships with certain disorders, including recessive osteogenesis imperfecta and high myopia. However, no direct evidence for the physiological and pathological roles of 3-Hyp has been presented. In this study, we first estimated the overall alterations in prolyl hydroxylation in collagens purified from skin, bone, and tail tendon of 0.5–18-month-old rats by LC-MS analysis with stable isotope-labeled collagen, which was recently developed as an internal standard for highly accurate collagen analyses. 3-Hyp was found to significantly increase in tendon collagen until 3 months after birth and then remain constant, whereas increased prolyl 3-hydroxylation was not observed in skin and bone collagen. Site-specific analysis further revealed that 3-Hyp was increased in tendon type I collagen in a specific sequence region, including a previously known modification site at Pro^{707} and newly identified sites at Pro^{716} and Pro^{719}, at the early ages. The site-specific alterations in prolyl 3-hydroxylation with aging were also observed in bovine Achilles tendon. We postulate that significant increases in 3-Hyp at the consecutive modification sites are correlated with tissue development in tendon. The present findings suggest that prolyl 3-hydroxylation incrementally regulates collagen fibril diameter in tendon.

Type I collagen is the major component of connective tissues, such as skin, bone, and tendon. Collagens consist of repeating Gly-X-Y triplets and have characteristic post-translational modifications (PTMs),[2] including 3-hydroxyproline (3-Hyp) and 4-hydroxyproline (4-Hyp). Almost all Pro residues lying in the Y position are hydroxylated to 4-Hyp (~100 residues/1000 amino acid residues in type I collagen), which stabilizes the collagen triple helix (1). In contrast, Pro residues at the X position are rarely hydroxylated to 3-Hyp, which was identified in collagen more than 50 years ago (2). Although 3-Hyp was reported to slightly increase the stability of the triple-helical structure of collagen (3), the biological function of this minor modification is not well understood (4). The 3-Hyp levels in type I collagen vary according to tissue type, *e.g.* 0.5 residues in skin, 0.7 residues in bone, and 2.4 residues in tail tendon per 1000 amino acid residues in our previous study on Sprague-Dawley rats at 5 weeks of age (5).

Originally, α1(I) Pro^{986} was identified as the primary 3-Hyp site, and is usually almost fully hydroxylated (6). Prolyl 3-hydroxylase (P3H) 1 is responsible for the reaction by forming a ternary complex with cartilage-associated protein and cyclophilin B (7, 8). Mutations in the complex components were recently reported to cause severe forms of recessive osteogenesis imperfecta (9–11), thus indicating the importance of prolyl 3-hydroxylation and attracting increased attention to the modification. MS has enabled high-sensitive and site-specific analysis of collagen PTMs, including 3-Hyp (12–16). Using LC-MS, Eyre and colleagues (12, 17, 18) identified novel 3-Hyp sites in type I collagen, including α1(I) Pro^{707}, α2(I) Pro^{707}, and a C-terminal $(GPP)_n$ motif, which are mainly modified by P3H2 (19). Detailed analyses using LC-MS have also provided clues about the function of 3-Hyp. For example, P3H2 mutations were reported to be associated with non-syndromic severe high myopia in humans (20, 21). Site-specific LC-MS analysis further revealed that prolyl 3-hydroxylation was decreased at specific sites in collagens from eye tissues of P3H2-null mice, suggesting that the under 3-hydroxylation causes high myopia (19). In another P3H2 KO mouse model, a lack of 3-Hyp in type IV collagen resulted in embryonic lethality, because 3-Hyp in type IV collagen showed a crucial role in preventing maternal platelet aggregation (22).

A recent study implied developmental regulation of prolyl 3-hydroxylation (23). The C-terminal $(GPP)_n$ motif in type I collagen had a high 3-Hyp content in adult human tendon compared with that in fetal human tendon, although such age-dependent alterations were not observed for α1(I) Pro^{986} and α2(I) Pro^{707}, which were completely hydroxylated in fetal tendon. We considered that variations in 3-Hyp levels with aging could be important clues for elucidating the function of 3-Hyp. Age-related alterations in collagen PTMs, such as hydroxylation of Pro and Lys (24) and glycosylation of hydroxylysine (Hyl) to galactosyl-hydroxylysine (GHL) and subsequent gluco-

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[2] The abbreviations used are: PTMs, post-translational modifications; Hyp, hydroxyproline; P3H, prolyl 3-hydroxylase; Hyl, hydroxylysine; GHL, galactosyl-hydroxylysine; GGHL, glucosyl-galactosyl-hydroxylysine; SI-collagen, stable isotope-labeled collagen.

syl-galactosyl-hydroxylysine (GGHL) (25), have been examined. However, no studies have thoroughly estimated the effects of age on prolyl 3-hydroxylation, because of analytical difficulties for such a minor modification. Recently, we developed stable isotope-labeled collagen (SI-collagen), in which Pro, Lys, Arg, and collagen PTMs are all substituted with stable isotopically heavy ones, to enable highly accurate collagen analyses through its use as an internal standard in LC-MS (5, 26). Here, we first estimated the 3-Hyp contents in collagens from skin, bone, and tail tendon of Sprague-Dawley rats over a wide age distribution using the new method. Rapid and significant increases in 3-Hyp were observed for tendon collagen until 3 months after birth, whereas such marked variations were not observed for skin and bone collagens. In further experiments analyzing tryptic peptides by LC-MS, we identified novel 3-Hyp sites at Pro^{716} and Pro^{719} adjacent to Pro^{707} in tail tendon type I collagen, and site-specific analysis revealed that 3-Hyp increased at the consecutive modification sites only in tendon at the early ages.

Experimental Procedures

Ethics Statement—All animal studies were approved by the Experimental Ethical Committee of Nippi Research Institute of Biomatrix.

Extraction and Purification of Tissue Collagens—Skin, bone, and tail tendon were dissected from male Sprague-Dawley rats at 0.5, 1, 2, 3, 6, 12, and 18 months of age (Charles River Laboratories Japan). Fetal bovine Achilles tendon was obtained from Japan Bio Serum, and adult bovine Achilles tendon was obtained from Shibaura Zoki. The femurs were demineralized in 0.5 M EDTA (pH 7.8) for 3 days at 4 °C, and the demineralization procedure was repeated once more after cutting off both sides. The tissues were then digested with pepsin (5 mg/ml in 0.5 M acetic acid) at 4 °C for 16 h. The extracted collagens were purified by salt precipitation (1 M NaCl) and subsequent isoelectric precipitation (pH 8.0, adjusted with Tris-HCl). Further purification was performed by salt precipitation (0.7 M NaCl in 0.5 M acetic acid) to exclude type V collagen, and the precipitates were dissolved in 5 mM acetic acid.

Preparation of SI-collagen—SI-collagen was prepared from fibroblast cultures as described previously (5). Briefly, human embryonic lung fibroblasts, which possess a high capacity for type I collagen secretion (27, 28), were cultured with 100 mg/liter of [$^{13}C_6$]Lys (Thermo Scientific), 100 mg/liter of [$^{13}C_6{}^{15}N_4$]Arg (Thermo Scientific), and 200 mg/liter of [$^{13}C_5{}^{15}N_1$]Pro (Cambridge Isotope Laboratories) in SILAC DMEM (Thermo Scientific) supplemented with 0.5% dialyzed FBS (Thermo Scientific) and 200 μM L-ascorbic acid phosphate magnesium salt *n*-hydrate (Wako Chemicals). After discarding the culture medium of the first 3 days, the culture medium was collected every 3 days and digested with pepsin-agarose (0.1 mg/ml in 0.1 N HCl; Sigma) under gentle mixing at 4 °C for 16 h. Following removal of the immobilized pepsin by centrifugation, SI-collagen was purified by salt precipitation (1 M NaCl), and dissolved in 5 mM acetic acid.

Total and Site-specific Analysis of Collagen Prolyl 3-Hydroxylation by LC-MS—The total 3-Hyp content in the collagen samples was analyzed by acid hydrolysis (6 N HCl, 110 °C for 20 h in the gas phase under N_2) using SI-collagen as an internal standard (5). The acid hydrolysate was subjected to LC-MS analysis using a hybrid triple quadrupole/linear ion trap 3200 QTRAP mass spectrometer (AB Sciex) coupled to an Agilent 1200 Series HPLC system (Agilent Technologies). Sample separation was performed using a ZIC-HILIC column (3.5 μm particle size, length × inner diameter 150 mm × 2.1 mm; Merck Millipore), and the peak areas of the eluted amino acids were quantified in multiple reaction monitoring mode with Analyst software 1.6.2 (AB Sciex). The relative value of the 3-Hyp content was calculated according to the following formula: (3-Hyp/[$^{13}C_5{}^{15}N_1$]3-Hyp)/(Arg/[$^{13}C_6{}^{15}N_4$]Arg). Pro, 4-Hyp, Lys, total Hyl (Hyl + GHL + GGHL), Hyl, GHL, and GGHL were also analyzed by acid hydrolysis and alkaline hydrolysis (2 N NaOH, 110 °C for 20 h in the gas phase under N_2) with SI-collagen in a similar manner. The absolute 3-Hyp content expressed as residues/1000 total residues was calculated using the predetermined 3-Hyp content in SI-collagen (5).

For site-specific analysis of 3-Hyp, collagen samples were digested with sequencing grade-modified trypsin (Promega) at 37 °C for 16 h following heat denaturation at 60 °C for 30 min. The tryptic peptides were analyzed by LC-MS using an Ascentis Express C18 HPLC column (5 μm particle size, length × inner diameter 150 mm × 2.1 mm; Supelco) at a flow rate of 500 μl/min with a binary gradient as follows: 98% solvent A (0.1% formic acid) for 2.5 min, linear gradient of 2–50% solvent B (100% acetonitrile) for 12.5 min, 90% solvent B for 2.5 min, and 98% solvent A for 2.5 min. Peptide identification was performed by searching the acquired MS/MS spectra against the UniProtKB/Swiss-Prot database (release 2011_08, July 2011) with ProteinPilot software 4.0 (AB Sciex). The relative abundance of prolyl 3-hydroxylation at each modification site was semi-quantitatively estimated by the relative peak area ratio of extracted ion chromatograms for each 3-Hyp variant of the peptides containing the sites.

N-terminal Amino Acid Sequence Analysis—A tryptic peptide containing the novel 3-Hyp sites was purified using an Ascentis Express F5 HPLC column (2.7 μm particle size, length × inner diameter 150 mm × 2.1 mm; Supelco) with MS monitoring. N-terminal amino acid sequence analysis of the collected fraction was performed by a Procise 492 protein sequencer (Applied Biosystems) in pulsed-liquid mode.

Results

3-Hyp Increases in Tendon Collagen at Young Ages—The age-related alterations in prolyl hydroxylation in skin, bone, and tail tendon collagens from Sprague-Dawley rats (0.5–18 months of age) were estimated by LC-MS analysis following acid hydrolysis, using SI-collagen as an internal standard (Fig. 1). The Pro and 4-Hyp contents were revealed to remain definitely constant over the entire period in all three tissues. In contrast, the 3-Hyp content notably varied with age in tendon collagen (Fig. 1*C*). The 3-Hyp level increased by 3-fold (from ~0.8 to 2.4 residues/1000 total residues) from 0.5 to 3 months of age, and subsequently remained high until 18 months of age. The absolute 3-Hyp contents are summarized in Table 2. In contrast to tendon collagen, 3-Hyp slightly decreased in skin collagen until 3 months of age (from ~0.6 to 0.5 residues/1000 total residues)

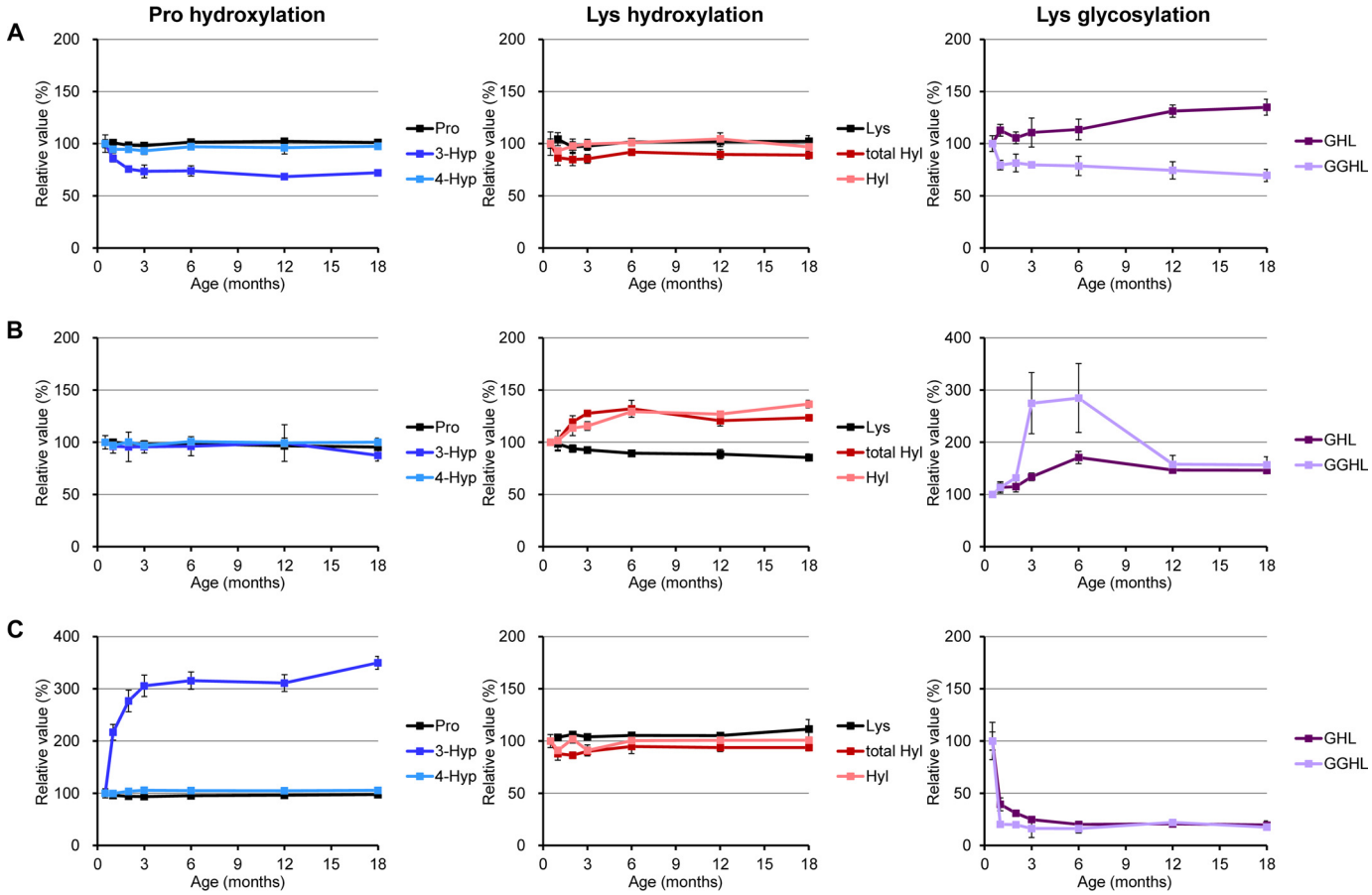

FIGURE 1. **Age-related alterations in the overall extent of post-translational modifications in collagens from different tissues.** Collagens purified from skin (*A*), bone (*B*), and tail tendon (*C*) of Sprague-Dawley rats (0.5, 1, 2, 3, 6, 12, and 18 months of age) were subjected to acid hydrolysis (Pro, 3-Hyp, 4-Hyp, Lys, total Hyl, and Arg) and alkaline hydrolysis (Hyl, GHL, and GGHL) following addition of SI-collagen as an internal standard. The peak areas of the generated amino acids were quantified by LC-MS, and the relative contents of the amino acids per collagen molecules were calculated according to the following formula expressing 3-Hyp as an example: (3-Hyp/[$^{13}C_5{}^{15}N_1$]3-Hyp)/(Arg/[$^{13}C_6{}^{15}N_4$]Arg), with 0.5 months of age set at 100%. The data represent the mean ± S.D. ($n = 3$).

(Fig. 1*A*). The 3-Hyp level in bone collagen was almost unchanged with age (~0.7 residues/1000 total residues) (Fig. 1*B*). Although we focused on prolyl 3-hydroxylation in the present study, Lys hydroxylation/glycosylation also showed tissue-specific alterations with aging (Fig. 1).

Novel 3-Hyp Sites in Type I Collagen Specific for Tendon—The dramatic and tendon-specific increases in the overall extent of prolyl 3-hydroxylation led us to predict that there were some unidentified 3-Hyp sites in tendon. We thoroughly searched for novel 3-Hyp sites in tryptic digests of collagen from tail tendon of 18-month-old rats by LC-MS. The MS/MS-based peptide identification raised two prospective 3-Hyp sites at Pro^{716} and Pro^{719} in type I collagen. Tryptic peptides containing the known 3-Hyp sites at α1(I) Pro^{707} (VGP^{707}OGPSGNAGPOGPOGPVGK; O indicates 4-Hyp) and α2(I) Pro^{707} (TGP^{707}OGPSGITGPOGPOGAAGK) were found to have two further hydroxylation forms (Fig. 2). Pro^{716} and Pro^{719} lying in the Gly-*Pro*-Hyp motif sequence were hydroxylated in both α1(I) (Fig. 2*A*) and α2(I) (Fig. 2*B*), judging from the +16 Da mass shift in the MS/MS spectra.

The presence of 3-Hyp at the newly identified sites was verified by N-terminal amino acid sequence analysis of a purified double-substituted (2 × 3-Hyp) peptide containing α2(I) Pro^{707}, Pro^{716}, and Pro^{719} (Fig. 3). Three split 3-Hyp peaks were observed at cycle 3 corresponding to the known 3-Hyp site at α2(I) Pro^{707}. Three 3-Hyp peaks were similarly observed at cycle 12 corresponding to α2(I) Pro^{716}. Furthermore, one of the three 3-Hyp peaks was obviously detected at cycle 15 corresponding to α2(I) Pro^{719}, although it was near the detection limit. We were unable to purify a sufficient amount of the 2 × 3-Hyp peptide containing α1(I) Pro^{707}, Pro^{716}, and Pro^{719} for N-terminal sequence analysis because of its low abundance. However, from the results for α2(I), it was conceivable that the +16 Da mass shifts at α1(I) Pro^{716} and Pro^{719} (Fig. 2*A*) were also attributed to prolyl 3-hydroxylation.

As shown in Fig. 2, the MS/MS fragmented y_{10} ion included Pro^{716} and Pro^{719}, but not Pro^{707}. In the MS/MS spectrum of the 3 × 3-Hyp peptide containing α2(I) Pro^{707}, Pro^{716}, and Pro^{719} (*lower spectrum* in Fig. 2*B*), a single +32 Da mass variant (2 × 3-Hyp) was observed for the y_{10} ion (*m/z* 912.44), indicating the presence of 3-Hyp at both Pro^{716} and Pro^{719}. On the other hand, in the case of the 2 × 3-Hyp peptide (*upper spectrum* in Fig. 2*B*), only a +16 Da mass variant (1 × 3-Hyp) was observed for the y_{10} ion (*m/z* 896.45), indicating only one 3-Hyp at either Pro^{716} or Pro^{719}. There was no double-substituted peptide without 3-Hyp at Pro^{707}, meaning that prolyl 3-hydroxylation first occurred at Pro^{707} in the consecutive 3-Hyp sites in α2(I). Furthermore, Pro^{716} appeared to be secondarily hydrox-

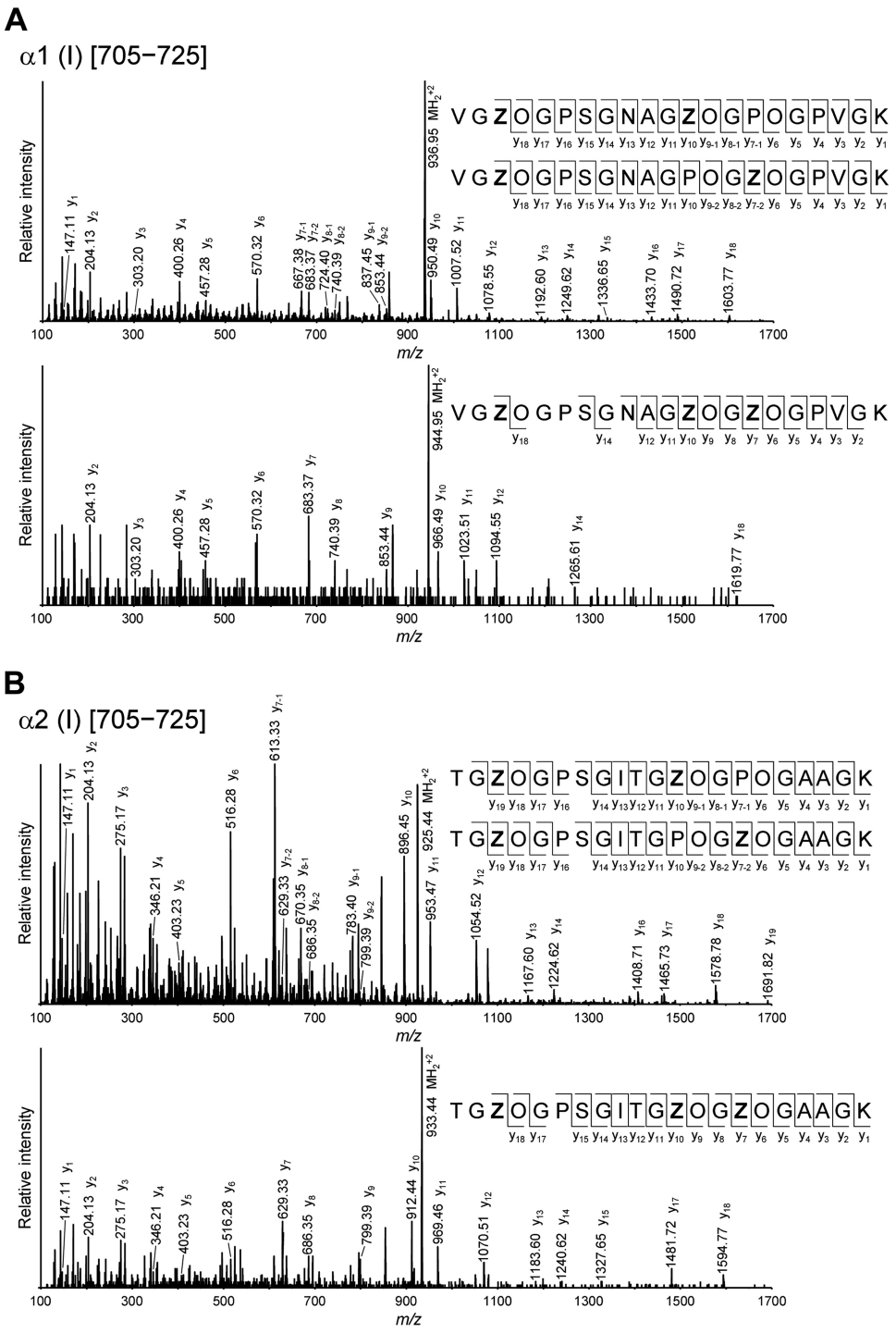

FIGURE 2. **Identification of novel 3-Hyp sites in tendon type I collagen.** Tail tendon type I collagen purified from 18-month-old Sprague-Dawley rats was digested with trypsin and subjected to LC-MS/MS analysis. *A*, MS/MS spectra of α1(I) VGP^{707}OGPSGNAGP^{716}OGP^{719}OGPVGK containing two 3-Hyp (*upper spectrum*; *m/z* 936.95, *z* = 2) and three 3-Hyp (*lower spectrum*; *m/z* 944.95, *z* = 2). *z* indicates 3-Hyp. *B*, MS/MS spectra of α2(I) TGP^{707}OGPSGITGP^{716}OGP^{719}OGAAGK containing two 3-Hyp (*upper spectrum*; *m/z* 925.44, *z* = 2) and three 3-Hyp (*lower spectrum*; *m/z* 933.44, *z* = 2).

ylated as seen in the example of the y_9 ion, which included Pro^{719}, in the 2 × 3-Hyp peptide (*upper spectrum* in Fig. 2*B*). The 0 × 3-Hyp fragment ($y_{9\text{-}1}$ ion; *m/z* 783.40) was dominantly detected compared with the 1 × 3-Hyp fragment ($y_{9\text{-}2}$ ion; *m/z* 799.39). These MS/MS fragmentation patterns were similarly observed for α1(I) (Fig. 2*A*). Taken together, α1/α2(I) Pro^{716} and Pro^{719} are secondary 3-Hyp sites adjacent to Pro^{707} in tendon type I collagen. Prolyl 3-hydroxylation at the sites was also observed in collagen from Achilles and patellar tendons (data not shown).

Prolyl 3-Hydroxylation at Specific Sites Increases in Tendon Type I Collagen at Young Ages—The tryptic peptides containing the previously known and newly identified 3-Hyp sites in type I collagen are summarized in Table 1. Fig. 4 shows the extracted ion chromatograms of the 3-Hyp-containing peptides from tail tendon type I collagen derived from young rats (0.5 months)

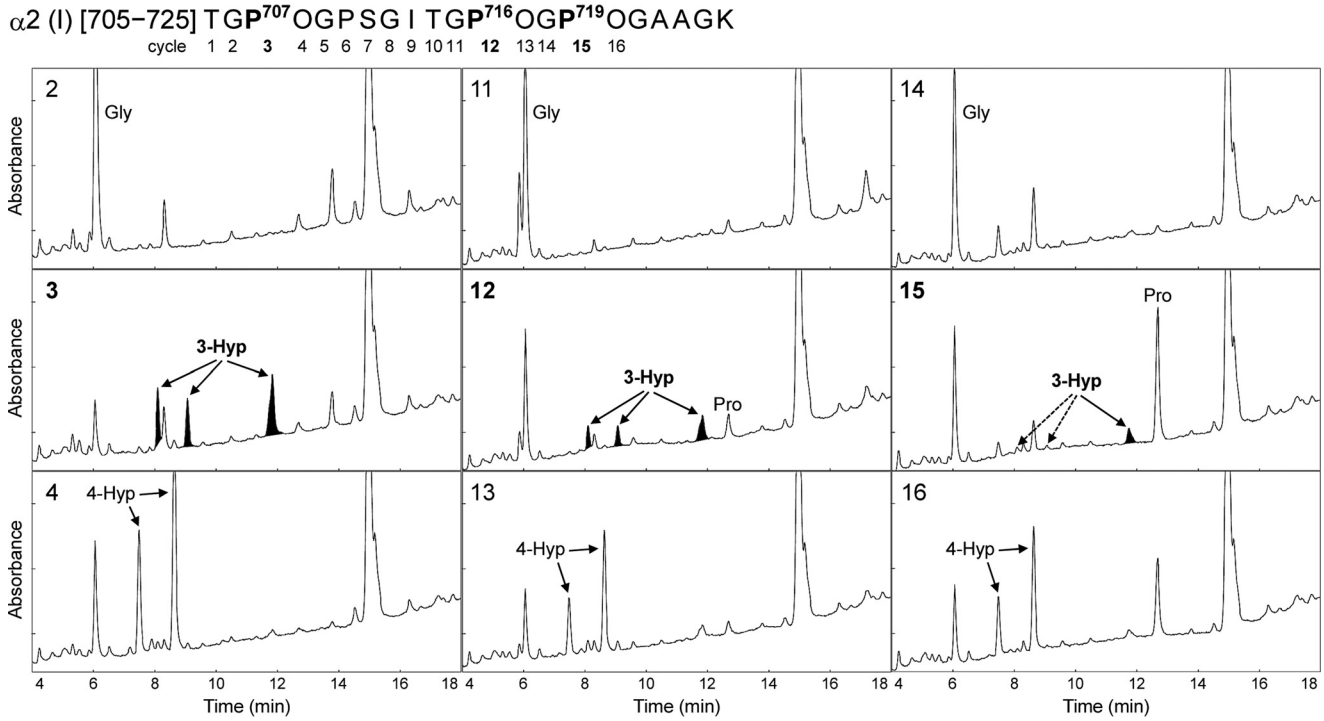

FIGURE 3. **N-terminal amino acid sequence analysis of a tryptic peptide containing novel 3-Hyp sites.** A tryptic peptide, α2(I) TGP^{707}OGPSGITGP^{716}OGP^{719}OGAAGK, containing two 3-Hyp at the three modification sites was obtained from tail tendon type I collagen of 18-month-old Sprague-Dawley rats and subjected to N-terminal amino acid sequence analysis. HPLC chromatograms of three Gly-Pro-Hyp motifs (cycles 2–4, 11–13, and 14–16) are shown with highlighting of the 3-Hyp residues.

TABLE 1
Summary of tryptic peptides containing 3-Hyp sites of type I collagen

Chain	Position	Sequence[a]	3-Hyp	Molecular weight	*z*	*m/z*	Time
							min
α1	975–990	DGLNGLOGPIGP^{986}OGPR	0	1544.79	2	773.40	9.1
			1	1560.79	2	781.39	8.9
	705–725	VGP^{707}OGPSGNAGP^{716}OGP^{719}OGPVGK	0	1839.91	2	920.96	7.5
			1	1855.91	2	928.95	7.3
			2	1871.90	2	936.95	7.1
			3	1887.90	2	944.95	6.9
α2	705–725	TGP^{707}OGPSGITGP^{716}OGP^{719}OGAAGK	0	1816.90	2	909.45	7.7
			1	1832.89	2	917.45	7.5
			2	1848.89	2	925.44	7.3
			3	1864.88	2	933.44	7.1

[a] P in boldface with a number indicates the potential 3-Hyp site.

and adult rats (18 months). Although α1(I) Pro^{986} was almost completely hydroxylated in both young and adult rats (Fig. 4*A*), varied prolyl 3-hydroxylation was observed for α1(I) Pro^{707,716,719} (0–2 × 3-Hyp; 3 × 3-Hyp peptide was under the detection limit) and α2(I) Pro^{707,716,719} (0–3 × 3-Hyp) (Fig. 4, *B* and *C*). It was evident that the prolyl 3-hydroxylation level at α1/α2(I) Pro^{707,716,719} was significantly higher in adult rats compared with young rats. Prolyl 3-hydroxylation at the newly identified modification sites (Pro^{716} and Pro^{719}) was not detected for skin and bone type I collagens (Fig. 5).

We further estimated the influence of aging on prolyl 3-hydroxylation at each modification site in tendon type I collagen by analyzing tail tendons from 0.5–18-month-old rats (Fig. 6*C*). At the primary site, α1(I) Pro^{986}, the relative abundance of 3-Hyp was extremely high in 0.5-month-old tendon (96.9%) and remained at a high level during aging until 18 months (90.3%). In contrast, α1(I) Pro^{707,716,719} was rarely occupied by 3-Hyp in the 0.5-month-old tendon (8.3% for 1 × 3-Hyp, 1.4% for 2 × 3-Hyp). However, significant increases in 3-Hyp at the sites were observed until 3 months of age (48.2% for 1 × 3-Hyp, 7.2% for 2 × 3-Hyp). The basal level of prolyl 3-hydroxylation at α2(I) Pro^{707,716,719} in the young tendon was higher than that at α1(I) Pro^{707,716,719}. The relative abundance of 3-Hyp at 0.5 months of age was 42.0% for 1 × 3-Hyp, 0% for 2 × 3-Hyp, and 3.4% for 3 × 3-Hyp. The actual 3-Hyp level in the 3 × 3-Hyp peptide was probably close to 0% in the 0.5-month-old tendon, because the peptide peak overlapped with the nonspecific peak indicated in Fig. 4*C*. Higher resolution of LC separation or MS detection is required to accurately analyze the minor hydroxylation form. Similar to α1(I) Pro^{707,716,719}, the 3-Hyp level increased until 3 months of age (68.3% for 1 × 3-Hyp, 16.9% for 2 × 3-Hyp, and 8.2% for 3 × 3-Hyp). The absolute 3-Hyp content at each modification site is summarized in Table 2. Unlike tendon, the peptides containing 2 × or 3 × 3-Hyp, which represent prolyl 3-hydroxylation at α1/α2(I) Pro^{716} and Pro^{719}, were not detected for type I collagens from skin and bone (Fig.

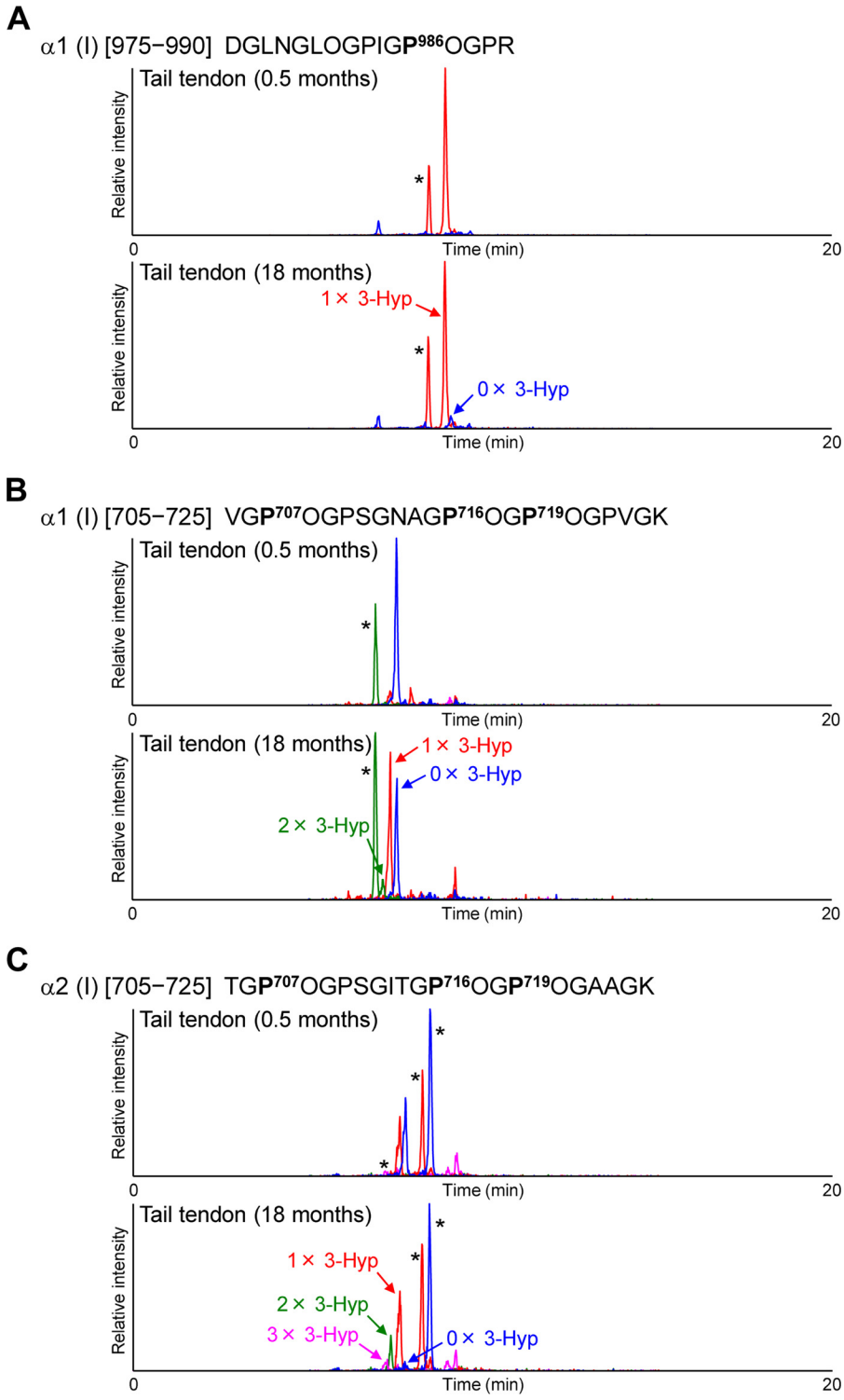

FIGURE 4. **Extracted ion chromatograms of tryptic peptides containing 3-Hyp sites of tail tendon type I collagen.** Tail tendon type I collagens from 0.5- and 18-month-old Sprague-Dawley rats were analyzed by LC-MS following trypsin digestion. Monoisotopic extracted ion chromatograms of peptides containing 0 × 3-Hyp (*blue*) and 1 × 3-Hyp (*red*) were extracted for (*A*) α1(I) DGLNGLOGPIGP^{986}OGPR, and those containing 0 × 3-Hyp (*blue*), 1 × 3-Hyp (*red*), 2 × 3-Hyp (*green*), and 3 × 3-Hyp (*pink*) were extracted for (*B*) α1(I) VGP^{707}OGPSGNAGP^{716}OGP^{719}OGPVGK and (*C*) α2(I) TGP^{707}OGPSGITGP^{716}OGP^{719}OGAAGK. Detailed information for these 3-Hyp-containing peptides is summarized in Table 1. The 3 × 3-Hyp peptide in *B* was under the detection limit. *Asterisks* indicate nonspecific peaks or other collagen-derived peptide peaks whose *m/z* overlapped with the monoisotopic *m/z* ranges of the 3-Hyp-containing peptides.

6, *A* and *B*). Consistent with the amino acid analysis shown in Fig. 1, *A* and *B*, the 3-Hyp levels at both α1(I) Pro^{986} and α1/α2(I) Pro^{707} were slightly decreased in skin (Fig. 6*A*) and nearly unchanged in bone (Fig. 6*B*) with age.

The increases in prolyl 3-hydroxylation at α1/α2 (I) Pro^{707}, Pro^{716}, and Pro^{719} during young ages were similarly observed in collagen from Achilles tendon and patellar tendon (data not shown). In addition, the site-specific altera-

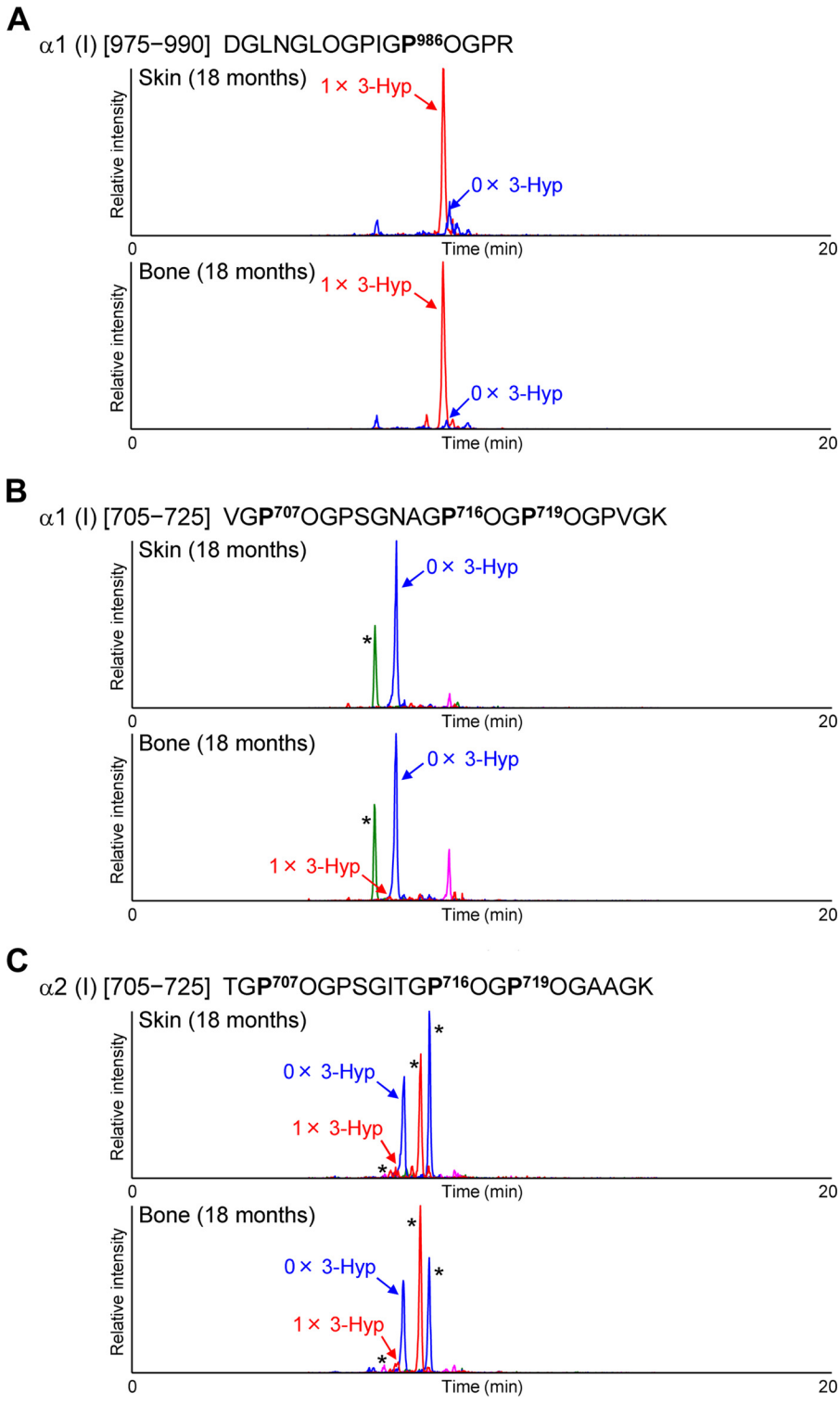

FIGURE 5. **Extracted ion chromatograms of tryptic peptides containing 3-Hyp sites of type I collagens from skin and bone of 18-month-old rats.** Monoisotopic extracted ion chromatograms of peptides containing 0 × 3-Hyp (*blue*) and 1 × 3-Hyp (*red*) were extracted for (*A*) α1(I) DGLNGLOGPIGP^{986}OGPR. In a similar manner, peptides containing 0 × 3-Hyp (*blue*), 1 × 3-Hyp (*red*), 2 × 3-Hyp (*green*), and 3 × 3-Hyp (*pink*) were extracted for (*B*) α1(I) VGP^{707}OGPSGNAGP^{716}OGP^{719}OGPVGK and (*C*) α2(I) TGP^{707}OGPSGITGP^{716}OGP^{719}OGAAGK. *Asterisks* indicate nonspecific peaks or other collagen-derived peptide peaks, whose *m/z* overlapped with the monoisotopic *m/z* ranges of the 3-Hyp-containing peptides.

tions in the 3-Hyp level were also observed in bovine tendon (Fig. 7). The total 3-Hyp content in adult Achilles tendon collagen was ~2.5 times higher than that in fetal Achilles tendon collagen (Fig. 7*A*). Furthermore, similar to rat collagen, prolyl 3-hydroxylation increased with aging at α1/α2(I) Pro^{707,716,719} in bovine Achilles tendon (Fig. 7, *C* and *D*), whereas the 3-Hyp level at α1(I) Pro^{986} slightly decreased during the same period (Fig. 7*B*).

Contamination with other tissue collagens, such as those from skin having low 3-Hyp contents, critically influences

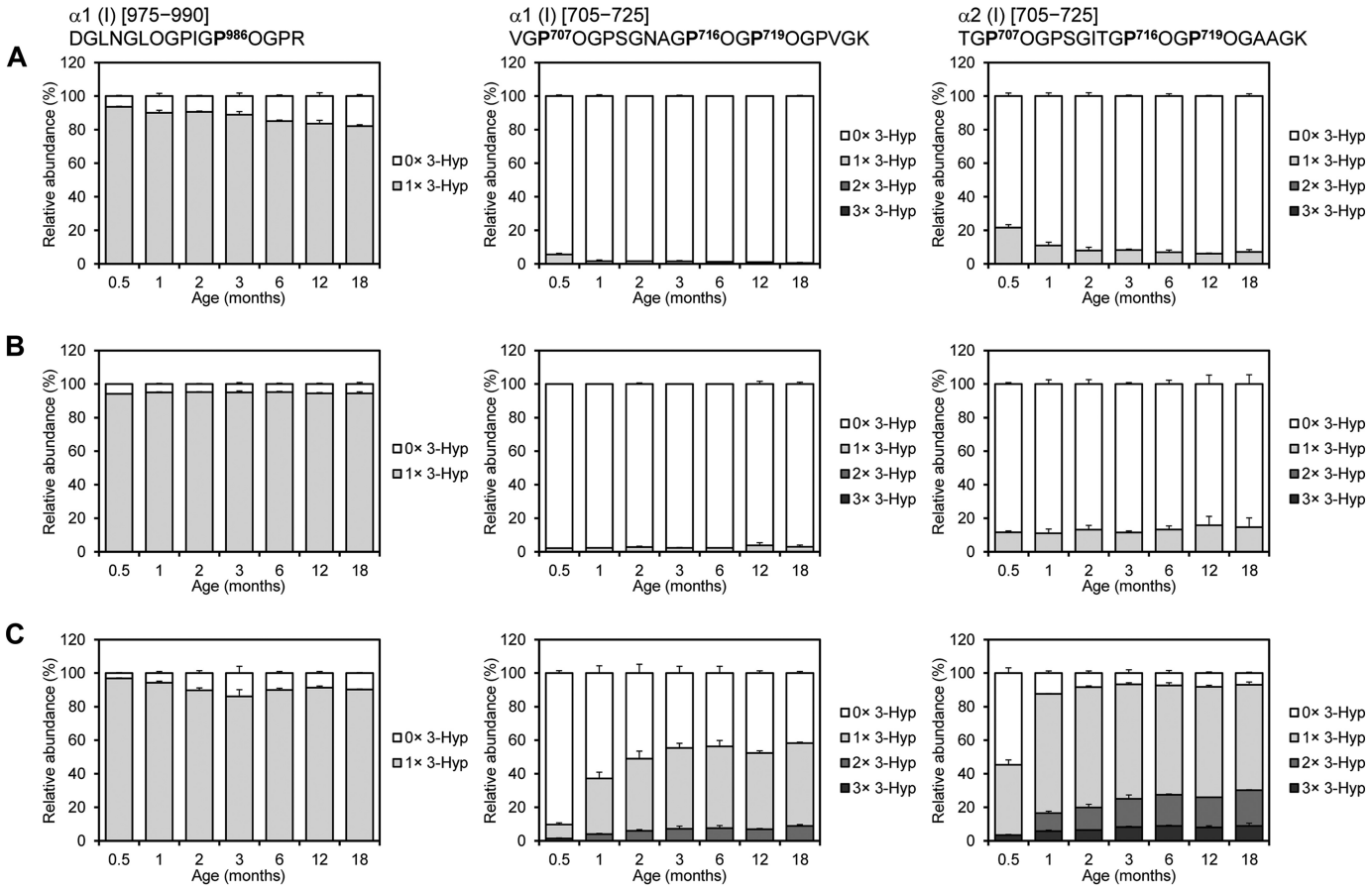

FIGURE 6. **Age-related alterations in prolyl 3-hydroxylation at specific sites in type I collagens from different tissues.** Tryptic peptides of skin (*A*), bone (*B*), and tail tendon (*C*) collagens from 0.5–18-month-old Sprague-Dawley rats were analyzed by LC-MS. The relative abundance of prolyl 3-hydroxylation in tryptic peptides containing α1(I) Pro^{986} (0–1 × 3-Hyp) was semi-quantitatively estimated from the peak area ratio of the extracted ion chromatograms of each species shown in Fig. 4. The relative abundance of prolyl 3-hydroxylation at α1(I) $Pro^{707,716,719}$ (0–2 × 3-Hyp) and α2(I) $Pro^{707,716,719}$ (0–3 × 3-Hyp) was estimated in a similar manner. The data represent the mean ± S.D. ($n = 3$).

TABLE 2

Absolute 3-Hyp contents in tail tendon collagen from 0.5–18-month-old rats obtained by two analytical approaches

		Site-specific 3-Hyp content			
	Total 3-Hyp content[a]	α1(I) Pro^{986}[b]	α1(I) $Pro^{707,716,719}$[b]	α2(I) $Pro^{707,716,719}$[b]	Sum[c]
	residues/1000 total residues		*residues/site*		*residues/chain*
0.5 months	0.77	0.97	0.11	0.52	0.89
1 month	1.68	0.94	0.41	1.10	1.27
2 months	2.14	0.90	0.55	1.18	1.36
3 months	2.37	0.86	0.63	1.27	1.41
6 months	2.44	0.90	0.64	1.29	1.45
12 months	2.41	0.91	0.59	1.26	1.42
18 months	2.71	0.90	0.67	1.32	1.49

[a] Total 3-Hyp content was calculated from the results in Fig. 1 using the predetermined 3-Hyp content in SI-collagen, and expressed as residues/1000 total residues.
[b] Site-specific 3-Hyp content at each modification site was calculated from the results shown in Fig. 6.
[c] Sum of the site-specific 3-Hyp contents was estimated according to the following formula: (2 × [α1(I) Pro^{986}] + 2 × [α1(I) $Pro^{707,716,719}$] + [α2(I) $Pro^{707,716,719}$])/3, and expressed as residues/chain, which nearly corresponds to residues/1000 total residues for the total 3-Hyp content. The data represent the mean ($n = 3$).

the quantitative accuracy of the estimation of 3-Hyp contents in tendon samples. To ensure the purity of the tissue collagen samples in the present study, we monitored a tryptic peptide from type I collagen α2 chain in which Pro^{891} is potentially hydroxylated to 4-Hyp. We found tissue-specific and stationary hydroxylation rates at α2(I) Pro^{891} that were ~100% in skin, 60–80% in bone, and 20–40% in tendon (Fig. 8). The tendon samples from rats at 0.5–18 months of age were confirmed to be pure tendon collagen using the tissue marker peptide (Fig. 8*D*).

Discussion

In this study, our highly accurate analytical approaches using LC-MS demonstrated that 3-Hyp is significantly increased in type I collagen from rat tail tendon between 0.5 and 3 months after birth at consecutive modification sites, including Pro^{707}, Pro^{716}, and Pro^{719}. Except for these increases at young ages in tendon, no other notable increases in 3-Hyp were observed for collagen from skin, bone, and tendons. The maintained level of 3-Hyp after the drastic increases in tendon indicates that the

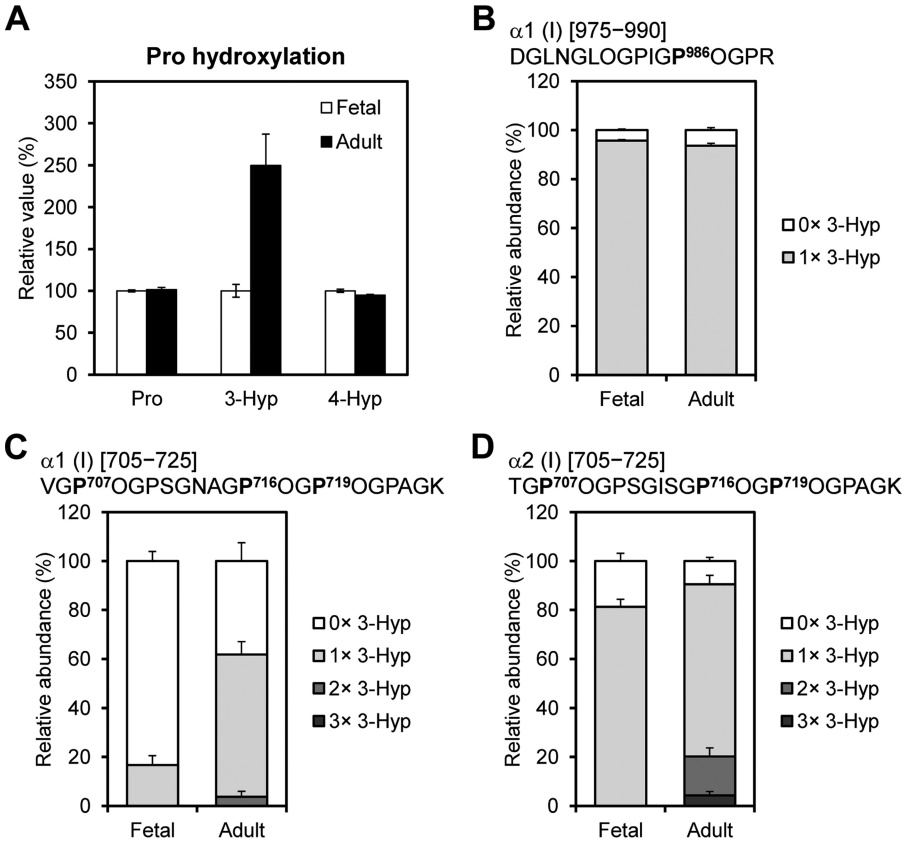

FIGURE 7. **Age-related alterations in prolyl 3-hydroxylation in bovine tendon collagen.** Total and site-specific prolyl 3-hydroxylation in collagen were compared between fetal and adult bovine Achilles tendons. *A,* Pro, 3-Hyp, and 4-Hyp were quantified by LC-MS following acid hydrolysis with SI-collagen. The data represent the mean ± S.D. ($n = 3$), with fetal tendon set at 100%. *B–D,* the relative abundance of prolyl 3-hydroxylation in tryptic peptides containing α1(I) Pro^{986} (0–1 × 3-Hyp), α1(I) $Pro^{707,716,719}$ (0–2 × 3-Hyp), and α2(I) $Pro^{707,716,719}$ (0–3 × 3-Hyp) was semi-quantitatively estimated from the peak area ratio of the monoisotopic extracted ion chromatograms of each species in LC-MS. The data represent the mean ± S.D. ($n = 3$).

alterations are correlated with tissue development in tendon, rather than with aging. 3-Hyp was suggested to be involved in collagen fibril assembly (12, 18, 29). Previous studies indicated that the collagen fibril diameter in rat tail tendon increases rapidly until 3–4 months after birth (30–32). These observations correspond well to the specific increases in 3-Hyp during 0.5–3 months of age obtained in the present study. Although KO of P3H1, which is responsible for prolyl 3-hydroxylation at α1(I) Pro^{986}, was reported to result in a slight increase in collagen fibril diameter in newborn tendon (15), we suggest that at least 3-Hyp at α1/α2(I) Pro^{707}, Pro^{716}, and Pro^{719} contributes to the incremental regulation of fibril diameter. The 3-Hyp content in tendon is known to be higher than those in skin and bone (5, 33); however, the 3-Hyp level did not differ much among these tissues at 0.5 months of age (Fig. 6). Subsequently, until 3 months of age, 3-Hyp was significantly increased in tendon, slightly decreased in skin, and constant in bone. The unique tissue-specific properties of collagen fibrils may partly arise through the alterations in prolyl 3-hydroxylation at the early stage of development. The bovine data comparing fetal Achilles tendon and adult Achilles tendon (Fig. 7) suggest that the developmental alterations in prolyl 3-hydroxylation are common to other animals, probably including humans.

Developmental regulation of prolyl 3-hydroxylation in the C-terminal $(GPP)_n$ motif of tendon type I collagen was mentioned in a recent paper comparing young and adult tendons (23). We were not able to detect tryptic peptides containing the C-terminal $(GPP)_n$ motif, because pepsin-extracted collagen shows heterogeneous C-terminal cleavage sites that may prevent reliable analysis of 3-hydroxylation at these sites (23). Other collagen extraction methods using acid, heating, or cyanogen bromide are required to analyze the C-terminal modification sites. However, the modification level at the C-terminal sites can be roughly estimated by subtracting the sum of the site-specific 3-Hyp contents (Fig. 6) from the total 3-Hyp content (Fig. 1). For example, in the 3-month-old tendon, the total 3-Hyp content and sum of the site-specific 3-Hyp contents were ~2.4 and 1.4 residues/1000 total residues, respectively (Table 2). The 3-Hyp content in the C-terminal $(GPP)_n$ motif is thus approximated to 1.0 residue/1000 total residues, provided there are no more unidentified 3-Hyp sites. In contrast, the total 3-Hyp content and sum of the site-specific 3-Hyp contents in the 0.5-month-old tendon were ~0.8 and 0.9 residues/1000 total residues, respectively (Table 2), indicating almost no 3-Hyp occupies the C-terminal region. Thus, it seems that prolyl 3-hydroxylation also increases in the C-terminal $(GPP)_n$ motif during the developmental stage in tendon.

α1(I) Pro^{986} is hydroxylated by P3H1 (8), whereas α1(I)/α2(I) Pro^{707} and the C-terminal $(GPP)_n$ motif are mainly hydroxylated by P3H2 (19). In the present study, the order of prolyl 3-hydroxylation at the consecutive 3-Hyp sites appeared to be $Pro^{707} > Pro^{716} > Pro^{719}$ based on the MS/MS fragmentation

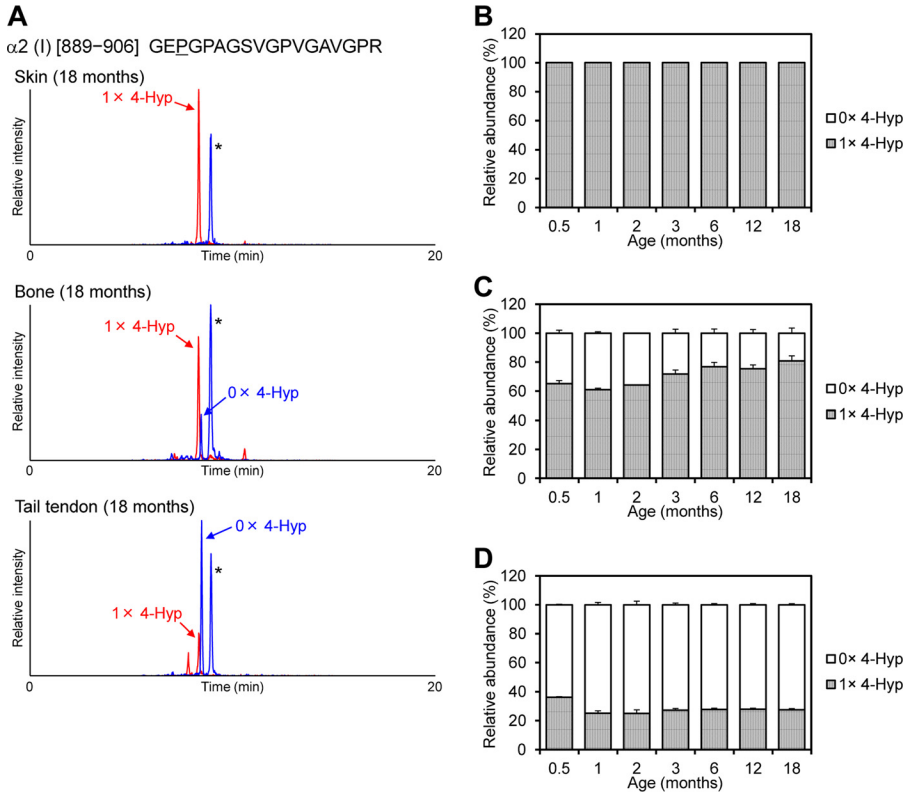

FIGURE 8. **Relative abundance of prolyl 4-hydroxylation in the tryptic tissue marker peptide from different tissues.** Tryptic digests of collagens from skin, bone, and tail tendon were analyzed by LC-MS. *A*, monoisotopic extracted ion chromatograms of α2(I) GEP[891]GPAGSVGPVGAVGPR containing 0 × 4-Hyp (*m/z* 780.90–781.40, *z* = 2) and 1 × 4-Hyp (*m/z* 788.90–789.40, *z* = 2) are shown for collagens from skin, bone, and tail tendon of 18-month-old Sprague-Dawley rats. *Asterisks* indicate nonspecific peaks or other collagen-derived peptide peaks, whose *m/z* overlapped with the monoisotopic *m/z* ranges of the tissue marker peptide. The relative abundance of prolyl 4-hydroxylation (0–1 × 4-Hyp) at α2(I) Pro[891] was semi-quantitatively estimated for collagen from skin (*B*), bone (*C*), and tail tendon (*D*) of 0.5–18-month-old Sprague-Dawley rats. The data represent the mean ± S.D. (*n* = 3).

patterns, as mentioned under "Results" (*upper spectra* in Fig. 2, *A* and *B*). The definitive order of prolyl 3-hydroxylation suggests that Pro[716] and Pro[719] are modified by P3H2 after the hydroxylation of Pro[707]. In addition, the 3-Hyp level at the highly hydroxylated α1(I) Pro[986] in tendon decreased slightly with age, especially until 3 months of age, in contrast to the consecutive modification sites at which the 3-Hyp level increased significantly during the same period. We suspect that opposite outcomes arise through the difference in the responsible P3H enzyme. This hypothesis can be confirmed by analyzing the P3H2-null tendon.

In this study, we focused on prolyl 3-hydroxylation of collagen. However, amino acid analysis showed significant alterations in glycosylation of Hyl in skin, bone, and tail tendons with different profiles (Fig. 1), which would partly arise through cross-link formation (34, 35). Hyl glycosides (GHL and GGHL) are minor modifications similar to 3-Hyp, and are thus difficult to analyze accurately. However, we previously developed a specific purification method for GHL/GGHL-containing peptides to allow highly sensitive analysis of these minor glycosylations (13, 36). Although age-related analysis of GHL/GGHL has been already conducted at the amino acid level (25), site-specific analysis using this method may lead to further novel findings.

We have observed that the 3-Hyp level in collagen shows individual variability and is relatively easy to alter in response to physiological and pathological conditions compared with 4-Hyp (data not shown). Prolyl 3-hydroxylation might be responsible for temporal regulation of the mechanical/structural properties of connective tissues, and the 3-Hyp level could be an indicator of the qualitative status of tissue collagens. Although the biological significance of the increases in 3-Hyp in tendon remains to be clarified, the tissue-, developmental stage-, and modification site-specific alterations imply its key role in the tissue. Further studies are warranted for this minor, but important, modification.

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References

1. Berg, R. A., and Prockop, D. J. (1973) The thermal transition of a non-hydroxylated form of collagen. Evidence for a role for hydroxyproline in stabilizing the triple-helix of collagen. *Biochem. Biophys. Res. Commun.* **52,** 115–120
2. Ogle, J. D., Arlinghaus, R. B., and Lgan, M. A. (1962) 3-Hydroxyproline, a new amino acid of collagen. *J. Biol. Chem.* **237,** 3667–3673
3. Mizuno, K., Peyton, D. H., Hayashi, T., Engel, J., and Bächinger, H. P. (2008) Effect of the -Gly-3(S)-hydroxyprolyl-4(R)-hydroxyprolyl-tripeptide unit on the stability of collagen model peptides. *FEBS J.* **275,** 5830–5840
4. Hudson, D. M., and Eyre, D. R. (2013) Collagen prolyl 3-hydroxylation: a major role for a minor post-translational modification? *Connect. Tissue Res.* **54,** 245–251
5. Taga, Y., Kusubata, M., Ogawa-Goto, K., and Hattori, S. (2014) Stable

isotope-labeled collagen: a novel and versatile tool for quantitative collagen analyses using mass spectrometry. *J. Proteome Res.* **13,** 3671–3678
6. Fietzek, P. P., Rexrodt, F. W., Wendt, P., Stark, M., and Kühn, K. (1972) The covalent structure of collagen. Amino-acid sequence of peptide 1-CB6-C2. *Eur. J. Biochem.* **30,** 163–168
7. Vranka, J. A., Sakai, L. Y., and Bächinger, H. P. (2004) Prolyl 3-hydroxylase 1, enzyme characterization and identification of a novel family of enzymes. *J. Biol. Chem.* **279,** 23615–23621
8. Vranka, J. A., Pokidysheva, E., Hayashi, L., Zientek, K., Mizuno, K., Ishikawa, Y., Maddox, K., Tufa, S., Keene, D. R., Klein, R., and Bächinger, H. P. (2010) Prolyl 3-hydroxylase 1 null mice display abnormalities in fibrillar collagen-rich tissues such as tendons, skin, and bones. *J. Biol. Chem.* **285,** 17253–17262
9. 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, U., Byers, P. H., Weis, M., Fernandes, R. J., Eyre, D. R., Yao, Z., Boyce, B. F., and Lee, B. (2006) CRTAP is required for prolyl 3-hydroxylation and mutations cause recessive osteogenesis imperfecta. *Cell* **127,** 291–304
10. Cabral, W. A., Chang, W., Barnes, A. M., Weis, M., Scott, M. A., Leikin, S., Makareeva, E., Kuznetsova, N. V., Rosenbaum, K. N., Tifft, C. J., Bulas, D. I., Kozma, C., Smith, P. A., Eyre, D. R., and Marini, J. C. (2007) Prolyl 3-hydroxylase 1 deficiency causes a recessive metabolic bone disorder resembling lethal/severe osteogenesis imperfecta. *Nat. Genet.* **39,** 359–365
11. van Dijk, F. S., Nesbitt, I. M., Zwikstra, E. H., Nikkels, P. G., Piersma, S. R., Fratantoni, S. A., Jimenez, C. R., Huizer, M., Morsman, A. C., Cobben, J. M., van Roij, M. H., Elting, M. W., Verbeke, J. I., Wijnaendts, L. C., Shaw, N. J., Högler, W., McKeown, C., Sistermans, E. A., Dalton, A., Meijers-Heijboer, H., and Pals, G. (2009) PPIB mutations cause severe osteogenesis imperfecta. *Am. J. Hum. Genet.* **85,** 521–527
12. Weis, M. A., Hudson, D. M., Kim, L., Scott, M., Wu, J. J., and Eyre, D. R. (2010) Location of 3-hydroxyproline residues in collagen types I, II, III, and V/XI implies a role in fibril supramolecular assembly. *J. Biol. Chem.* **285,** 2580–2590
13. Taga, Y., Kusubata, M., Ogawa-Goto, K., and Hattori, S. (2012) Development of a novel method for analyzing collagen *O*-glycosylations by hydrazide chemistry. *Mol. Cell Proteomics* **11,** M111.010397
14. Yang, C., Park, A. C., Davis, N. A., Russell, J. D., Kim, B., Brand, D. D., Lawrence, M. J., Ge, Y., Westphall, M. S., Coon, J. J., and Greenspan, D. S. (2012) Comprehensive mass spectrometric mapping of the hydroxylated amino acid residues of the α1(V) collagen chain. *J. Biol. Chem.* **287,** 40598–40610
15. Pokidysheva, E., Zientek, K. D., Ishikawa, Y., Mizuno, K., Vranka, J. A., Montgomery, N. T., Keene, D. R., Kawaguchi, T., Okuyama, K., and Bächinger, H. P. (2013) Posttranslational modifications in type I collagen from different tissues extracted from wild type and prolyl 3-hydroxylase 1 null mice. *J. Biol. Chem.* **288,** 24742–24752
16. Perdivara, I., Yamauchi, M., and Tomer, K. B. (2013) Molecular characterization of collagen hydroxylysine: glycosylation by mass spectrometry: current status. *Aust. J. Chem.* **66,** 760–769
17. Hudson, D. M., Weis, M., and Eyre, D. R. (2011) Insights on the evolution of prolyl 3-hydroxylation sites from comparative analysis of chicken and *Xenopus* fibrillar collagens. *PLoS ONE* **6,** e19336
18. Eyre, D. R., Weis, M., Hudson, D. M., Wu, J. J., and Kim, L. (2011) A novel 3-hydroxyproline (3Hyp)-rich motif marks the triple-helical C terminus of tendon type I collagen. *J. Biol. Chem.* **286,** 7732–7736
19. Hudson, D. M., Joeng, K. S., Werther, R., Rajagopal, A., Weis, M., Lee, B. H., and Eyre, D. R. (2015) Post-translationally abnormal collagens of prolyl 3-hydroxylase-2 null mice offer a pathobiological mechanism for the high myopia linked to human LEPREL1 mutations. *J. Biol. Chem.* **290,** 8613–8622
20. Mordechai, S., Gradstein, L., Pasanen, A., Ofir, R., El Amour, K., Levy, J., Belfair, N., Lifshitz, T., Joshua, S., Narkis, G., Elbedour, K., Myllyharju, J., and Birk, O. S. (2011) High myopia caused by a mutation in LEPREL1, encoding prolyl 3-hydroxylase 2. *Am. J. Hum. Genet.* **89,** 438–445
21. Guo, H., Tong, P., Peng, Y., Wang, T., Liu, Y., Chen, J., Li, Y., Tian, Q., Hu, Y., Zheng, Y., Xiao, L., Xiong, W., Pan, Q., Hu, Z., and Xia, K. (2014) Homozygous loss-of-function mutation of the LEPREL1 gene causes severe non-syndromic high myopia with early-onset cataract. *Clin. Genet.* **86,** 575–579
22. Pokidysheva, E., Boudko, S., Vranka, J., Zientek, K., Maddox, K., Moser, M., Fässler, R., Ware, J., and Bächinger, H. P. (2014) Biological role of prolyl 3-hydroxylation in type IV collagen. *Proc. Natl. Acad. Sci. U.S.A.* **111,** 161–166
23. Hudson, D. M., Werther, R., Weis, M., Wu, J. J., and Eyre, D. R. (2014) Evolutionary origins of C-terminal (GPP)n 3-hydroxyproline formation in vertebrate tendon collagen. *PLoS ONE* **9,** e93467
24. Barnes, M. J., Constable, B. J., Morton, L. F., and Royce, P. M. (1974) Age-related variations in hydroxylation of lysine and proline in collagen. *Biochem. J.* **139,** 461–468
25. Murai, A., Miyahara, T., and Shiozawa, S. (1975) Age-related variations in glycosylation of hydroxylysine in human and rat skin collagens. *Biochim. Biophys. Acta* **404,** 345–348
26. Taga, Y., Kusubata, M., Ogawa-Goto, K., and Hattori, S. (2014) Highly accurate quantification of hydroxyproline-containing peptides in blood using a protease digest of stable isotope-labeled collagen. *J. Agric. Food Chem.* **62,** 12096–12102
27. Ueno, T., Tanaka, K., Kaneko, K., Taga, Y., Sata, T., Irie, S., Hattori, S., and Ogawa-Goto, K. (2010) Enhancement of procollagen biosynthesis by p180 through augmented ribosome association on the endoplasmic reticulum in response to stimulated secretion. *J. Biol. Chem.* **285,** 29941–29950
28. Ueno, T., Kaneko, K., Sata, T., Hattori, S., and Ogawa-Goto, K. (2012) Regulation of polysome assembly on the endoplasmic reticulum by a coiled-coil protein, p180. *Nucleic Acids Res.* **40,** 3006–3017
29. Hudson, D. M., Kim, L. S., Weis, M., Cohn, D. H., and Eyre, D. R. (2012) Peptidyl 3-hydroxyproline binding properties of type I collagen suggest a function in fibril supramolecular assembly. *Biochemistry* **51,** 2417–2424
30. Parry, D. A., and Craig, A. S. (1977) Quantitative electron microscope observations of the collagen fibrils in rat-tail tendon. *Biopolymers* **16,** 1015–1031
31. Parry, D. A., and Craig, A. S. (1978) Collagen fibrils and elastic fibers in rat-tail tendon: an electron microscopic investigation. *Biopolymers* **17,** 843–845
32. Scott, J. E., Orford, C. R., and Hughes, E. W. (1981) Proteoglycan-collagen arrangements in developing rat tail tendon. An electron microscopical and biochemical investigation. *Biochem. J.* **195,** 573–581
33. Stoltz, M., Furthmayr, H., and Timpl, R. (1973) Increased lysine hydroxylation in rat bone and tendon collagen and localization of the additional residues. *Biochim. Biophys. Acta* **310,** 461–468
34. Yamauchi, M., and Sricholpech, M. (2012) Lysine post-translational modifications of collagen. *Essays Biochem.* **52,** 113–133
35. Terajima, M., Perdivara, I., Sricholpech, M., Deguchi, Y., Pleshko, N., Tomer, K. B., and Yamauchi, M. (2014) Glycosylation and cross-linking in bone type I collagen. *J. Biol. Chem.* **289,** 22636–22647
36. Taga, Y., Kusubata, M., Ogawa-Goto, K., and Hattori, S. (2013) Site-specific quantitative analysis of overglycosylation of collagen in osteogenesis imperfecta using hydrazide chemistry and SILAC. *J. Proteome Res.* **12,** 2225–2232