Heterogeneity in Proline Hydroxylation of Fibrillar Collagens Observed by Mass Spectrometry

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Abstract

Collagen is the major protein in the extracellular matrix and plays vital roles in tissue development and function. Collagen is also one of the most processed proteins in its biosynthesis. The most prominent post-translational modification (PTM) of collagen is the hydroxylation of Pro residues in the Y-position of the characteristic (Gly-Xaa-Yaa) repeating amino acid sequence of a collagen triple helix. Recent studies using mass-spectrometry (MS) and tandem MS sequencing (MS/MS) have revealed unexpected hydroxylation of Pro residues in the X-positions (X-Hyp). The newly identified X-Hyp residues appear to be highly heterogeneous in location and percent occupancy. In order to understand the dynamic nature of the new X-Hyps and their potential impact on applications of MS and MS/MS for collagen research, we sampled four different collagen samples using standard MS and MS/MS techniques. We found considerable variations in the degree of PTMs of the same collagen from different organisms and/or tissues. The rat tail tendon type I collagen is particularly variable in terms of both over-hydroxylation of Pro in the X-position and under-hydroxylation of Pro in the Y-position. In contrast, only a few unexpected PTMs in collagens type I and type III from human placenta were observed. The reproducibility of the different sequencing efforts of the same sample is also limited especially when the modified species are present at a low population, presumably due to the unpredictable nature of the ionization process. Additionally, despite the heterogeneous preparation and sourcing, collagen samples from commercial sources do not show elevated variations in PTMs compared to samples prepared from a single tissue and/or organism. These findings will contribute to the growing body of information regarding the PTMs of collagen by MS technology, and culminate to a more comprehensive understanding of the extent and the functional roles of the PTMs of collagen.

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27 **Abbreviations page:**

28 Both the single letter and the three letter abbreviations of an amino acid will be used with the
 29 following additions: Hyp or O stands for 4R-hydroxylated proline and 3Hyp stands for 3-
 30 hydroxylated proline. When needed for clarity, the lower case single letter abbreviation will be
 31 used to represent the genomic DNA sequence, and upper case ones the sequence seen in the
 32 peptides.

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Introduction

The new technologies in mass spectrometry (MS) have transformed collagen research in recent years, and even expanded the field of collagen research to include archeology for the study of ancient species (1, 2). The high sensitivity of MS and tandem MS sequencing (MS/MS) also led to the identification of new post-translational modifications (PTMs) in fibrillar collagen (3-10). Fibrillar collagen is the major protein of bone, skin, cartilage, and blood vessel walls, and plays critical roles in many physiological and pathological events (11, 12). The newly discovered PTMs that are of particular interest is the 3-hydroxyproline residues (3Hyp, or 3O) in unexpected locations, since mutations in the enzymes involved in the formation of 3Hyp have been linked to severe cases of Osteogenesis Imperfecta (the brittle bone diseases) (13, 14). Yet, emerging from further studies of 3Hyp is an increasingly more heterogeneous pattern in terms of number, location, and the percent occupancy of this PTM (4, 10). Such varied patterns of 3Hyp make it challenging to pin down the specific molecular interactions involving 3Hyp. At the same time, collagens have been used as biomarkers for disease detection, species identification, and investigations of the involvement of collagen in cancer metastasis, tissue remodeling, the homeostasis of extracellular matrix and cell signaling, and the mineralization process of bones, to name just a few (15-18). The unpredictable nature of prolyl-3-hydroxylation has hampered the application of MS in these and other related research that rely on a precise knowledge of the genomic sequence and the PTMs of specific segments of collagen. Both the functional research of PTMs and the applications of MS for a broad range of quantitative studies of collagen depend on a comprehensive understanding of the extent and the variations of PTMs.

Collagen is a highly processed protein during its biosynthesis (11, 12). The gene products of collagen (often referred to as the α chains) usually comprise a triple helix domain together

with propeptides at both the N- and C-termini. The triple helix domain of fibrillar collagens, which include collagens type I-III, V and XI, often contains more than 1000 amino acid residues in the uninterrupted (Gly-Xaa-Yaa) repeating amino acid sequence; while the Xaa and Yaa can be any amino acid. Collagen is known for its exceedingly high content of Pro: about 10-12% of the residues at each of the X- and Y-positions are Pro(19). In addition to the processing of the N- and C-propeptides, the residues of the triple helix domain are also heavily hydroxylated and glycosylated during the post-translational modification (20, 21). The major PTM of collagen is the prolyl-4-hydroxylation of Pro residues in the Y-position of the (Gly-Xaa-Yaa) triad (11). This is a stable, invariant modification; nearly all Pro residues in the Y-position are hydroxylated to 4R-hydroxyproline (4Hyp). Some Lys residues in the Y-position are also hydrolyzed to hydroxylysine (Hyl), which are often glycosylated and/or form covalent cross-links through an oxidation process catalyzed by lysyl oxidase during tissue maturation (22, 23). Until recently, only one Pro residue in the X-position, Pro⁹⁸⁶ of the $\alpha 1$ chain of type I and type II collagen (the $\alpha 1(I)$ chain and the $\alpha 1(II)$ chain, respectively) was known to be a 3R-hydroxyproline (3Hyp), in which the hydroxyl group is appended on the β -carbon (the 3-position) of the pyrrole ring of the Pro instead of on the γ -carbon (the 4-position) of the pyrrole ring as in the case of 4Hyp (24). A more recent study using MS and MS/MS found several other Pro in the X-position of fibrillar collagens were also hydroxylated, including Pro⁷⁰⁷ of $\alpha 2(I)$ chain, Pro⁹⁴⁴ of $\alpha 1(II)$ and $\alpha 2(V)$ chains, and Pro⁴⁷⁰ of $\alpha 2(V)$ chain (9, 10). Some of the X-Hyps were later confirmed to be a 3Hyp by Edman sequencing. Multiple 3Hyps were also found in the repeating genomic sequence of gly-pro-pro (gpp) at the C-terminal end of the $\alpha 1(I)$ and $\alpha 2(I)$ chains, although their exact locations could not be specified (6). Differing from the 3Hyp⁹⁸⁶, which is invariant and has a nearly 100% occupancy, the newly discovered X-Hyp and 3Hyp residues are often found in a

mixed population having the percentage of hydroxylated moiety ranging from 10% to 80% depending on the location, the type of collagen, the tissue, and the organism (4, 6, 9, 10).

The PTMs of collagen are considered to be essential for its secretion and self-assembly, and the immune responses of tissues (10, 20, 25-29), although much of the molecular mechanisms of their involvements remain unclear. Impaired prolyl-4-hydroxylation is the major cause of the condition of scurvy linked to the fragility in skin, blood vessels, and dentine (20). In this case, the tissue fragility was linked to the decreased stability of the collagen triple helix due to the lack of 4Hyp. Studies using triple helical peptides have firmly established the significant stabilizing effects of a 4Hyp in the Y-position compared to that of a Pro (30-33). Organisms having a higher physiological temperature often have a higher content of 4Hyp (34). The Hyl related glycosylation and cross-links were also considered an important part of the fibril stability, and the extent of the modification increases with the advance of the developmental stages(35-37). The understanding of the function of 3Hyp is more limited, except it is important for bone health (13, 38, 39).

Determining the biological functions of PTMs in fibrillar collagen is often confounded by the complex structural hierarchy of collagen fibrils (11, 12). The processed α chains of fibrillar collagen frequently have over 1000 amino acid residues. Three such α chains, they can be identical or different, come together in parallel to form the rod-shaped collagen triple helix, each about 300 nm in length. The Gly residues at every third position are packed at the center of the helix, while the side chains of the X and Y residues are largely exposed to solvent. The triple helix, however, is not the functional unit of collagen. The triple helices further self-associate laterally in a specific manner to form collagen fibrils having a unique 67 nm axially repeating structure known as the *D*-period. Any modifications of residues in X or Y positions can

potentially impact the stability of the triple helix, the molecular recognition process during fibrillogenesis, and the interactions of collagen fibrils with cell receptors and other macromolecules during tissue development and function. Eyre and colleagues postulate the newly discovered 3Hyps are involved in fibrillogenesis because the locations of some of them are approximately a *D*-period apart (6, 8). However, considering the low occupancy at some of the locations, it remains to be evaluated at what extent of the hydroxylation the purported interactions involving 3Hyp will have a sustained impact on the fibril assembly. A systematic MS/MS characterization of rat type I collagen found an increased occupancy of 3Hyp with the developmental stages in rat-tail tendon, but the same study also reported a relatively constant extent of hydroxylation of type I collagen in bones and in skin (4). If the prevalent presence of 4Hyp is consistent with its structural role on the overall stability of the triple helix and the collagen fibrils, the highly diverged and sporadic presence of 3Hyp may suggest a more dynamic role for this unique collagen PTM, which may involve the hydroxylation of specific X-Pro residues at specific stages of development and/or in response to specific cues of the extracellular matrix. Similar dynamic PTMs were reported to be part of the ‘epigenetic code’ of histone and other proteins (40). Under such a dynamic regulation, 3Hyps are likely to present in low quantity: a few selected X-positions (comparing to more than 100 4Hyp residues in the Y-positions) in each α chain, and only in a subpopulation of the α chain. Both the locations of 3Hyp and the occupancy at a specific location may also vary depending on the physiological conditions of the tissues. Deciphering such dynamic patterns of 3-hydroxylation would require both robust protocols of quantitative detection and extensive sampling of collagens from different sources.

It is often difficult to delineate the variations of a PTM as part of the dynamic epigenetic regulation from the statistical nature of the techniques used for detection and/or for sample

handling. MS and MS/MS are the technique of choice for studying PTMs of a protein, yet quantitative analysis of protein samples is often complicated (41, 42). The situation is particularly challenging for collagen due to the repetitive sequences and the high content of Pro residues. Fragmentation of Pro containing peptides is often inefficient due to the well characterized ‘proline effect’ in MS/MS which predicts a biased, sequence dependent potential to fragment N-terminal to a Pro bond during collision induced dissociation (CID) (3, 10, 43-45). In the case of the detection of 3Hyp the high frequency of the genomic sequence of pro-gly-pro-pro moiety further complicated the precise localization of the hydroxyl group without a good series of fragmented ions. Aside from functional dynamics, there is often an innate level of variations in the PTMs of a protein between individuals and between different organisms. Without a known priori on the statistical distribution of the PTMs in a specific tissue at a specific developmental stage, even a carefully designed study can only reflect a statistical snapshot. Collagens produced by recombinant systems may appear to be a well-controlled source of more homogeneous collagens. However, the expression of a foreign gene(s) can skew the PTM processes in a host cell leading to a different PTM pattern (2).

To gain a better understanding of the average impact of the prolyl-hydroxylation in both the X and the Y positions and the reliability of the detection by standard MS techniques, we carried out MS/MS sequencing of several samples of collagen from commercial sources and of collagen isolated from tissues. Our study revealed a more varied nature of the hydroxylation of proline residues in the type I collagen and substantial differences in the hydroxylation pattern among different collagens. The commercial collagens are often scorned as being impure because their productions often rely on batch collection of samples from mixed sources. However, this ‘mixed’ nature of commercial collagens can be a good statistical representation of the overall

extent of different PTMs. Additionally, commercial collagens are very frequently used as standards for analytical analyses, and as extracellular matrix substitutes in various biological and biomedical studies that rely on specific interactions with residues on collagen including the PTMs. A better understanding of the PTMs of commercial collagen samples will enhance the sensitivity and reliability of these research efforts. Mapping out all the 3Hyp residues with the highest sensitivity and accuracy is not the main focus of this work. Rather, we seek to understand the reliability and reproducibility of the detection of unexpected hydroxylations using the standard MS approach. Furthermore, the uncertainties of PTMs on the X position complicate the fundamental premises of MS studies of collagen that assumes Pro in X-positions are unmodified, while those in the Y-position will inevitably have a mass increase of 16 due to the addition of the hydroxyl group. The finding of this work will, thus, contribute to both the understanding of the dynamics of 3Hyp and the applications of MS in other areas of collagen research.

Materials and Methods

Collagen preparation – Human collagen type III, human collagen type I, and rat collagen type I were purchased from Sigma. According to Sigma, human collagen type I and type III was purified from placenta, and rat collagen type I from tail tendon. The purchased collagens were solubilized in 20 mM acetic acid, pH 3, at 4 °C and 2.4 mg/mL. Fresh rat collagen type I was prepared from a single rat tail tendon following a procedure published by Dr. Sergey Leikin's group, and the precipitated collagen was solubilized in 20 mM acetic acid, pH 3, at 4 °C and 3.0 mg/mL (46). Collagen was mixed with 5X SDS sample loading buffer containing 60 mM Tris-HCl pH 6.8, 25% glycerol, 2% SDS, 350 mM DTT, and 0.1% bromophenol blue, and was run on

a 4-20% Precise gel or a 7.5% SDS PAGE gel and stained with coomassie blue. Bands of alpha chains were excised from the gels and submitted to The Rockefeller University for in-gel digestion and mass spectrometry analysis.

In-gel Trypsin Digestion – The gel bands were reduced with dithiothreitol for 45 minutes at 55 °C, and alkylated with iodoacetamide for 30 minutes at room temperature in the dark. 10 µL of 0.02 µg/µL trypsin in 50 mM NH₄HCO₃/0.1% octyl glucopyranoside (OGP)/ 5 mM calcium chloride was used to digest each sample overnight at 37 °C in 50 mM NH₄HCO₃. The digestion was stopped with the addition of 5 µL of 10% acetic acid, and the peptides were extracted first with 30% ACN/5% TFA and then with 50% ACN/5% TFA. The samples were dried down in a Speed Vac to a few microliters.

Trypsin In-Solution Digestion – The collagen solution was heat denatured and digested overnight with trypsin in 25 mM ammonium bicarbonate at 37 °C. The reaction was terminated by bringing the solution to a final concentration of 0.1% trifluoroacetic acid

MALDI-TOF MS Analysis - Matrix, α -cyano-4-hydroxycinnamic acid, was prepared as a saturated solution in 50% acetonitrile/0.1% trifluoroacetic acid. Solution digests of collagen were spotted 1:1 with matrix onto a sample plate and allowed to dry. All spectra were acquired using a Voyager-DE STR mass spectrometer (PE Biosystems, Foster City, CA) equipped with a pulse nitrogen laser (λ = 337 nm, 3 Hz frequency) in the reflectron positive ion, delayed extraction mode. Spectra from 100 individual laser shots were averaged.

LC-MS/MS Analysis – The in-gel digests and solution digest samples were chromatographed using a C₁₈ column on a Dionex HPLC eluted with a gradient of 0.1% formic

acid and 100% ACN and introduced into a mass spectrometer. The sequencing was done at The Rockefeller University Proteomics Center.

Data Base Search and Analysis – For peptide identification a Mascot (Matrix Science) search was performed. The .raw data files were converted to .dta or .mgf files which were then used to perform a search. The database User 0710 (in house database at Rockefeller Proteomic Center) containing human collagen type I and type III, and rat collagen type I, was used with oxidized methionine, proline and lysine as variable modifications. The Swiss Prot database was used for protein identification confirmation. For the freshly prepared rat tail, a summary report file was created using Discoverer version: 1.3.0.339, with the signal to noise threshold set to 1.5. Peaks6 software was also used. The reliability of the MS/MS largely depends on the detection of the fragmented ions. All the sequencing was determined using the Mascot search engine with a score of 40 or higher, and a probability of false identification of $<10^{-5}$; an effort was made to manually check the identified ions in the sequencing outcome.

Results

1. The variations in the hydroxylation of collagen

The heterogeneity of the hydroxylation of collagen α -chains can be observed at different levels by mass-spectrometry (MS). The sequencing by MS is carried out on trypsin digested peptides of collagen which usually range from 6-30 amino acid residues in length. The observed mass of one tryptic peptide that has undergone heterogeneous hydroxylation will resemble a mixture of species with mass differences of 16 (replacing –H by –OH) or multiples of 16. The presence of such mixed species was frequently observed in MALDI MS spectra of a total

collagen digest (Fig. 1). The exact number of such peak-clusters varies among different collagens, and/or the different preparations of the same collagen. The observation of such peaks by MALDI MS depends on the trypsin digestion reaction and the signal level in the MALDI MS spectra. Only those peaks with the highest level of signal are labeled in Fig. 1; many others are present with low signal levels. Although it is not possible to identify these peptides by MALDI MS *per se*, given the widespread existence of peaks with mass variants of 16, it is unlikely for these peaks to be caused by the coincidental mass variations of different tryptic peptides. Rather, these peaks point to a mixed population of tryptic peptides with incomplete and/or ‘over’ hydroxylation of amino acid residues. The MS/MS sequencing study further supports this conclusion.

Fig 1. MALDI-TOF spectra of total trypsin digest. (A) Human collagen type III, (B) Human collagen type I, (C) Rat collagen type I. Peaks A – E in panel (A) and panel (B) and A-F in panel (C) are tryptic peptides of the corresponding collagens identified based on the agreement of their molecular weight (+1 ion) with that of the ‘theoretical value’ (assuming all Y-Pro as Hyp). The mass variants of 16 of each peak are labeled based on their mass differences from that of the ‘theoretical value’.

2. The O_x and the P_y residues identified by MS/MS sequencing of the collagens

For the clarity and the convenience of data presentation we will use O_x (or X-Hyp) and P_y (or Y-Pro), respectively, for a hydroxylated Pro in the X position and an unhydroxylated Pro in

237 the Y-position to highlight the unusual hydroxylation results; the normal symbols of P (or Pro)
238 and O (or Hyp) are used for Pro and 4-hydroxyproline in X and Y positions, respectively. Even
239 by MS/MS sequencing, the exact modification often cannot be resolved with certainty with the
240 mass information alone. In case a +16 mass is observed for a fragment of pro-gly-pro or pro-pro-
241 gly sequence, for example, it is generally necessary to assume the hydroxylation of Pro is in the
242 Y-position and not in the X-position in order to resolve the mass variations. Such fragments are
243 common because of the high content of Pro in collagen and the proline-effect of MS/MS-
244 sequencing (3, 10, 43-45). In compiling the sequencing data the ‘theoretical mass’ is calculated
245 assuming *all* Pro residues in the Y-position are hydroxylated. Thus, a mass variation of -16
246 reflects an incomplete hydroxylation of Y-Pro residues, while that of +16 indicates an *additional*
247 hydroxylation beyond the usual Hyp at Y-positions. In addition to Pro in an X position, a
248 modified Lys (Hyl) in a Y-position or an oxidized Met (M_{ox}) would also cause a mass change of
249 +16 compared to their unmodified counterpart (47). A C-terminal Y-position Lys of the peptides
250 can potentially be a hydroxylated Lys and contribute to the +16 mass variation. Considering the
251 7-fold decrease in trypsin susceptibility to Hyl relative to that of Lys, however, a C-terminal Hyl
252 of a tryptic digest is an unlikely event (48). The C-terminal Lys residues are, thus, usually taken
253 as one that is not hydroxylated with one exception: Hyl⁸⁷ of the $\alpha 2$ chain of rat tail type I
254 collagen (see later sections). The hydroxylation of this Lys was supported unambiguously by the
255 +16 data of fragmented ions: all Y-Pro residues of this tryptic peptide are all fully occupied and
256 there is no Pro in the X-position. The Lys in the equivalent position of human collagen was
257 found to be hydroxylated by other methods (3). Hyl will not be seen in the sequencing results if
258 glycosylated or cross-linked to neighboring peptides.

In the following, we only report the O_x and P_y residues that are unambiguously supported by a series of fragmented b- and/or y- ions. One typical example of unusual hydroxylation is given in Fig 2. There are 3 Pro residues in this 19-residue peptide from position 435-453 of the α₁(I) chain of collagen isolated from a single rat tail tendon (srtt): two in the X-position (p⁴⁴⁶, p⁴⁴⁹) and one in Y-position (p⁴⁴⁴). The tryptic digest of this particular region of the α₁(I) chain was partitioned into three populations with different hydroxylation. The sequencing outcome in Fig 2A reflects the expected hydroxylation pattern, with the p⁴⁴⁴ being hydroxylated and the mass of the peptide the expected value of 1680.76. The Hyp⁴⁴⁴ is supported by the identification of a nearly complete series of y- and b-ions and most directly by the y₁₀ ion with a very strong signal. The sequencing outcome of the second population indicate the peptide carries a mass variant of +16 compared to the theoretical value (Fig. 2B), and the extra hydroxylation is unambiguously located on the p⁴⁴⁹ at the X-position, directly supported by the +16 values of y₅ and y₆ ions comparing to that in Fig 2A (*i.e.*, the theoretical value). In the third case (Fig. 2C) the mass has a -16 variant, and again the strong y₁₀ ion, as well as the -16 mass of the y₁₂ and y₁₃ ions demonstrated that the p⁴⁴⁴ at the Y-position was not hydroxylated. Thus, there are three different scenarios regarding Pro hydroxylation in this region: the one with expected Hyp⁴⁴⁴ in the Y-position, the one with O_x⁴⁴⁹, and the one with P_y⁴⁴⁴. Since this particular collagen sample is isolated from a single rat tail tendon, the heterogeneous patterns of the hydroxylation reflects the natural variations of the Pro-hydroxylases and/or the variations accumulated over the development of this organism.

Fig 2. The MS/MS spectra of tryptic peptide ⁴³⁵dgeagaqgagpgpagpger of rat tail tendon α₁(I) chain (lower case stands for the sequence from the genes). Sequencing outcome from ion

840.37762⁺ (1680.7551⁺) (upper panel), ion 848.37612⁺ (1696.7521⁺) (middle panel) and ion 832.38122⁺ (1664.7623⁺) (lower panel). The hydroxylation sites are shown as ^{ox}P. For clarity, only selected ions, those most relevant to the identification of residues are labeled.

The MS and MS/MS sequencing were carried out for the α 1(I) and the α 2(I) chains of type I collagen of rat tail tendon of a commercial sample (crt) and of a sample that is purified from a single rat tail tendon (srtt), the α 1(I) and the α 2(I) chains of a commercial sample of human type I collagen from placenta, and the α 1(III) chain of human type III collagen from a commercial sample. The results of the peptides with unexpected hydroxylations for all five α chains of collagens are summarized in Tables 1-3 (details below); complete sequencing results of all collagen samples are given in the tables of the supplemental material S1. All the samples are sequenced at least twice in order to evaluate the reproducibility of the results. The reproducible findings are shown in boldface in the tables. In addition to the fragmentation data, the unusual hydroxylation of the peptides in Tables 1-3 is unequivocally identified by the variations of their masses (Δm).

3. The over hydroxylation and under-hydroxylation of type I collagen from rat tail tendon

The results in Table 1 revealed a range of variations of the hydroxylation in both the α 1 and the α 2 chains of a commercial sample of the rat tail tendon type I collagen. The +16 or +32 mass confirmed the unexpected hydroxylations in 6 segments of the α 1 chain at residues 145-174, 193-219, 238-252, 375-396, 658-684, and 705-725; three over-hydroxylated segments were found in the α 2 chain: at residues 76-87, 145-174 and 705-725. The hydroxylation sites of these

peptides except that of residues 76-87 (peptide mass 1238.6052) are assigned to a specific X-Hyp based on the fragmentation ions; the +16 mass between residues 76-87 is assigned to the C-terminal Lys since it is the only residue in that peptide that can be hydroxylated. The precise location of the extra hydroxylation of peptide mass 2605.2529 (residues 145-174) of the $\alpha 2(I)$ chain was difficult to resolve since the terminal pro-lys residues were not fragmented; both are the candidates for the hydroxylation (with a +16 mass). We are not aware of any other report on the hydroxylation of this particular Lys. Since Lys is more likely to be cleaved by trypsin than Hyl, we have tentatively assigned the hydroxylation site to be the Pro¹⁷³ in the X-position, and the ***O_x**¹⁷³* was shown in italic in Table 1 to highlight this uncertainty. Despite lacking a clear resolution of the location of the extra hydroxylation, the same peptide was observed more than once during multiple sequencing efforts as shown in the bold face in Table 1, which is presumably related to its measurable presence in this rat tail sample. The Lys residue in the equivalent position of the $\alpha 1(I)$ chain in the peptide of residues 145-174 was resolved unambiguously, and for multiple times by the observation of the fragment having a terminal unhydroxylated Lys. This peptide, however, was found to have an over hydroxylation site on X-Pro¹⁵⁵ (***O_x**¹⁵⁵*). Similarly, for peptide mass 2323.1313 (residues 658-684) of $\alpha 1(I)$ chain (Table 1), the ***O_x**⁶⁸³* was assigned with uncertainty because the non-fragmented terminal Pro-Lys prevented an unambiguous assignment of the unexpected hydroxylation site.

Table 1. Peptides of rat type I collagen with mass variants of 16 (the commercial sample)

Ion mass ^a	Δm^b	Score ^c	Unusual Hydroxylation	Sequence of Peptide ^d
The $\alpha 1(I)$ chain of rat collagen (commercial sample)				
1306.6386		92		²³⁸ GPSGPQGPSGAOGPK

1322.6335	+16	65	O_x^{245}	²³⁸ GPSGPQGO _x SGAOGPK
1338.6284	+32	58	O_x^{239}, O_x^{245}	²³⁸ GO _x SGPQGO _x SGAOGPK
1328.6481	-16	74	P_y^{804}	⁷⁹³ GFOGLOGPSGEP _y GK
1344.6430		41		⁷⁹³ GFOGLOGPSGEOGK
1435.6812	-16	43	P_y^{297}	²⁹⁵ GEP _y GPSGLOGPOGER
1840.9188		50		⁷⁰⁵ VGPOGPSGNAGPOGPOGPVGK
1856.9137	+16	61	O_x^{707}	⁷⁰⁵ VGO _x OGPSGNAGPOGPOGPVGK
1872.9086	+32	49	O_x^{707}, O_x^{719} or O_x^{722}	⁷⁰⁵ VGO _x OGPSGNAGPOGO _x OGPVGK
1959.9519	-16	87	P_y^{273}	²⁷¹ GEP _y GPAGVQGPOGPAGEEGKR
1975.9468		94		²⁷¹ GEOGPAGVQGPOGPAGEEGKR
2014.9689		41		³⁷⁵ TGPOGPAGQDGRGPAGPOGAR
2030.9639	+16	50	O_x^{377}	³⁷⁵ TGO _x OGPAGQDGRGPAGPOGAR
2120.9955	-16	98	P_y^{771}	⁷⁵⁷ GSOGADGPAGSOGTP _y GPQGIAGQR
2136.9905		84		⁷⁵⁷ GSOGADGPAGSOGTOGPQGIAGQR
2169.0571	-16	57	P_y^{876}	⁸⁵⁹ GETGPAGPOGAOGAOGAP _y GPVGPAGK
2185.0520		45		⁸⁵⁹ GETGPAGPOGAOGAOGAOGPVGPAGK
2307.1364		58		⁶⁵⁸ GDAGPOGPAGPAGPOGPIGNVGAOGPK
2323.1313	+16	79	O_x^{683}	⁶⁵⁸ GDAGPOGPAGPAGPOGPIGNVGAOGO _x K
2316.0487		53		¹⁹³ GEOGPOGPAGAAGPAGNOGADGQOGAK
2332.0436	+16	79	O_x^{206}	¹⁹³ GEOGPOGPAGAAGO _x AGNOGADGQOGAK
2548.2063		101		¹⁴⁵ GNDGAVGAAGPOGPTGPTGPOGFOGAAGAK
2564.2012	+16	54	O_x^{155}	¹⁴⁵ GNDGAVGAAGO _x OGPTGPTGPOGFOGAAGAK
2679.2394	-16	42	P_y^{948}	⁹³⁴ GFSGLQGPOGSOGSP _y GEQGPSGASGPAGPR
2695.2343		127		⁹³⁴ GFSGLQGPOGSOGSEQGPSGASGPAGPR
The α2 (I) chain of rat collagen (commercial sample)				
1238.6052	+16	58	Hyl ⁸⁷	⁷⁶ GFOGTOGLOGFK _x
1560.8129	-16	102	P_y^{891}	⁸⁸⁹ GEP _y GPAGSVGPVGAVGPR
1576.8078		59		⁸⁸⁹ GEOGPAGSVGPVGAVGPR
1576.7714	-32	79	P_y^{294}, P_y^{297}	²⁹² GSP _y GEP _y GSAGPAGPOGLR
1592.7663	-16	58	P_y^{297}	²⁹² GSOGEP _y GSAGPAGPOGLR

1608.7612		76		²⁹² GSOGEOSAGPAGPOGLR
1781.7937	-16	69	P_y²⁷³	²⁷¹ GEP _y GSAGAQQPOGPSGEEGK
1937.8948	-16	115	P_y²⁷³	²⁷¹ GEP _y GSAGAQQPOGPSGEEGKR
2368.0648	-16	41	P_y²⁷³	²⁶⁵ GETGNKGEP _y GSAGAQQPOGPSGEEGK
2384.0597		60		²⁶⁵ GETGNKGEOGSAGAQQPOGPSGEEGK
1833.8977	+16	47	O_x⁷⁰⁷	⁷⁰⁵ TGO _x OGPSGITGPOGPOGAAGK
2605.2529	+16	80	O_x¹⁷³	¹⁴⁵ GSDGSVGPVGPAGPIGSAGPOGFOGAOG O_xK
2971.512	-16	76	P_y⁷⁶²	⁷⁵⁷ GPSGEP _y GTTGPOGTAGPQGLLGAOGILGLOGSR
2987.5069		48		⁷⁵⁷ GPSGEOGTTGPOGTAGPQGLLGAOGILGLOGSR

Footnote:

^a: The mass of the single charged ion used to identify each peptide. The sequencing results of most of the peptides are based on the fragmentation of the double charged species (see below).

^b: The mass differences compared to the ‘theoretical’ assuming all Pro in the Y positions are hydroxylated.

^c: The score of Mascot search engine (see Material and Methods).

^d: Unexpected hydroxylation of X-Pro and unhydroxylated Y-Pro are shown as O_x and P_y, respectively; those observed in more than one sequencing effort and/or in both the commercial and the rrt samples (see text) are in bold; those with uncertainties are shown in italic (see text).

In most cases a mixed population of variable hydroxylation was observed for a particular peptide. For example, the peptide with ion mass 1306.6386 (residues 238-252) coexists with the two over-hydroxylated species: a +16 species with mass 1322.6335 and a +32 species with mass 1338.6284 carrying, respectively, one and two extra hydroxylation sties. Due to the unpredictable and complex nature of the ionization process of MS and MS/MS, it is difficult to quantify the relative percentages of the various hydroxylated species by MS alone. In a few cases, such as for the α2 chain peptide with mass 2605.2529 (residues 145-174) only a single population with an extra hydroxylation was sequenced. Despite two sequencing efforts, a species with the theoretical mass was not observed. This lack of detection, however, does not rule out the existence of this population in the sample *per se*. This species may fail to be sequenced to an

acceptable quality either due to poor ionization and/or fragmentation, or may have failed to be identified due to unexpected post-translational modifications. One limitation of mass-spec data interpretation is the inability to draw conclusions about peptides that are not selected for fragmentation.

Concurrently, incomplete hydroxylation was found in six and four regions, respectively, of the $\alpha 1(I)$ and the $\alpha 2(I)$ chains, located in the regions of residues 271-291, 295-309, 757-780, 793-806, 859-884 and 934-963 of the $\alpha 1(I)$ chain, and of residues 271-290, 292-309 (having 2 different P_y residues), 757-789 and 889-906 of the $\alpha 2(I)$. Remarkably, among the 11 detected P_y residues in both α chains, seven of them were found in the triplet GEP_y .

One noticeable observation is the overlapping of the regions having unusual hydroxylation between the $\alpha 1(I)$ and $\alpha 2(I)$ chains. The region between residues 705 and 725 in both α chains contain multiple O_x residues: O_x^{707} and O_x^{722} in $\alpha 1(I)$ chain and O_x^{707} in $\alpha 2(I)$ chain. Similarly, some of the P_y residues appear located in similar regions of both α chains as well: P_y^{273} between residues 271-291, P_y^{294} and P_y^{297} between residues 295-309, P_y^{762} and P_y^{771} between residues 757-789. Combining the observations of different peptides from both α chains, the region of residues 271-309 in both $\alpha 1(I)$ and $\alpha 2(I)$ chains stands out as a particularly poorly hydroxylated region, missing two to three expected Hyp residues in the Y-positions of each α chain.

The finding of such a wide range of variations in hydroxylation of the type I chain is rather unexpected. The purity and the purification procedures of this commercial sample were called into question. In order to get a better understanding of the origin of the heterogeneity we purified the type I collagen from a single rat tail tendon (srtt). Interestingly, the sequencing result

of this srtt sample turns out to be remarkably similar (Table 2). The 2 observed O_x residues in the $\alpha 2(I)$ of the commercial sample and all but two (O_x^{206} and O_x^{377}) in the $\alpha 1(I)$ chain (Table 1) were reproduced in the srtt sample. Similarly, more than half of the P_y residues found in the commercial sample were also observed in the srtt sample. This srtt sample appeared to be particularly over-hydroxylated having 11 O_x residues in each α chain. The content of P_y is also higher: 10 and 9 P_y residues, respectively, were found in the $\alpha 1(I)$ and $\alpha 2(I)$ chains. The noticeably more heterogeneous hydroxylation pattern of this srtt sample, especially that for the $\alpha 2(I)$ chain, may relate to the better overall sequencing outcome of the sample reflected, in part, by the better sequence coverage of this $\alpha 2$ chain (Fig. 3 and S1 Tables S1-S6). The identified O_x residues of the $\alpha 1(I)$ chain of the srtt sample include the well-known 3-Hyp O_x^{986} ; this section of the $\alpha 1(I)$ chain of the commercial sample was not sequenced. Another interesting peptide with an additional Hyp of interest is the seven-residue peptide from the N-telopeptide region preceding the triple helical domain (ion mass 1452.7264). The Met residue in this fragment appears to be oxidized based on the detection of M_{ox} with neutral loss. In addition to the M_{ox} , the Pro in the N-telopeptide appears to be hydroxylated (the P^*). Since this Pro proceeds a Gly residue, which characterizes the canonical hydroxylation site of the prolyl-hydroxylase (C-P4H), its hydroxylation, although never reported before, probably does not come as too much of a surprise.

Table 2A. Peptides of type I collagen from a *single* rat tail tendon with mass variants of 16

Ion mass ^a	Δm^b	Score ^c	Unusual Hydroxylation	Peptide ^d
The $\alpha 1$ chain of type I collagen isolated from a single rat tail tendon (srtt)				
1242.5961	-16	63	P_y^{369}	³⁶¹ GLTGSOGSP _y GPDGK
1322.6335	+16	76	O_x^{239}	²³⁸ GO _x SGPQGPGSGAOGPK

1338.6284	+32	76	O_x^{239}, O_x^{251}	$^{238}GO_xSGPQGPSGAOGO_xK$
1354.6233	+48	49	$O_x^{239}, O_x^{245}, O_x^{251}$	$^{238}GO_xSGPQGO_xSGAOGO_xK$
1452.7264		65		$^{-7}SAGVSVPGPMGPSGPR$
1468.7213	+16	51	M_{ox}^3	$^{-7}SAGVSVPGPM_{ox}GPSGPR$
1484.7162	+32	40	P^{-1*}, M_{ox}^3	$^{-7}SAGVSVPGPM_{ox}GPSGPR$
1574.8173	-16	86	P_y^{600}	$^{586}GLTGPiGPOGPAGAP_yGDK$
1590.8122		42		$^{586}GLTGPiGPOGPAGAOGDK$
1561.7969	+16	40	O_x^{986}	$^{975}DGLNGLOGPIGO_xOGPR$
1872.9086	+32	49	O_x^{707}, O_x^{719}	$^{705}VGO_xOGPSGNAGPOGO_xOGPVGK$
1888.9035	+48	48	$O_x^{707}, O_x^{716}, O_x^{719}$	$^{705}VGO_xOGPSGNAGO_xOGO_xOGPVGK$
1664.7623	-16	88	P_y^{444}	$^{435}DGEAGAQGAP_yGPAGPAGER$
1680.7572		96		$^{435}DGEAGAQGAOGPAGPAGER$
1696.7521	+16	92	O_x^{449}	$^{435}DGEAGAQGAOGPAGO_xAGER$
1800.8697	-32	105	P_y^{825}, P_y^{831}	$^{817}GPOGPMGPP_yGLAGPP_yGESGR$
1816.8647	-16	125	$P_y^{825}, P_y^{831}, M_{ox}^{822}$	$^{817}GPOGPM_{ox}GPP_yGLAGPP_yGESGR$
1803.8508	-16	77	P_y^{273}	$^{271}GEP_yGPAGVQGPOGPAGEEGK$
1819.8457		82		$^{271}GEOGPAGVQGPOGPAGEEGK$
2105.0006	-32	78	P_y^{759}, P_y^{771}	$^{757}GSP_yGADGPAGSOGTP_yGPQGIAGQR$
2339.1262	+32	40	O_x^{668}, O_x^{683}	$^{658}GDAGPOGPAGO_xAGPOGPiGNVGAPGO_xK$
2548.2063		58		$^{145}GNDGAVGAAGPOGPTGPTGPOGFOGAAGAK$
2564.2012	+16	96	O_x^{155}	$^{145}GNDGAVGAAGO_xOGPTGPTGPOGFOGAAGAK$
2663.2444	-32	96	P_y^{945}, P_y^{948}	$^{934}GFSGLQGPOGSP_yGSP_yGEQGPSGASGPAGPR$
2679.2394	-16	105	P_y^{948}	$^{934}GFSGLQGPOGSOGSP_yGEQGPSGASGPAGPR$
2695.2343		62		$^{934}GFSGLQGPOGSOGSOGEQGPSGASGPAGPR$

Footnote:

^a: The mass of the single charged ion used to identify each peptide. The sequencing results of most of the peptides are based on the fragmentation of the double charged species (see below).

^b: The mass differences compared to the ‘theoretical’ assuming all Pro in the Y positions are hydroxylated.

^c: The score of Mascot search engine (see Material and Methods).

^d: Unexpected hydroxylation of X-Pro and unhydroxylated Y-Pro are shown as O_x and P_y , respectively; those observed in more than one sequencing effort and/or in both the commercial and the rrt samples (see text) are in bold; those with uncertainties are shown in italic (see text).

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394 Table 2B. Peptides of type I collagen from a *single* rat tail tendon with mass variants of 16

Ion mass ^a	Δm^b	Score ^c	Unusual Hydroxylation	Peptide ^d
The $\alpha 2$ chain of type I collagen isolated from a single rat tail tendon (srtt)				
868.4635		51		⁹⁰⁷ GPSGPQGIR
884.4585	+16	46	O _x ⁹⁰⁸	⁹⁰⁷ GO _x SGPQGIR
937.5102		52		⁹⁶⁴ GPAGPSGPIGK
953.5051	+16	51	O _x ⁹⁶⁸	⁹⁶⁴ GPAGO _x SGPIGK
1068.5684	-16	71	P _y ²⁵⁸	²⁵³ GLVGEP _y GPAGSK
1084.5633		70		²⁵³ GLVGEOGPAGSK
1175.5691	+16	79	O _x ⁴⁰⁷	³⁹⁷ GEAGNIGFOGPK
1187.6280	-16	54	P _y ⁹⁸¹	⁹⁷⁸ SGHP _y GPVGPAGVR
1238.6052	+16	64	Hyl ¹⁸⁷	⁷⁶ GFOGTOGLOGFK _{ox}
1437.7485	-16	79	P _y ⁴⁸⁶	⁴⁸⁴ GLP _y GEFGLOGPAGPR
1453.7434		75		⁴⁸⁴ GLOGEFGLOGPAGPR
1469.7383	+16	110	O _x ⁴⁹⁴	⁴⁸⁴ GLOGEFGLOGO _x AGPR
1490.7122		59		⁷⁴¹ TGEIGASGPOGFAGEK
1506.7071	+16	67	O _x ⁷⁴⁹	⁷⁴¹ TGEIGASGO _x OGFAGEK
1560.8129	-16	102	P _y ⁸⁹¹	⁸⁸⁹ GEP _y GPAGSVGPVAVGPR
1576.8078		110		⁸⁸⁹ GEOGPAGSVGPVAVGPR
2025.0373	-16	82	P _y ⁸⁹¹	⁸⁸⁵ HGNRGE _y GPAGSVGPVAVGPR
2041.0322		76		⁸⁸⁵ HGNRGEOPAGSVGPVAVGPR
1576.7714	-32	76	P _y ²⁹⁴ , P _y ²⁹⁷	²⁹² GSP _y GEP _y GSAGPAGPOGLR
1592.7663	-16	85	P _y ²⁹⁴	²⁹² GSP _y GEOGSAGPAGPOGLR
1608.7612		88		²⁹² GSOGEOGSAGPAGPOGLR
1624.7562	+16	94	O _x ³⁰²	²⁹² GSOGEOGSAGO _x AGPOGLR
1748.8674	-16	69	P _y ²⁹⁷	²⁹¹ RGSOGE _y GSAGPAGPOGLR
1764.8624		50		²⁹¹ RGSOGEOPAGPAGPOGLR

1599.8238	-16	87	P_y^{183}	¹⁷⁵ GELGPVGNP _y GPAGPAGPR
1615.8187		105		¹⁷⁵ GELGPVGNNOGPAGPAGPR
1781.7937	-16	85	P_y^{273}	²⁷¹ GEP _y GSAGAQQPOGPSGEEGK
1797.7886		94		²⁷¹ GEOGSAGAQQPOGPSGEEGK
1817.9028		52		⁷⁰⁵ TGPOGPSGITGPOGPOGAAGK
1833.8977	+16	69	O_x^{707}	⁷⁰⁵ TG O_x OGPSGITGPOGPOGAAGK
1849.8926	+32	89	O_x^{707}, O_x^{716}	⁷⁰⁵ TG O_x OGPSGITG O_x OGPOGAAGK
1865.8876	+48	97	$O_x^{707}, O_x^{716}, O_x^{719}$	⁷⁰⁵ TG O_x OGPSGITG O_x OG O_x OGAAGK
2589.2580		112		¹⁴⁵ GSDGSVGPVGPAGPIGSAGPOGFOGAOGPK
2605.2529	+16	103	O_x^{173}	¹⁴⁵ GSDGSVGPVGPAGPIGSAGPOGFOGAOG O_x K
2621.2478	+32	51	O_x^{164}, O_x^{173}	¹⁴⁵ GSDGSVGPVGPAGPIGSAGO _x OGFOGAOG O_x K
3045.4257	-16	78	P_y^{552}	⁵²⁰ GPSGAOGPDGNKGEAGAVGAOGSAGASGPGGLP _y GER
3077.4155	+16	76	O_x^{548}	⁵²⁰ GPSGAOGPDGNKGEAGAVGAOGSAGASGO _x GGLOGER

Footnote:

^a: The mass of the single charged ion used to identify each peptide. The sequencing results of most of the peptides are based on the fragmentation of the double charged species (see below).

^b: The mass differences compared to the ‘theoretical’ assuming all Pro in the Y positions are hydroxylated.

^c: The score of Mascot search engine (see Material and Methods).

^d: Unexpected hydroxylation of X-Pro and unhydroxylated Y-Pro are shown as O_x and P_y , respectively; those observed in more than one sequencing effort and/or in both the commercial and the srtt samples (see text) are in bold; those with uncertainties are shown in italic (see text).

The regions identified in the commercial sample where both $\alpha 1(I)$ and $\alpha 2(I)$ chains are over-hydroxylated, residues 238-252 and residues 705-725 (Table 1), also have multiple O_x in this srtt sample. The region of residues 705-725 revealed a particularly varied hydroxylation pattern having 1 to 3 O_x residues in both chains. By comparing to the commercial sample, another highly variable region stands out: residues 238-252 of $\alpha 1(I)$ chain having up to 2 and 3 O_x residues in the commercial sample and the srtt sample, respectively. The over-hydroxylation, however, is not seen for the equivalent region of the $\alpha 2(I)$ chain because of the non-homologous

sequences: none of the equivalent X-residues of the $\alpha 2(I)$ chain where an O_x is observed in the $\alpha 1(I)$ chain is Pro. The poorly hydroxylated region of residues 271-309 is also under-hydroxylated in this srtt sample, lacking 1 and 3 expected Y-Hyps, respectively, in the $\alpha 1(I)$ and the $\alpha 2(I)$ chains. Other P_y residues that are frequently observed in both samples are P_y^{771} and P_y^{948} of the $\alpha 1(I)$ chain, and P_y^{891} of the $\alpha 2(I)$ chain.

In fact, the sequenced peptides of the commercial sample in Table 1 appear almost as a subset of that included in Table 2A and 2B of the srtt sample. Thus, on this account, the commercial samples are quite representative of the averaged features of PTMs of collagens from the type I collagen of the rat tail tendon. All together combining the results of the two samples of type I collagen we have identified a total of 13 O_x in the $\alpha 1(I)$ chain and 11 in the $\alpha 2(I)$ chains, also 13 and 10 P_y residues, respectively, in the $\alpha 1(I)$ and $\alpha 2(I)$ chains. The abnormal hydroxylation sites detected from *both* samples are mapped out on the sequences of the α -chains arranged in D-periodicity in Figure 3. The sites of O_x appear to be scattered rather uniformly throughout the α -chains except the two identified regions of highly variable hydroxylation patterns (HVRs): the N-terminal highly variable region (N-HVR) of residues 238-252 of the $\alpha 1(I)$ chain, and the C-terminal highly variable region (C-HVR) of residues 705-725 of both α chains. The O_x^{155} of the $\alpha 1(I)$ chain is the only unexpected hydroxylation outside the HVRs that has been observed multiple times in both samples. The P_y residues also seem to cluster: in addition to residues 271-302 mentioned above, regions of residues 941-950 and 821-840 of the $\alpha 1(I)$ chain, and 757-780 of both $\alpha 1$ and the $\alpha 2$ chains all have multiple P_y residues (Figure 3, Tables 1, 2A and 2B).

Figure 3. The mapping of individual unexpected hydroxylation sites on the α chains of collagen. Sequences of the α_1 (I) and α_2 (I) chains of human (H1A1 and H1A2, respectively), the α_1 (I) and the α_2 (I) chains of rat (R1A1 and R1A2, respectively), and the $\alpha 1$ (III) chains of human (H3A1) were arranged by the *D*-periodicity according to Di Lullo *et al* (49): the 4 *D*-periods are highlighted by a colored bar of grey, yellow, cyan, and magenta, respectively; the 0.6 *D* is marked by the colored bar of green. The Gly-X-Y triplets including an O_x are in grey highlight. The P_y residues are lighted in red in the tripeptide unit of Y-Gly-X in order to reflect the potential connection with the enzyme selectivity of C-P4H (see text). The entire segment of the three highly variable regions (N- or C-HVR, see text) with multiple O_x residues are boxed. The hydroxylated proline in the telopeptide is P*. Hydroxylysines and the Gly-Pro-Lys tripeptide where the hydroxylation could not be precisely located between the Pro-Lys residues at the C-terminus of a peptide (see text) are highlighted in yellow with Lys in green font. The oxidized methionines are in yellow colored font. In all cases the PTMs observed from more than one detection/sample preparation were shown in bold font. Not sequenced regions are in faint grey. The amino acid sequence of the five collagen α chains were adapted from UniProt database.

4. Human collagen type I and type III.

The detected unusual hydroxylation sites of human collagen type I and type III are summarized in Table 3. Only the primary 3-Hyp at position 986 (O_x^{986}) of $\alpha 1$ (I) was found as the X-Hyp in human type I collagen (Table 3A). Despite multiple sequencing efforts the peptide containing Pro⁷⁰⁷ of $\alpha 2$ (I), one of the class-2 X-Hyp reported by Eyre and colleagues, was not sequenced. The Pro⁷⁰⁷ of $\alpha 1$ (I) was sequenced but was found not hydroxylated in spite of the

nearly identical amino acid sequences in this region between the two α -chains (Fig 3). The residue Met⁸²² appears to be oxidized (Table 3A). The oxidation of Met is not a regular post-translational modification but an oxidation event usually found in cells under stress; it can also occur with sample handling (47, 50) (51). A few cases of incomplete hydroxylations were observed for both the α 1(I) chain and the α 2(I) chain. In general, the sequencing of the human type I collagen sample appeared to be rather clean, with only a low degree of unexpected modifications.

Table 3. Peptides with mass variants of 16 in human collagen

Ion mass ^a (m/z ⁺¹)	Δm^b	Score ^c	Unusual Hydroxylation	Peptide ^d
A. Human collagen type I				
α 1 chain				
1561.7969	+16	65	O _x ⁹⁸⁶	⁹⁷⁵ DGLNGLOGPIGO _x OGPR
1832.8596		78	M _{ox} ⁸²² , P _y ⁸²⁵	⁸¹⁷ GPOGPM _{ox} GPP _y GLAGPOGESGR
1848.8545	+16	41	M _{ox} ⁸²²	⁸¹⁷ GPOGPM _{ox} GPOGLAGPOGESGR
2105.0006	-16	66	P _y ⁷⁷¹	⁷⁵⁷ GSOGADGPAGAOGTP _y GPQGIAGQR
2120.9955		58		⁷⁵⁷ GSOGADGPAGAOGTOGPQGIAGQR
2497.2066	-16	81	P _y ⁸⁷⁶	⁸⁵⁶ GDRGETGPAGPOGAOGAOGAP _y GPVGPAGK
2513.2015		109		⁸⁵⁶ GDRGETGPAGPOGAOGAOGAOGPVGPAGK
2703.2394	-16	63	P _y ⁶⁴⁵	⁶¹⁹ GAOGDRGEOGPOGPAGFAGPOGADGQP _y GAK
2149.9785		73		⁶²⁵ GEOGPOGPAGFAGPOGADGQOGAK
α 2 chain				
1168.4978	-16	61	P _y ⁸⁴⁰	⁸³⁷ DGNP _y GNDGPOGR
B. Human type III collagen				
α 1 chain				

949.5102	-16	45	P_y^{981}	$^{973}GPVGPSGPP_yGK$
965.5051		44		$^{973}GPVGPSGPOGK$
1138.5674		65		$^{790}GLAGPOGMOGPR$
1154.5623	+16	41	M_{ox}^{797}	$^{790}GLAGPOGM_{ox}OGPR$
1203.5827		57		$^{406}GQOGVMGFOGPK$
1219.5776	+16	42	O_x^{416}	$^{406}GQOGVMGFOGO_xK$
1514.7346	-16	50	P_y^{990}	$^{984}DGTSGHP_yGPIGPOGPR$
1530.7295		57		$^{984}DGTSGHOGPIGPOGPR$
1670.7989		42	P_y^{240}, M_{ox}^{243}	$^{229}GEMGPAGIOGAP_yGLM_{ox}GAR$
2088.0832	-16	94	P_y^{777}	$^{766}GSOGAQGPOGAP_yGPLGIAGITGAR$
2104.0782		137		$^{766}GSOGAQGPOGAOGPLGIAGITGAR$
2283.1000		79		$^{667}GEGGPOGVAGPOGGSGPAGPOGPQGVK$
2299.0949	+16	106	O_x^{686} or O_x^{689} or K_{ox}^{693}	$^{667}GEGGPOGVAGPOGGSGPAGPOGPQGVK$
2950.4653		82		$^{595}GPTGPIGPOGPAGQOGDKGEGGAOGLOGIAGPR$
2966.4603	+16	58	O_x^{605}	$^{595}GPTGPIGPOGO_xAGQOGDKGEGGAOGLOGIAGPR$

Footnote:

^a: The mass of the single charged ion used to identify each peptide. The sequencing results of most of the peptides are based on the fragmentation of the double charged species (see below).

^b: The mass differences compared to the ‘theoretical’ assuming all Pro in the Y positions are hydroxylated.

^c: The score of Mascot search engine (see Material and Methods).

^d: Unexpected hydroxylation of X-Pro and unhydroxylated Y-Pro are shown as O_x and P_y ; those observed in more than one sequencing effort are in bold; those with uncertainties are shown in italic (see text).

Additional hydroxylation was seen in two regions of the $\alpha 1(III)$ chain of the type III collagen (Table 3B): peptides of residues 406-417 (mass 1219.5776) and of residues 595-627 (peptide mass 2966.6403). The peptide with mass 2966.4603 contains an internal Lys⁶¹², the +16 mass was tentatively assigned to the hydroxylation of O_x^{605} for lacking complete fragmentation between O_x^{605} and Lys⁶¹². The peptide 406-417 (mass 1219.5776) carries the Met⁴¹¹, which can potentially be oxidized with a mass increase of 16; the fragmentation data has ruled out this

possibility. Overall five P_y residues were detected in α1(III) samples, and the P_y⁹⁸¹ was seen with a very strong signal in every sequencing outcome.

In summary, the MS/MS sequencing results are mapped out on the sequences of the five α-chains arranged in *D*-periodicity in Fig. 3. Using the rather stringent sequencing criteria outlined in Materials and Methods the sequence coverage is about 35% for type III collagen and around 56% for the α₁(I) and 46% for the α₂(I) chains of human type I collagen, and about 62% and 67% for α₁(I) and α₂(I) chains, respectively, of rat tail tendon type I collagen combining both samples. Some observations of the O_x are consistent between the two different rat tail tendon samples, such as those in the HVRs; the others appeared sporadic. Most of the over-hydroxylations seen in the rat tail tendon type I collagen are not present in human placental type I collagen. The consistent observations between the human type I collagen and that of the rat tail tendon include the Hyp⁹⁸⁶ of the α1 chain and P_y residues at positions 771 and 876 of the α1(I) chain.

Discussion

By carrying out this study of the selected collagen samples we are hoping to gain a better understanding of the variations in the hydroxylation of fibrillar collagen in MS studies. Because of the high sensitivity of the MS and MS/MS approach, observing unusual hydroxylation of collagen proves to be a common event. Using a standard protein mass-spec sequencing technique we have detected unusual hydroxylation at several levels in rat type I collagen, human type I collagen and human type III collagen. The variations of the hydroxylation were supported by the spectrometry data for both the fragmented ions and the overall mass of the tryptic peptides. The

over-hydroxylation was largely attributed to the hydroxylation of Pro in the X-positions, which is especially prevalent in both the $\alpha 1(I)$ and the $\alpha 2(I)$ chains of the type I collagen of rat tail tendon. The heterogeneity in the hydroxylation of human collagen type I and type III is much lower, reflecting the variations in enzyme selectivity of the hydroxylase among different species and/or tissues. As expected, most of the hydroxylated proline residues in the X-position are detected as a mixture; some may be present at a relatively low level, while others, as those in the highly variable regions (HRVs), are more prevalent and representative.

The repeated sequencing outcomes of the same collagen sample often carry high levels of variations as shown in Tables 1-3; detections of about half of the O_x and P_y residues are seen in multiple sequencing efforts (in boldface), while that of the others are less reproducible. In fact, the variations in the sequencing results of the two very different rat tail tendon samples are not in any way more substantial than that of the repeated sequencings of the same collagen samples. Such varied outcomes reflected the complex and unpredictable nature of the ionization process of MS and sample handling (52). Each sequencing outcome often represents no more than a single sampling of a population consisting of heterogeneous modifications. The unpredictable ionization process is one of the major concerns for quantitative estimation of the populations of the sequenced peptides using MS/MS, especially when the sample is heterogeneous and the scope of the PTMs of the protein is not fully characterized. Sequence coverage will also affect the detection of PTMs, and this may be the reason that the canonical 3Hyp⁹⁸⁶ of the $\alpha 1(I)$ chain was detected only once among multiple sequencing attempts of samples from human placenta and from srtd; this tryptic peptide containing position 986 was not sequenced at all in the commercial rat tail sample. Protocols using multiple proteases will result in a better sequence coverage, especially in cases like type III collagen where the tryptic peptides are often either too

large or too small for reliable MS/MS results. On the other hand, despite the low sequence coverage, several unusual hydroxylations were observed rather consistently (Table 3, Fig 3). The observations of the O_x residues, in the highly variable regions of the rat tail tendon type I collagen is quite robust and consistent even among samples prepared from different sources.

The over hydroxylation observed in the C-HVR of the $\alpha 1(I)$ and $\alpha 2(I)$ chains of type I collagen in rat tail tendon is in keeping with the unusually high 3Hyp content of this collagen (53). The amino acid composition analysis estimated three to four 3Hyp in each of the $\alpha 1(I)$ and the $\alpha 2(I)$ chains of the rat tail tendon type I collagen, compared to only one 3Hyp, the Hyp⁹⁸⁶, in the rat $\alpha 1(I)$ chain of type I collagen from bones or skin. The O_x⁷⁰⁷, O_x⁷¹⁶ and O_x⁷¹⁹ of the $\alpha 2(I)$ chains were subsequently identified as 3Hyp by N-terminal sequencing (4). The C-HVR also includes the location of the ‘class-2’ 3Hyp⁷⁰⁷ in the human $\alpha 2(I)$ chain observed previously (9). Unfortunately this segment of the human $\alpha 2(I)$ chain was not sequenced in our study despite repeated attempts; the same region in the human $\alpha 1(I)$, which has the same amino acid sequence as that in the $\alpha 2(I)$ chain, was sequenced, but no X-Hyp was found. The over-hydroxylation in the N-HVR of residue 238-252 of rat tendon $\alpha 1(I)$ has never been reported before. Multiple O_x residues in this region in both samples of the rat tendon collagen were observed with reproducible results. Interestingly, the amino acid sequences of the two highly variable regions share limited homology; they are also rather different from the sequence surrounding the 3Hyp⁹⁸⁶. The two O_x residues of the N-HVR, O_x²³⁹ and O_x²⁴⁵, appeared in the peptide triad of GO_xS, while the O_x⁷⁰⁷ and O_x⁷¹⁶ of C-HVR in both $\alpha 1(I)$ and $\alpha 2(I)$ chains are in the more common GO_xO moiety; but the O_x⁷²² of the $\alpha 1(I)$ chain is in a GO_xV triplet. The GOA and GOS are two moieties identified for X-Hyp of type V collagen (10). It is also worth noting that, similar to the 3Hyp

residues identified by Eyre and colleagues, the two HVRs of rat tail tendon type I collagen are located exactly a $2D$ -period apart (Fig. 3), although the significance of it remains to be evaluated (9). No further effort was made to confirm the 3Hyp identity of the identified O_x in this study. While most of the newly identified X-Hyp residues have been confirmed to be 3Hyp, at least in one occasion an X-Hyp was later confirmed to be a 4R-Hyp (54).

The unhydroxylated Pro residues in the Y-position appear to be more common than X-Hyp among all α chains, with the highest content seen in the rat tail tendon type I collagen. Most of the detected Y-Pro residues are present as a mixed population having varied occupancies. Combining all the five α chains, the P_y residues were observed in 29 peptides. It is tempting to postulate the region of residues 273-302 of rat tail tendon type I collagen, where up to 5 P_y residues were found within a short stretch of 38-residues, to have unique conformational dynamics, since a Pro in the Y-position is known to significantly destabilize the triple helix compared to a Hyp (55). The real impact will, of course, depend on the percent of occupancy in these sites.

While incomplete hydroxylation has been known for some time, the site-specific data and the sequence motif of the missed-hydroxylations have not been reported before. The sequence information of the P_y residues may relate to the substrate selectivity of the prolyl-4-hydroxylase (C-P4H). Studies using short peptides established that the enzyme recognizes Pro-Gly-Xaa triplets during hydroxylation, where the Pro is the residue to be hydroxylated, and the selection of the Pro in a Y-position is affected by the conformation around the -Gly-Xaa residues (56-58). The hydroxylation takes place on the nascent polypeptide chains before the formation of the triple helix. Despite the higher than normal content of the Pro residues, the unfolded α -chains of collagen are not known to assume any well-defined conformation, although isolated segments

may temporarily adapt to polyproline II (PP II) like or β -turn like ϕ and φ angles. Specifically, the type II β -turn bent between the -Gly-Xaa was considered to favor the binding of C-P4H and thus, the hydroxylation of the Pro proceeding the Gly; while the PPII conformation in -Gly-Xaa was considered inhibitory (57, 58). Residues Ala, Leu, Ile, and Phe in the position of Xaa were found to favor a β -turn around the Gly, and a Pro favors a PPII ϕ and φ angles (58). Our finding here appears to reflect this conformational preference of C-P4H in vivo. If we consider the P_y as a *miss* of the C-P4H in selecting a Pro in a Y-position for hydroxylation, the hydroxylation action appears to be particularly slippery in the sequence context of Pro-Gly-Pro. Nineteen out of the 36 P_y residues identified in the 5 α chains are in the P_y -Gly-Pro moiety. On the other hand, this high occurrence of P_y -Gly-Pro moiety may simply reflect the higher frequencies of genomic sequence pro-gly-pro in fibrillar collagen. The other frequent *misses* include P_y in Pro-Gly-Glu (5/33), Pro-Gly-Ser (4/30) triplets, and Pro-Gly-Leu (3/33) triplet. Among the identified P_y residues, the P_y^{771} of $\alpha 1(I)$ in a P_y -Gly-Pro moiety is the only one that is identified in both human and the two rat tail tendon samples. The P_y^{876} , also in P_y -Gly-Pro moiety, of $\alpha 1(I)$ was detected in human and the commercial sample of rat tail tendon, but not in the srtt sample. There also appears to be an overrepresentation of P_y residues in a GEP_y tripeptide in rat type I collagen: 8 out of the 23 identified P_y residues are in a GEP moiety. It is not clear if the Glu preceding the Pro in the Y-position affects the selectivity of C-P4H in rat tail tendon. Other more common sequence motifs for P_y are GAP_y , GPP_y and GSP_y . It is also unclear if the missed hydroxylation of these residues has any functional roles for collagen.

Conclusions

The high sensitivity of MS/MS sequencing has revealed a subpopulation of collagen that bares unexpected hydroxylated Pro in the X-positions, and unhydroxylated Pro in the Y-positions. The detection of X-Hyp and the Y-Pro by mass-spec sequencing is impacted by both the tissue/organism dependent variations of the Pro-hydroxylation reaction and the statistic nature of the technique. The detection of some modifications, however, is rather robust and can be used as a biomarker for general applications using MS/MS. A more thorough understanding of the dynamics of the specific PTM of 3-Hyp in the X-position and its role in epigenetic regulation will require a knowledge base that is broad enough to reflect the statistical nature of both the variations of the PTMs in different tissues and organisms, and the reproducibility of their detections by MS/MS.

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Supporting Information

S1: The Complete Sequencing Results

Figure 1

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Figure 1

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Figure 2

Figure 3

The sequence of collagen α -chains in D-periodicity

D1	-7	1	21	41	61	81								
		GPMGSPGPRG	LPGPPGAPGP	QGFQGPPEP	GEPGASGPMG	PRGPPGPPGK	NGDDGEAGKP	GRPGERGPPG	PQGARGLPQT	AGLPGMKGHR	GFSGLD	H1A1		
		SAGVSV*GP	GPSGPRG	LPGPPGAPGP	QGFQGPPEP	GEPGASGPMG	PRGPPGPPGK	NGDDGEAGKP	GRPGERGPPG	PQGARGLPQT	AGLPGMKGHR	GFSGLD	R1A1	
		GVGLGP	GP*GL	GPRG	PPGAAGAPGP	QGFQGPAGEP	GEPQGTGPAG	ARSPAGPPGK	AGEDGHGPKP	GRPGERGVVG	PQARGFPFGT	PGLPGFKGIR	GHNGLD	H1A2
		GVSAGP*GP	GL	GPRG	PPGAVGAPGP	QGFQGPAGEP	GEPQGTGPAG	SRSPAGPPGK	AGEDGHGPKP	GRPGERGVVG	PQARGFPFGT	PGLPGFKGIR	GHNGLD	R1A2
		GLAGYPPGAP	PPGPPGPPGT	SGHPGSPGSP	GYQGPFPGEF	QAGPSGPPGP	PGAIGPSGPA	GKDGESGRPG	RFGERGLPGP	PGIKGPAGIP	GFFPMK	H3A1		
D1	101	121	141	161	181	201								
GAKG	DAGPAGPKGE	PGSPGENGAP	QMGPRGLPG	ERGRPGAPGP	AGARGNDGAT	GAAGPPGPTG	PAGPPGPPGA	VGAKGEAGPQ	GPRGSEGPQG	VRGEPGPPGP	AGAAGPAGNP	GA	H1A1	
GAKG	DTGPAGPKGE	PGSPGENGAP	QMGPRGLPG	ERGRPGPPGS	AGARGNDGAV	GAAGPPGPTG	PTGPPGPPGA	AGAKGEAGPQ	GARGSEGPQG	VRGEPGPPGP	AGAAGPAGNP	GA	R1A1	
GLKG	QPGAPGVKGE	PGAPGNGTF	QGTGARGLPG	ERGRVGAAPGP	AGARGSDGVS	GPVGPAGPIG	SAGPPGPPGA	PGPKGEIGAV	GNAGPAGPAG	PRGEVGLPGL	SGPVGPPGPNP	GA	H1A2	
GLKG	QPGACGVKGE	PGAPGNGTF	QGTGARGLPG	ERGRVGAAPGP	AGARGSDGVS	GPVGPAGPIG	SAGPPGPPGA	PGPKGEIGAV	GNAGPAGPAG	PRGEVGLPGL	SGPVGPPGPNP	GA	R1A2	
CHRG	FDGRNCEKGE	TGAPGLKGEN	GLPGENGAPG	PMGPRGAPGE	RGRPGLPGAA	GARGNDGARG	SDGQGPFPGP	PGTAGFPSP	GAKGEVGPAG	SPGSGNAPQ	RGEFGPQCHA	GA	H3A1	
D2	221	241	261	281	301	321								
DGQPGAKG	ANGAPGIAGA	PGFPARGPS	GPGGPGPPG	PKNSGEPGA	PGSKGDTGAK	GEPGPVGVQG	PPGPAGEEGK	RGARGEPPGT	GLPGPPGERG	GPGRGPPGA	DGVAGPKGP	H1A1		
DGQPGAKG	ANGAPGIAGA	PGFPARGPS	GPGGPGPPG	PKNSGEPGA	PGSKGDTGAK	GEPGPVGVQG	PPGPAGEEGK	RGARGEPPGT	GLPGPPGERG	GPGRGPPGA	DGVAGPKGP	R1A1		
NGLTGAKG	AAGLPGVAGA	PGLPGPPGIP	GPVGAAGATG	ARGLVGEPGP	AGSKGESGNK	GEPGSAGPQG	PPGSGGEGK	RGPNGEAGSA	GPPGPPGLRG	SPGSRGLPGA	DGVAGV*GP	H1A2		
NGLTGAKG	ATGLPGVAGA	PGLPGPPGIP	GPVGAAGATG	ARGLVGEPGP	AGSKGETGNK	GEPGSAGPQG	PPGSGGEGK	RGS*PGEPQSA	GPPGPPGLRG	SPGSRGLPGA	DGRAGV*GP	R1A2		
QGPFPGPG	INGSPGKGE	MGAPGIPGAP	GLMGARPPG	PAGANGAPGL	RGGAGEPGKN	GAKGEPGPRG	ERGEAGIPGV	PGAKGEDGKD	GSPGEPGANG	LPGAAGERJA	PGFRGPAGP	H3A1		
D2	341	361	381	401	421	441								
A	GERGSPGPAG	PKGSPGEAGR	PGEAGLPKAK	GLTGSPGSPG	PDGKTGPPGP	AGQDGRPGPP	GPPGARGQAG	VHGFPGPKGA	AGEPGKAGER	GVPGPPGAVG	PAGKDGEAGA	QGPPG	H1A1	
A	GERGSPGPAG	PKGSPGEAGR	PGEAGLPKAK	GLTGSPGSPG	PDGKTGPPGP	AGQDGRPGPA	GPPGARGQAG	VHGFPGPKGT	AGEPGKAGER	GVPGPPGAVG	PAGKDGEAGA	QGA*PG	R1A1	
P	GSRGASGPAG	VRGPNGDAGR	PGEPLMGPR	GLPGSPGNIG	PAGKEGPPVL	PGIDGRPGPI	GPAGARGEPG	NIGFPGPKGP	TGDPGKNGDK	GHAGLAGARG	APGPDGNGA	QGPPG	H1A2	
P	GNRSTGPAG	VRGPNGDAGR	PGEPLMGPR	GLPGSPGNIG	PAGKEGPPVL	PGIDGRPGPI	GPAGARGEPG	NIGFPGPKGP	TGDPGKNGDK	GHAGLAGARG	APGPDGNGA	QGPPG	R1A2	
N	GIPGKGPAG	ERGAPGPAG	RGAAGEPRGD	GVPGGPGMRG	MPGSPGPGS	DGKPGPPGSG	GESGRPGPPG	PSGPPGQPGV	MGFPFGKND	GAPGKNGERG	GPGGPGPQGP	PGKNG	H3A1	
D3	461	481	501	521	541	561								
PAGPA	GERGEQGPAG	SPGFQGLPGP	AGPPGEAGKP	GEQGVPGDLG	APGPGSARGE	RGFPGERGVQ	GPPGPAGPRG	ANGAPGNDGA	KGDAGAPGAP	GSQCAPGLQG	MPGERGAAGL	PGP	H1A1	
PAGPA	GERGEQGPAG	SPGFQGLPGP	AGPPGEAGKP	GEQGVPGDLG	APGPGSARGE	RGFPGERGVQ	GPPGPAGPRG	ANGAPGNDGA	KGDAGAPGAP	GSQCAPGLQG	MPGERGAAGL	PGP	R1A1	
PQGVQ	GKGGEQGPFG	PPGFQGLPGP	SGPAGEVGKP	GERGLHGEFG	LPGPAGPRGE	RGPPGESGAA	GPTGPIGSRG	PSGPPGPDGN	KGEPGVVAVG	GTAGPSGPGS	LPGERGAAGI	PGG	H1A2	
PQGVQ	GKGGEQGPAG	PPGFQGLPGP	SGTAGEVGKP	GERGLHGEFG	LPGPAGPRGE	RGPPGESGAA	GPSGPIGIRG	PSGAPGPDGN	KGEAGAVGAP	GSAGASGPGG	LPGERGAAGI	PGG	R1A2	
ETGPQ	GPPGPTGPGG	DKGDTGPPGP	QGLQGLPQTG	GPPGNGKPG	EPGPKGDAGA	PGAPGCKGDA	GAPGERGPPG	LAPAGLRRG	AGPPGPEGGK	GAAGPPGPPG	AAGTPGLQGM	PGE	H3A1	
D3	581	601	621	641	661	681								
KGDRGDA	GPKGADGSPG	KDGVRLTGP	IGPPGPAGAP	GDKGESGSPG	PAGPTGARGA	PGDRGEPGPP	GPAGFAGPPG	ADGQPGAKGE	PGDAGAKGDA	GPPGPAGPAG	PPGPIGNVGA	P	H1A1	
KGDRGDA	GPKGADGSPG	KDGVRLTGP	IGPPGPAGAP	GDKGETGSPG	PAGPTGARGA	PGDRGEPGPP	GPAGFAGPPG	ADGQPGAKGE	PGDTGVFVGA	GPPGPAGPAG	PPGPIGNVGA	P	R1A1	
KGEKGE	GLRGEIGNPG	RDGARGAPGA	VGAPGPAGAT	GDRGEAGAAG	PAGPAGPRGS	PGERGEVGPA	GPNGFAGPAG	AAGQPGAKGE	RGAKGPKGEN	GUVGPTGPVG	AAGPAGPNPG	P	H1A2	
KGEKGE	GLRGEIGNPG	RDGARGAPGA	IGAPGPAGAS	GDRGEAGAAG	PSGPAGPRGS	PGERGEVGPA	GPNGFAGPAG	AAGQPGAKGE	KGTGPKGEN	GIVGPTGPVG	AAGPSGPNPG	P	R1A2	
RGLGSP	GPKGDGEPG	GPGADGVPGK	DGPRGPTGPI	GPPGPAQPG	DKGEGGAPGL	PGIAGPRGSP	GERGETGPPG	PAGFPAGAPQ	NGEPGKGER	GAPGKGEKG	PPGVAGPPGG	S	H3A1	
D4	701	721	741	761	781									
GAKGARGSA	GPPGATGFPG	AAGRVGPPGP	SGNAGPTGPP	GPAGKEGKGG	PRGETGPAGR	PGEVGPPGPP	GPAGEKGSFG	ADGPAGAPGT	PDPQGIAGQR	GVVGLPQQRG	ERGFTGLP	H1A1		
GPK*SRGAA	GPPGATGFPG	AAGRVGPPGP	SGNAGPTGPP	GPVGRGEGKG	PRGETGPAGR	PGEVGPPGPP	GPAGEKGSFG	ADGPAGSPGT	PDPQGIAGQR	GVVGLPQQRG	ERGFTGLP	R1A1		
GPAGSRGDG	GPPGATGFPG	AAGRVGPPGP	SGNAGPTGPP	GPAGKEGKGG	PRGQDQPVGR	TGEIVGAVGP	GFAGEKGPSG	EAGTAGPPGT	PGPQGLLAGP	GILGLPGSRG	ERGFLPVA	H1A2		
GPAGSRGDG	GPPGATGFPG	AAGRVGPPGP	SGITGPPGPP	GAAGRGIRG	PRGQDQPVGR	TGEIGASGPP	GFAGEKGPSG	EPGTTGPPGT	AGPQGLLAGP	GILGLPGSRG	ERGQPGIA	R1A2		
GPAG*PGGQ	GVK*GEIGSPG	GPGACPFCA	RULPGFPGRH	GRFPVGPSPG	SPGKDGPFGP	AGNTGAPGSP	GVSGPKDAG	QPGKEGSPGA	QGPPGAPGGL	GIAGITGARG	LAPPPGMP	H3A1		
D4	801	821	841	861	881	901								
GP	SGEPGKQGPS	GASGERGPPG	P*GP	PGLAGP	PGESGREGAP	GAEGSPGRDG	SPGAKGDRGE	TGPAGPPGAP	GAPGAPGVG	PAGKSGDRGE	TGPAGTGPV	GPVGARGPAG	PQGP	H1A1
GP	SGEPGKQGPS	GASGERGPPG	P*GP	PGLAGP	PGESGREGSP	GAEGSPGRDG	APGAKGDRGE	TGPAGPPGAP	GAPGAPGVG	PAGKSGDRGE	TGPAGTGPV	GPVGARGPAG	PQGP	R1A1
GA	VGEPLGLIA	GPPGARGPPG	AVGSPGVNGA	PGEAGRDGNP	GNDGPPGRDG	QPGHKGERGY	PGNIGPVGAA	GAPGPHGPGV	PAGKHGMRGE	TGSPGVGPA	GAVGPRGPGS	PQGI	H1A2	
GA	VGEPLGLIA	GPPGARGPPG	AVGSPGVNGA	PGEAGRDGNP	GSDGPPGRDG	QPGHKGERGY	PGNIGPVGAA	GAPGPHGPGV	PAGKHGMRGE	PDPAGSVGPV	GAVGPRGPGS	PQGI	R1A2	
GP	RSPGPGQGVK	GESGKPGANG	LSGERGPPGP	QGLPGLAGTA	GEPGRDGNPG	SDGLPGRDGS	PGGKGDGRGN	GSPGAPGAPG	HPGPPGVGP	AGKSGDRGES	GPAGPAGAPG	PAGS	H3A1	
D4-6	921	941	961	981	1001									
RSDKGE	TGEQGDRIK	GHRGFSGLQG	PPGPPGSPGE	QGPSGASGPA	GPRGPPGSAG	APGKDGLNGL	PGPIGPPGPR	GRTGDAGPVG	PPGPPGPPGP	PGPP	H1A1			
RSDKGE	TGEQGDRIK	GHRGFSGLQG	PPGS*PGSPGE	QGPSGASGPA	GPRGPPGSAG	SPGKDGLNGL	PGPIGPPGPR	GRTGDSGPAG	PPGPPGPPGP	PGPP	R1A1			
RSDKGE	PGKGPRLPL	GLKGNGLQG	LPGIAGHNGD	QAGPSGVGPA	GPRGPPGSPG	PAGKDRGTGH	PGTVGPAGIR	GPQGHQGPAG	PPGPPGPPGP	PGVS	H1A2			
RSDKGE	PGDKGARGLP	GLKGNGLQG	LPLAGLHNGD	QAGCPGVGPA	GPRGPPGSPG	PICKDRSGH	PGTVGPAGVR	GSQCSQGPAG	PPGPPGPPGP	PGVS	R1A2			
RCAPGP	QGPGRDKGET	GERGAAGIKG	HRGPPGNPGA	PGSPGPAQGG	GAIGSPGAPG	PRGPVGPSPG	PGKDDTSGHP	GPIGPPGPRG	NRGERGSEGS	PGHPGQPGPP	GPPGAPGPG	H3A1		