Fibril-forming Collagens in Lamprey

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Five types of collagen with triple-helical regions approximately 300 nm in length were found in lamprey tissues which show characteristic D-periodic collagen fibrils. These collagens are members of the fibril-forming family of this primitive vertebrate. Lamprey collagens were characterized with respect to solubility, mobility on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, carboxymethyl-cellulose chromatography, peptide digestion patterns, composition, susceptibility to vertebrate collagenase, thermal stability, and segment long spacing-banding pattern. Comparison with fibril-forming collagens in higher vertebrates (types I, II, III, V, and XI) identified three lamprey collagens as types II, V, and XI. Both lamprey dermis and major body wall collagens had properties similar to type I but not the typical heterotrimeric composition. Dermis molecules had only α1(I)-like chains, while body wall molecules had α2(I)-like chains combined with chains resembling lamprey type II. Neither collagen exhibited the interchain disulfide linkages or solubility properties of type III. The conservation of fibril organization in type II/type XI tissues in contrast to the major developments in type I and type III tissues after the divergence of lamprey and higher vertebrates is consistent with these results. The presence of type II and type I-like molecules as major collagens and types V and XI as minor collagens in the lamprey, and the differential susceptibility of these molecules to vertebrate collagenase is analogous to the findings in higher vertebrates.

The family of collagen molecules in higher animals with uninterrupted (Gly-X-Y), triple-helical regions 300 nm in length and with the capacity to aggregate into fibrils with an axial period of D = 67 nm includes five distinct types of collagens: types I, II, III, V, and XI (1α, 2α, 3α) (1–3). These five collagens have been designated the fibril-forming or group I collagens (1). Although these collagens show some sequence homology, especially in the distribution of charged residues (4–6), each genetic type has identifying characteristics, such as chain composition, amino acid composition, and the amount of hydroxylysine and hydroxylysine-linked disaccharides resulting from post-translational modification (7–9, see Table V). Group I collagens also differ in their susceptibility to vertebrate collagenase (10, 11): types I and III are cleaved rapidly and completely, while type II undergoes a slower, incomplete degradation and types V and XI are resistant to any cleavage.

Type I and II collagens are always major tissue components, while types V and XI constitute minor collagens in a tissue (1, 9). Type I is the predominant collagen in bone, tendon, and cornea, while type V is found as a minor component (usually less than 5%). Type II is the major collagen in cartilage, vitreous, and notochord, with type XI present in small amounts (less than 10%). Type III is found together with type I in skin, reticular tissues, and vascular tissues, where it constitutes 15–40% of total collagen.

Fibril-forming collagens evolved early in the development of multicellular animals. D-periodic fibrils containing molecules with a characteristic distribution of charged residues are found in many invertebrates (12–14). In the specialized tissues of vertebrates, a family of distinct fibril-forming types is seen. Lampreys, a member of the most primitive vertebrate class, have a collagen with α1(I)-like chains in the dermis (15, 16), and have type II and XI collagens in the notochord (17, 18). Types I and II have been identified in sharks and bony fish (12, 19, 20), but it is only in avian and mammalian species that all five types (I, II, III, V, XI) have been reported (7, 8, 21, 22).

We report here the collagens present in three tissues of the lamprey which contain D-periodic collagen fibrils: the dermis, notochord, and body wall. Five types of collagen with triple-helical regions 300 nm in length were found. Of these, three types were identifiable as II, V, and XI by their solubility, chain composition, amino acid composition, and susceptibility to vertebrate collagenase. The other two lamprey collagen molecules did not correspond to types I and III of higher vertebrates in their chain composition.

MATERIALS AND METHODS

Extraction and Purification—Mature lampreys (Petromyzon marinus) were obtained from the New Hampshire Fish and Game Commission. Tissues were dissected and diced on ice and all procedures were done at 4 °C (unless otherwise stated). Acid extraction of dermis and pepsin extraction of notochord and subcutaneous tissues were carried out as previously described (17, 23). Salt fractionation at neutral pH was performed on collagen solutions in 1 M NaCl, 0.05 M Tris, pH 7.5, by bringing the NaCl concentration in steps to 1.7, 2.0, 2.6, and 5.0 M, with precipitates collected by centrifugation at 15,000 rpm for 1 h in a Sorvall SS-34 rotor. Differential salt fractionation at acid pH was performed by bringing collagen solutions in 0.5 M acetic acid to 0.9 M NaCl and then to 1.2 M NaCl, with precipitates collected by centrifugation as above (7).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described (24, 25). Gels were stained with 25% Coomassie Blue, 10% trichloroacetic acid and destained with 7.5% acetic acid, 15% methanol. To reduce disulfide cross-links, 5% β-mercaptoethanol was added to the SDS sample buffer and the sample boiled for 3–4 min prior to electrophoresis.

1 The abbreviations used are: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; CNBr, cyanogen bromide; SLS, segment long spacing; CMC, carboxymethylcellulose chromatography.
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To separate polypeptide chains, carboxymethyl-cellulose chromatography (CMC) was carried out at 42 °C as described (7). Molecular sieve chromatography was performed on a 2.7 × 60-cm column of Sepharose CL-6B (Pharmacia LKB Biotechnology Inc.) (16). Samples were dissolved in 6 M urea, 1.0 M CaCl₂, 0.05 M Tris, pH 7.5, applied to the column, and eluted with 1.0 M CaCl₂, 0.05 M Tris, pH 7.5 at a flow rate of 15 ml/h.

Cyanogen Bromide and V-8 Protease Digestion—For cyanogen bromide (CNBr) digestion, 500 μg of lyophilized collagen was dissolved in 100 μl of nitrogen-purged 70% formic acid containing 50–100 mg/ml CNBr, and incubated for 4 h at 30 °C. The samples were evaporated to dryness then rehydrated with cold distilled water and lyophilized three times.

For V-8 protease digestion, 500 μg of lyophilized collagen was dissolved in 125 μl of buffer containing 0.125 M Tris, pH 6.8, 10% glycerol, 1% SDS, 0.0005% Bromphenol Blue, 1 mM EDTA. Staphylococcus aureus V-8 protease (250 μl of 100 μg/ml from Miles Laboratories Inc.) was added and samples were incubated at 37 °C for 24 h (20). The reaction was terminated by adding 20% SDS and boiling for 3 min.

Vertebrate Collagen Digestion—Lyophilized collagen (100 μg) was dissolved in 100 μl of 100 mM Tris, 150 mM NaCl, 10 mM CaCl₂, pH 7.5. Thirty microliters of human skin fibroblast collagenase (120 μg/ml) generously provided by Dr. John Jeffrey was activated by incubation with 10 μl of 0.5 mg/ml trypsin at room temperature for 15 min; then 10 μl of 2 mg/ml soybean trypsin inhibitor was added and the sample kept at room temperature for 10 min (11). The samples were incubated with the enzyme at 18 or 25 °C, using the lower temperature for collagens with a lower thermal stability. Aliquots were examined at 6 and 30 h. The reaction was stopped by adding 20 μl of 0.125 M Tris, pH 6.8, 6% SDS, 30% glycerol, 0.015% Bromphenol Blue and boiling for 3 min. The samples were then dialyzed against 0.125 M Tris, pH 6.8, 2% SDS, 10% glycerol, 0.001% Bromphenol Blue before being applied to SDS-PAGE.

Circular Dichroism Spectroscopy—Circular dichroism spectra were recorded on a Cary 61 instrument. To obtain melting curves, the wavelength was fixed at the ellipticity maximum near 221 nm, and the temperature was increased at a rate of 20–30 °C/h. All spectra and melting profiles were recorded with samples dissolved in 0.01 M acetic acid.

Amino Acid Analysis—Amino acid analyses were performed by Dr. Michel van der Rest on a Dionex model D-500 amino acid analyzer using samples hydrolyzed in 6 M HCl under nitrogen for 6, 12, and 24 h at 110 °C. The resulting amino acid composition data were extrapolated to zero time of hydrolysis. If there was only enough purified collagen for one sample, it was hydrolyzed for 12 h.

Segment Long Spacings—Segment long spacing (SLS) crystallites were formed, stained, and examined as previously described (17).

RESULTS

We report the characterization of collagens in the dermis, body wall, and notochord of the lamprey.

Dermis Collagen

The skin of the lamprey consists of a surface epidermal layer, a dermis, and a subcutaneous layer (Fig. 1). The dermis is composed of lamellae of homogenous diameter collagen fibrils (27), and all dermis collagen is solubilized by acid or neutral salt solutions (15, 28, 29). The dermis of the mature lamprey is unusual in the high solubility of its collagen, and in containing only one collagen type, with no minor components. Sequential salt precipitation studies carried out on acid-extracted collagen at neutral pH indicated no precipitation at 1.7 M NaCl, while substantial amounts came out at 2.0 M NaCl (37% of total), 2.3 M NaCl (17%), and 2.6 M NaCl (46%). Raising the salt concentration to 5.0 M NaCl precipitated little additional collagen (1%). Dermis collagen shows on gel electrophoresis (Fig. 2, lane 1) a single major band in the α-region, running slightly faster than the α(1) chain of higher vertebrates, and one major band in the β region, which we assume to represent cross-linked dimers, as previously reported (15, 28, 29). The polypeptides corresponding to the α and β bands could not be resolved on carboxymethyl-cellulose chromatography, in contrast to a report on another lamprey species (15), but were separated on a Sepharose CL-6B molecular sieve column (not shown). Comparison of the cyanogen bromide digestion patterns and purified V-8 digestion patterns of purified α chains and purified β component showed distinct peptide patterns (Fig. 3A, lanes 3 and 4; Fig. 3B, lanes 4 and 5). The presence of peptides in the β component not seen for α indicates that β contains at least one polypeptide chain distinct from the dermis monomer chains. Since the β-component lacks a number of CNBr and V-8 peptides seen for the α component, the β dimers must not include one chain in the α pool. The amino acid compositions of α and β are, however, very similar (Table I), suggesting the chains, as expected from their similar elution position on carboxymethyl-cellulose, are closely related. The dermis collagen molecule is thus a heterotrimer with two closely related polypeptide chains, as reported for a different lamprey species by Kimura et al. (15, 16).

Dermis α and β have amino acid compositions expected for a primitive α(1) chain, showing similarities to other vertebrate α(1) chains, together with lower hydroxyproline and higher serine contents (Table II; 12, 15). Dermis collagen is digested very efficiently by human skin collagenase, at a rate comparable to that of rat tail tendon type I collagen, which also supports its type I-like or type III-like character (Fig. 4). Its SLS-banding pattern is indistinguishable from that of higher vertebrate type I collagen (Fig. 5). The molecule had a characteristic triple-helical circular dichroism spectrum and melted at 28.5 °C with a sharp transition.

Since higher vertebrate skin contains type III collagen, with disulfide bonds linking the three polypeptide chains in a molecule, as well as type I, we compared the gel electrophoresis patterns of dermis collagen with and without reduction and saw no difference. Similar experiments on the other
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**Body Wall (or Subcutaneous) Collagen**

The subcutaneous layer of lamprey skin joins the muscle layer and is continuous with tissue surrounding the spinal cord and notochord sheath. Collagen in this layer is not solubilized by acid or neutral salt solution but can be extracted by pepsin treatment. Pepsin extraction of subcutaneous tissue yielded one major collagen and a minor component. The major collagen had a solubility similar to type I, precipitating at 0.9 M NaCl at acid pH. During sequential salt fractionation at neutral pH, little collagen came out below 2.6 M NaCl (6%), with the majority precipitating at 2.6 M NaCl (47%) and 3.0 M NaCl (41%). On SDS-PAGE this collagen shows two bands running near $\alpha_1(I)$ which are often not resolved, and one band near $\alpha_2(I)$ (Fig. 2, lane 2). We denote these three chains as $\alpha_1$, $\alpha_1'$, and $\alpha_2$, respectively. A small amount of material was also seen in the $\beta$ region.

On carboxymethyl-cellulose chromatography, $\alpha_1'$ eluted just after $\alpha_1$ (at a salt concentration near 0.03 M NaCl), while $\alpha_2$ eluted at a significantly higher salt concentration (0.065 M NaCl) (Fig. 6). The individual chains were isolated chromatographically and further characterized. The amino acid compositions of $\alpha_1$ and $\alpha_1'$ are similar (Table I). The CNBr and V-8 protease digestion patterns of $\alpha_1'$ chains which appeared pure on electrophoresis (Fig. 6, inset), showed the major bands present in $\alpha_1$ but also had some distinct bands (Fig. 3A, lanes 7, and 8; Fig. 3B, lanes 8 and 9). These results suggest that $\alpha_1$ and $\alpha_1'$ are polypeptide chains related by degradation, variation in post-translational modification, or as products of duplicated genes. The amino acid compositions of the $\alpha_1$ and $\alpha_1'$ chains differ from lamprey dermis collagen and from $\alpha_1(I)$ chains of higher and lower vertebrates in their...
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TABLE I
Amino acid compositions of lamprey collagens

| Major body wall | Major body wall | Dermis | Minor body wall | Minor notochord component* |
|----------------|----------------|--------|----------------|----------------------------|
| al(II)         | a1             | a2     | α              | β                          |
| Hydroxyproline | 74             | 67     | 51             | 67                         | 59                         | 69                         | 61                         | 56                         | 79                         |
| Aspartic acid  | 47             | 42     | 59             | 53                         | 47                         | 57                         | 58                         | 52                         | 46                         |
| Threonine      | 27             | 20     | 23             | 18                         | 17                         | 27                         | 24                         | 28                         | 26                         |
| Serine         | 38             | 51     | 73             | 63                         | 69                         | 56                         | 42                         | 43                         | 42                         |
| Glutamic acid  | 99             | 103    | 97             | 74                         | 67                         | 68                         | 96                         | 105                        | 100                        | 102                        |
| Proline        | 115            | 116    | 99             | 69                         | 93                         | 91                         | 87                         | 106                        | 96                         | 121                        |
| Glycine        | 330            | 362    | 363            | 380                        | 367                        | 376                        | 345                        | 352                        | 360                        | 325                        |
| Alanine        | 104            | 84     | 94             | 75                         | 128                        | 122                        | 55                         | 69                         | 65                         | 102                        |
| Valine         | 12             | 10     | 12             | 21                         | 23                         | 26                         | 30                         | 30                         | 25                         | 12                         |
| Isolecucine    | 11             | 8      | 20             | 9                          | 10                         | 19                         | 19                         | 16                         | 9                          | 9                          |
| Leucine        | 29             | 30     | 32             | 42                         | 22                         | 24                         | 45                         | 46                         | 44                         | 27                         |
| Tyrosine       | 2              | <2     | <2             | 2                          | 2                          | 2                          | 4                          | <2                         | <2                         | 2                          |
| Phenylalanine  | 12             | 14     | 13             | 9                          | 10                         | 12                         | 8                          | 9                          | 9                          | 13                         |
| Histidine      | 4              | 4      | 5              | 3                          | 4                          | 10                         | 8                          | 8                          | 5                          |
| Hydroxylysine  | 22             | 11     | 11             | 6                          | 5                          | 34                         | 31                         | 37                         | 22                         |
| Lysine         | 16             | 23     | 25             | 18                         | 21                         | 20                         | 21                         | 17                         | 16                         |
| Arginine       | 51             | 48     | 50             | 49                         | 54                         | 52                         | 46                         | 42                         | 43                         | 52                         |

*This composition is for the slower mobility band of the minor subcutaneous component. The faster mobility band was contaminated with al(II).

These chains were previously called a1α, 2α, and 3α.

Methionine values were not measured because of the problem with oxidation.

TABLE II
Comparison of lamprey dermis α and body wall α chain with α1(I) chains of various vertebrates

| α1 lampey  | α1 lampey  | α1(I)  | α1(I)  | α1(I)  | α1(I)  |
|-------------|-------------|--------|--------|--------|--------|
| body wall*  | dermis*     | shark skin* | carp skin* | mackerel skin* | human skin* |
| al(I)       | al(I)       | al(I)  | al(I)  | al(I)  | al(I)  |
| Hydroxyproline | 72             | 67     | 60     | 71     | 68     | 108    | 92    | 127   |
| Aspartic acid  | 42             | 53     | 45     | 44     | 43     | 42     | 43    | 43    |
| Threonine     | 20             | 18     | 23     | 28     | 27     | 19     | 17    | 13    |
| Serine        | 51             | 65     | 51     | 36     | 43     | 27     | 37    | 39    |
| Glutamic acid | 103            | 67     | 69     | 75     | 76     | 77     | 77    | 72    |
| Proline       | 116            | 93     | 108    | 113    | 110    | 120    | 135   | 109   |
| Glycine       | 362            | 367    | 343    | 338    | 339    | 328    | 332   | 354   |
| Alanine       | 84             | 128    | 117    | 132    | 130    | 129    | 115   | 97    |
| Valine        | 10             | 21     | 26     | 16     | 18     | 14     | 21    | 14    |
| Methionine    | N.D.*          | N.D.   | 18     | 15     | N.D.   | N.D.   | N.D.  | N.D.  |
| Isolecucine   | 8              | 9      | 15     | 11     | 8      | 6      | 7     | 13    |
| Leucine       | 30             | 22     | 19     | 17     | 18     | 20     | 20    | 22    |
| Tyrosine      | <2             | 2      | 2      | 1      | 1      | 2      | 2     | 3     |
| Phenylalanine | 14             | 10     | 12     | 14     | 16     | 12     | 12    | 8     |
| Histidine     | 4              | 3      | 6      | 4      | 3      | 2      | 2     | 2     |
| Hydroxylysine | 11             | 6      | 9      | 4      | 5      | 5      | 4     | 5     |
| Lysine        | 23             | 21     | 28     | 31     | 27     | 30     | 30    | 30    |
| Arginine      | 48             | 54     | 51     | 53     | 53     | 50     | 50    | 47    |

*a This work.

b Ref. 30.

c Ref. 16.

d Ref. 31.

e Ref. 32.

f Ref. 33.

N.D., not determined.

high glutamic acid, leucine, and hydroxylysine and low alanine and valine content (Tables I and II). The unusual composition features (Table I) and overall digestion patterns (Fig. 3) of the α1 and α1' chains bear some resemblance to lamprey type II collagen.

The peptide maps of α2 differed markedly from those for α1 and α1' (Fig. 3A, lane 6; Fig. 3B, lane 7), and α2 had a very different composition, notable in its low content of imino acids and alanine and high content of histidine and hydrophobic residues (Table I). The α2 composition resembled that of the collagen chain in dogfish shark skin identified as α2(I) (30) (Table III). These experiments indicate for the first time that a chain with an electrophoretic mobility on SDS-PAGE, a high salt elution position on CMC chromatography, and an amino acid composition similar to α2(I) is present in the lamprey.

Pepsin digestion of the tissue surrounding the notochord (perinotochord), the body wall, and the muscle layer yielded collagen chains with electrophoretic mobilities and V-8 protease digestion patterns identical to that found in the subcutaneous layer, indicating this continuous connective tissue has a uniform collagen composition (data not shown). We shall refer to this collagen as body wall collagen.

The minor collagen component of the subcutaneous layer (less than 10% total collagen) showed two bands on SDS-PAGE, with the faster band slightly slower than rat α1(I) (Fig. 2, lane 5). The collagen was soluble at 2.6 M NaCl, but precipitated at 4.4 M NaCl at neutral pH. Most of the minor
collagen; and represents digestion at and tebrate collagenase digestion of the five lamprey collagens.

shown), and its amino acid composition showed the low alanine and high hydrophobic amino acid contents of a type a small amount of relatively pure minor collagen was soluble in the lamprey body wall tissue was digested at 18 °C for 0 and 30 h (lanes 10 and 11) and at 25 °C for 30 h (lane 12); chick type II collagen was digested at 25 °C for 0, 6, and 30 h (lanes 13-15); lamprey notochord type II was digested at 25 °C for 0, 6, and 30 h (lanes 16-18); and the lamprey notochord minor component (type XI) was digested at 25 °C for 0, 6, and 30 h (lanes 19-21).

collagen precipitated at 0.9 M NaCl in 0.5 M acetic acid, but a small amount of relatively pure minor collagen was soluble at this salt concentration and precipitated at 1.2 M NaCl. The chain with slower mobility could be separated on CMC (not shown), and its amino acid composition showed the low alanine and high hydrophobic amino acid contents of a type V collagen (Table IV, (5)) together with the lower hydroxyproline and higher serine expected for a primitive vertebrate (12). Studies of the faster moving band were hindered by contamination with the major a1 chain.

The major subcutaneous collagen underwent incomplete cleavage by human skin collagenase at a rate slower than that of type I and similar to that of type II collagen (Fig. 4, lanes 7-9). The minor component was not susceptible to digestion by this enzyme, a property which is characteristic of type V collagen (Fig. 4, lanes 10-12). The major collagen had a sharp thermal transition near 28.5 °C, while the minor subcutaneous collagen melted near 32 °C, with a broader melting profile. The major subcutaneous collagen formed SLS crystallites with characteristic type I-bandings patterns (Fig. 5). Although the minor collagen formed SLS crystallites, we were unable to obtain staining adequate for comparisons.

Notochord Collagen

Characterization of collagens in the notochord sheath of the lamprey was previously reported (17, 18). The major collagen was identified as type II, based on its electrophoretic mobility, solubility properties, and amino acid composition. We compared type II to other lamprey collagens, with respect to composition, CNBr peptide pattern, V-8 protease digestion patterns, and thermal stability (Fig. 3; and Table I). A minor collagen also present in notochord corresponds in its features to type XI collagen in higher vertebrate cartilage (Table IV).

Lamprey type II collagen was partially digested by human skin collagenase, at a rate similar to that seen for chick type II (Fig. 4), which is slower than that for mammalian and avian types I and III (10, 11). The minor notochord collagen was not digested, even after long time periods (Fig. 4, lanes 19-21). Both notochord collagens melted at 33 °C, showing broad melting transitions and both collagens showed SLS crystallite patterns similar to those seen for higher vertebrate types I and II collagens (Fig. 5).

DISCUSSION

Vertebrates first appeared in the fossil record about 500 million years ago. The earliest vertebrates (class Agnatha) were jawless fish with a notochord as the axial supporting structure (12), and lamprey and hagfish are contemporary survivors of this class. Lamprey tissues exhibiting D-periodic collagen fibrils were found to contain five collagen types with a pepsin-resistant triple-helical region of approximately 1000 residues as estimated from electrophoretic mobility on SDS-PAGE. These observations imply that at an early stage of vertebrate evolution the fibril-forming collagen family contained at least five members. The three major types form SLS crystallites with staining patterns indistinguishable from higher vertebrate type I. These observations support the hypothesis of a critical role for charge distribution and length

FIG. 6. Carboxymethyl-cellulose chromatography of the body wall major collagen. The column was equilibrated with 0.04 M sodium acetate containing 6 M urea, pH 4.8, at 42 °C and was eluted with an 0-0.10 M NaCl gradient. The a1 chains eluted at a lower salt concentration than a2 chains (0.032 M NaCl and 0.064 M NaCl, respectively). The inset shows SDS-PAGE (6% polyacrylamide gel) of selected fractions (circles on chromatogram): lane 1, rat tail tendon type I; lane 2, lamprey body wall collagen; lane 3, fraction 33; lane 4, fraction 43; lane 5, fraction 52; lane 6, fraction 64.

FIG. 5. SLS crystallite patterns of (a) lamprey body wall collagen; (b) lamprey dermis collagen; (c) lamprey notochord collagen; and (d) rat tail tendon type I collagen. The bar represents 50 nm.
of the triple-helical region in formation of D-periodic fibrils
(4).

The conserved features of charge distribution and triple-
helix length contrast with the variations in thermal stability
among lower vertebrate skin collagens, where melting tem-
perature is correlated with upper environmental temperature
(34). In lamprey the two notochord collagens and the minor
body wall collagens melt at 32–33 °C, while the major dermis
and body wall collagens melt at 28 °C. Fibril diameters and
organization are another characteristic which varies markedly
in the three lamprey tissues studied. Both fibril morphology
and thermal stability are important to function, and it is
likely that the presence of distinct genetic types is necessary
to mediate these tissue-specific properties.

Comparison of lamprey collagens with higher vertebrate
collagens with respect to tissue distribution, number of dis-
tinct chains per molecule, electrophoretic mobilities on SDS-
PAGE, amino acid composition, post-translational modifica-
tion, solubility properties, and susceptibility to vertebrate
collagenase allows us to identify the major collagen in noto-
chord as type II, the minor collagen in notochord, as type XI,
and the minor collagen in the body wall as type V (Table IV).

The conservation of so many features for these three collagens
implies that the characteristic features of types II, XI, and V
were already established in the earliest vertebrates and that
these features have been highly conserved in the divergent
evolutionary development of the modern lamprey and higher
vertebrates over 500 million years. The high degree of conserv-
ation of type II has been suggested by previous reports (19,
35). The relation of the major collagens in the lamprey dermis
and body wall to members of group I collagens in higher
vertebrates is less direct. Lamprey dermis collagen is related
to type I collagen on the basis of its presence in D-periodic
fibrils in skin, its SLS-banding pattern (12), its a1(I)-like

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**Table III**

| Lamprey body wall a2 | Dogfish shark skin | Carp skin | Mackerel skin | Chick skin | Human skin |
|----------------------|-------------------|-----------|---------------|------------|------------|
| Hydroxyproline       | 51                | 48        | 69            | 66         | 98         | 83         |
| Aspartic acid        | 59                | 41        | 56            | 53         | 50         | 47         |
| Threonine            | 23                | 26        | 25            | 24         | 20         | 20         |
| Serine               | 73                | 78        | 38            | 41         | 31         | 35         |
| Glutamic acid        | 74                | 67        | 64            | 63         | 67         | 68         |
| Proline              | 69                | 81        | 112           | 100        | 117        | 120        |
| Glycine              | 380               | 345       | 337           | 348        | 328        | 337        |
| Alanine              | 75                | 93        | 114           | 122        | 104        | 105        |
| Valine               | 25                | 32        | 27            | 22         | 28         | 33         |
| Methionine           | N.D.              | 17        | 10            | 13         | 5          | 5          |
| Isoleucine           | 20                | 19        | 10            | 11         | 16         | 15         |
| Leucine              | 42                | 37        | 31            | 27         | 32         | 30         |
| Tyrosine             | 2                 | 3         | 4             | 5          | 2          | 5          |
| Phenylalanine        | 9                 | 13        | 10            | 10         | 13         | 12         |
| Histidine            | 19                | 19        | 8             | 8          | 8          | 10         |
| Hydroxylysine        | 11                | 7         | 22            | 21         | 10         | 8          |
| Lysine               | 18                | 20        | 7             | 8          | 21         | 22         |
| Arginine             | 49                | 56        | 56            | 57         | 50         | 51         |

*This work.

**Table IV**

| Lamprey body wall, minor component | Human | Lamprey notochord | Human cartilage |
|-----------------------------------|-------|-------------------|----------------|
|                                   | a1(V) | a2(V)             | a1(II)         | a2(II) |
| Hydroxyproline                    | 69    | 107               | 61             | 56     |
| Aspartic acid                     | 57    | 48                | 58             | 52     |
| Threonine                         | 27    | 19                | 24             | 28     |
| Serine                            | 56    | 33                | 42             | 43     |
| Glutamic acid                     | 96    | 91                | 105            | 100    |
| Proline                           | 87    | 118               | 106            | 96     |
| Glycine                           | 343   | 334               | 332            | 360    |
| Alanine                           | 55    | 46                | 69             | 65     |
| Valine                            | 26    | 23                | 30             | 25     |
| Isoleucine                        | 19    | 18                | 19             | 16     |
| Leucine                           | 45    | 38                | 46             | 44     |
| Tyrosine                          | 4     | 1                 | <2             | <2     |
| Phenylalanine                     | 9     | 11                | 8              | 9      |
| Histidine                         | 10    | 8                 | 8              | 8      |
| Hydroxylysine                     | 34    | 35                | 31             | 37     |
| Lysine                            | 20    | 20                | 21             | 17     |
| Arginine                          | 46    | 44                | 42             | 43     |

*This work.

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2 B. Brodey, E. F. Eikenberry, K. BelBruno, and T. Hardt, manuscript in preparation.
amino acid composition (15), and its digestion by vertebrate collagenase. The molecular packing in lamprey dermis fibrils exhibits distinctive features found in mammalian skin fibrils, including a shorter axial period near 65 nm (36). Kimura (15, 16) has suggested that the presence of two distinct chain types in dermis collagen supports its type I nature, but this molecule differs from type I in not being associated with a minor type V component and in lacking the usual heterotrimeric nature of type I since both of its chains are similar to a1(1) rather than one chain having features of a2(I). Features of dermis collagen are consistent with a relationship to type III collagen, including its presence in skin, its nonheterotrimeric nature, and its rapid digestion by vertebrate collagenase. However, the distinctive properties that distinguish type III from type I, such as disulfide bonding within the triple-helix and precipitation at 1.7 M NaCl at neutral pH (9), are not found in dermis collagen, suggesting these characteristics may have developed after the lamprey evolved. Alternatively, it is possible that type III collagen in lamprey is particularly sensitive to degradation and was not solubilized intact in our preparations.

Studies of dermis collagen led to the suggestion that identifiable a2(I) chains did not evolve until after the divergence of the lamprey. However, we have identified a collagen type in the body wall which contains a chain with a2(I) characteristics. The presence of this a2(I)-like chain suggests that a2(I) as seen in skins of sharks, fish, and all higher vertebrates did not evolve from one of the dermis chains (15, 16) but was already present in recognizable form in the lamprey. In addition to its heterotrimeric nature, the major body wall collagen resembles type I in being widely distributed in different tissues, in being associated with a minor type V-like collagen, and in the similarity of its axial and lateral molecular packing to that seen in fibrils of bone and some tendons. However, its a1(a1') chains are distinct from a1(I) in amino acid composition and resemble lamprey type II chains in terms of composition, V-8 protease, and CNBr peptide patterns and digestion by vertebrate collagenase. Thus, dermis collagen and body wall collagen both have some characteristics resembling type I collagens, but neither has a traditional chain composition, since the dermis molecule contains only a1(I)-like chains while the body wall molecule contains an a2(I)-like chain in the same triple-helix as a chain different from a1(I).

Homology between the sequences of a1(I), a2(I), a1(II), a1(V) (or a2(V)), a1(III), a2(II), and a3(III) and a2(V) suggest these chains diverged at least 500 million years ago (37, 38). Our finding of a1(II), a2(II), a1(II), a1(I), a2(V), a2(II), and a3(III) suggests that the group 1 polyproline chains may have diverged and been present at the time vertebrates evolved, type I chains may not have been present in the same triple-helices we find today. The presence of a1(II) in lamprey collagen consists of an a2(I) chain closely related to the a chain in lamprey dermis and an a2(I) chain closely resembling the a2 chain of lamprey body wall raises the possibility that after the divergence of lamprey and higher vertebrates these chains may have joined to form the type I molecule of higher vertebrates (Table V). Such an association would require changes in the C-propeptides which are thought to govern chain selection (1).

The presence of type II collagen in notochord and type I-related molecules in the dermis and body wall suggests that different genetic types were involved in tissue specialization in early vertebrates. The status of type II and type I-related molecules as major collagenous components and types V and X as minor components is apparent only by the time the lamprey evolved and has been maintained in modern lampreys and mammals. Collagen molecules with amino acid compositions similar to type V are found as the major component in some invertebrate tissues with D-periodic fibrils (39), suggesting that relegation of type V to a minor status may have occurred as vertebrates developed. No lamprey collagen occupies the higher vertebrate type III niche, where type III comprises 15–40% of total collagen in specific tissues such as skin.

The degree of susceptibility of the five lamprey collagens to vertebrate collagenase digestion was a useful property to compare with mammalian collagen types. The resistance of the minor type V and XI collagens to cleavage and the digestibility of the three major lamprey collagens is strikingly similar to the situation in mammals and may relate to mechanisms of degradation established early in vertebrate evolution. We believe our data characterize the lowest vertebrate collagen known to be cleaved by vertebrate collagenase.

The data on lamprey and higher vertebrate collagens reflect proteins that have evolved separately for over 500 million years. We believe that the structure of lamprey collagens closely reflects early vertebrate forms since the body morphology of the lamprey today is similar to that seen in fossils from 300 million years ago (40). During the development of higher vertebrates from their primitive ancestors, there have been major changes in body morphology and connective tissues, with the appearance of bone and tendon (with largely type I collagen) and marked changes in collagen organization in skin. The conservation of fibril diameter, molecular packing, and morphology seen for type II fibrils throughout the vertebrates is consistent with the conservation of type II molecular features. In contrast, types I and III collagens have undergone substantial evolution in molecular composition since early vertebrates, and this is consistent with the dramatic changes in tissues containing these types.

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