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Biosynthesis of a disulphide-bonded short-chain collagen by calf growth-plate cartilage

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Collagen biosynthesis by organ cultures of the hypertrophic zone of calf growth-plate cartilage was studied. It was found that this tissue devotes a large portion of its biosynthetic commitment towards production of a collagen molecule comprising short collagen chains. This collagen is similar to short-chain collagens synthesized by chick-embryo tibiotarsus, rabbit growth-plate cartilage and chick chondrocytes grown in three-dimensional gels. However, in contrast with the collagen synthesized in these three systems, the short-chain collagen synthesized by calf growth-plate hypertrophic cartilage is stabilized by disulphide bonds localized within the pepsin-resistant triple-helical collagenous domains of these molecules.

The molecular heterogeneity of vertebrate collagens has now become a well-documented phenomenon (Bornstein & Traub, 1979). Although it had been thought that hyaline cartilage consisted exclusively of type II collagen, Burgeson & Hollister (1979) and Burgeson *et al.* (1982) have presented evidence for the existence of three additional collagen chains, designated 1α, 2α and 3α, in human and bovine articular cartilage. The presence of small amounts of these collagen chains has been reported in various other cartilaginous tissues, such as neonatal-pig hyaline cartilage (Shimokomaki *et al.*, 1980), bovine nasal cartilage (Ayad *et al.*, 1981) and chick hyaline cartilage (Reese & Mayne, 1981). In addition, these and other studies (Reese *et al.*, 1982) have identified disulphide-bonded collagens termed 'high molecular weight' (HMW) and 'low molecular weight' (LMW) collagens, which yield several smaller-M_r species after reduction.

More recently, we have reported the biosynthesis of a low-M_r collagen by rabbit growth-plate-cartilage organ cultures (Remington *et al.*, 1983). This collagen molecule appears similar to the low-M_r species synthesized by embryonic-chick sternal chondrocytes cultured in three-dimensional collagen gels (Gibson *et al.*, 1982, 1983) and by chondrocytes from the chick tibiotarsus (Schmid & Conrad, 1982*a*,*b*; Schmid & Linsenmayer, 1983). Characterization of the short-chain collagen from aged endochondrial chondrocytes cultured from the hypertrophic zone of embryonic-chick tibiotarsus has been accomplished (Schmid & Linsenmayer, 1983), and it was shown that these molecules comprise M_r-59 000 polypeptides containing a M_r-14 000 non-collagenous domain and a M_r-45 000 triple-helical collagenous region. One of the most salient features in the amino acid composition of this collagen was the absence of cysteine residues. Similar results have been obtained by Kielty *et al.* (1984). In the present paper we demonstrate that organ cultures of cartilage from the hypertrophic zone of calf growth plate biosynthesize large amounts of a similar short collagen molecule. In contrast with previous studies, however, the calf molecules are stabilized by interchain disulphide bonds localized in the pepsin-resistant triple-helical collagenous domains.

Abbreviation used: SDS, sodium dodecyl sulphate.

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Experimental

Biosynthetic studies

Ribs were removed from 1-week-old calves and carefully dissected from surrounding soft tissue. After the perichondrium had been scraped off with a scalpel, the growth-plate cartilage was easily separated from the bony metaphysis with a mild bending force. With a precision guillotine-type

cutting device (Stambaugh & Brighton, 1980), four 1 mm slices of growth-plate cartilage were cut sequentially, starting at the metaphyseal border. The cartilage slices corresponding to the hypertrophic zone were then placed in separate culture dishes and incubated for 24 h (at 37°C, in a 5%-CO_2 atmosphere) in tissue-culture medium (NCTC 135/Mimimum Eagle's Medium/Earle's Basic Salt solution) containing 10% foetal-calf serum, ascorbic acid (100 μg/ml), β-aminopropionitrile (2.5 mM) and [U-^{14}C]proline (10 μCi/ml; 275 Ci/mol; Amersham).

After incubation, the media were removed and frozen after a solution of proteinase inhibitors was added to give final concentrations of 5.0 mM-disodium EDTA, 0.2 mM-phenylmethanesulphonyl fluoride, 5.0 mM-*N*-ethylmalemide and 1.0 mM-*p*-aminobenzamidine hydrochloride. The cartilage slices were homogenized with a glass/glass tissue grinder in 1.0 M-NaCl/50 mM-Tris/HCl buffer (pH 7.4) at 4°C, containing the same concentrations of proteinase inhibitors listed above. Solubilized material was removed by centrifugation at 15 000*g* for 30 min. Media samples and solubilized material from the tissues were dialysed at 4°C against 0.1 M-NH_4HCO_3 containing 0.1 mM-phenylmethanesulphonyl fluoride and 0.5 mM-*N*-ethylmaleimide and then freeze-dried.

Proteolytic digestion

Freeze-dried samples used for collagenase digestion were resuspended in a buffer containing 0.15 M-NaCl, 5.0 mM-$CaCl_2$, 50 mM-Tris/HCl (pH 7.4) 10 mM-*N*-ethylmaleimide and 0.2 mM-phenylmethanesulphonyl fluoride at 37°C. Purified bacterial collagenase (form III; Advanced Biofactures, Lynbrook, NY, U.S.A.) was added (150 units/ml), and the material was digested for 6 h at 37°C. The reaction was terminated by the addition of EDTA (final concn. 10 mM) and cooling at 4°C. For digestion with pepsin, freeze-dried samples were suspended in 0.5 M-acetic acid containing 300 μg of pepsin/ml and incubated for 6 h at 15°C. The digested samples were then dialysed at 4°C against 0.1 M-NH_4HCO_3 and freeze-dried.

Gel electrophoresis and fluorography

Labelled proteins were examined by SDS/polyacrylamide-slab-gel electrophoresis. The buffer system used was the continuous Tris/borate system described by Benya (1981) and Benya & Shaffer (1982). Electrophoresis was performed on 3.5–10%-acrylamide exponential gradient gels, which were prepared as described previously to give a constant concentration of acrylamide at the top of the gel. Freeze-dried samples were dissolved in sample buffer [20 mM-Tris/borate (pH 8.5)/2.0 M-urea/2.0% SDS/1.0% glycerol] and heated for 1 h at 55°C. Reduced samples were treated in the same manner, except that the sample buffer contained 5.0% (v/v) 2-mercaptoethanol. Samples were electrophoresed for 2.5–3 h at 250 V constant voltage. After electrophoresis, gels were processed for fluorography with EN^{3}HANCE (New England Nuclear) and exposed to X-Omat AR film (Kodak). Two-dimensional CNBr-peptide mapping was performed by a modification of the method of Barsh & Byers (1981) as described previously (Remington *et al.*, 1983).

Results

Electrophoretic and fluorographic analysis as well as proteolytic digestion were performed on samples from media and 1.0 M-NaCl extracts. The results did not show appreciable differences between these samples, and therefore only the data obtained with the 1.0 M-NaCl extracts are presented below. When the 1.0 M-NaCl extracts were electrophoresed without disulphide-bond reduction (Fig. 1, lane *a*), it was found that, in addition to the prominent α_1(II)-chains, a large proportion of the radioactivity migrated in the high-M_r region, above β-chains. The relative amount of radioactivity in this region was 1.3–1.5 times that migrating in the region of α_1(II)-chains as determined from densitometric scanning of the fluorographs. In addition, a set of three bands was detected above the α_1(II)-chains. On the basis of other experiments involving proteolytic digestion and CNBr-peptide mapping, these bands have been identified as the pre-pepsin forms of 1α-, 2α- and 3α-chains (results not shown).

When similar samples were electrophoresed after disulphide-bond reduction (Fig. 1, lane *b*), there was a marked decrease in the high-M_r radioactivity, accompanied by the appearance of three new bands, one migrating just below β-chains, with an apparent M_r of approx. 180 000 (upper arrow), and two others migrating below α_1(II)-chains, with apparent M_r values of 85 000 and 60 000 (arrowhead and bottom arrow). The fact that these bands were only seen after reduction, coupled with the observation that a large amount of the high-M_r collagenous material was no longer present after reduction, suggests that these chains normally occur in the form of disulphide-bonded aggregates. These chains are sensitive to collagenase digestion (Fig. 1, lane *c*), and their electrophoretic behaviour is similar to that of collagen molecules previously described in studies of rabbit growth-plate biosynthesis (Remington *et al.*, 1983). Although the exact nature of these molecules is not completely clarified, in order not to add to the existing confusion regarding collagen nomen-

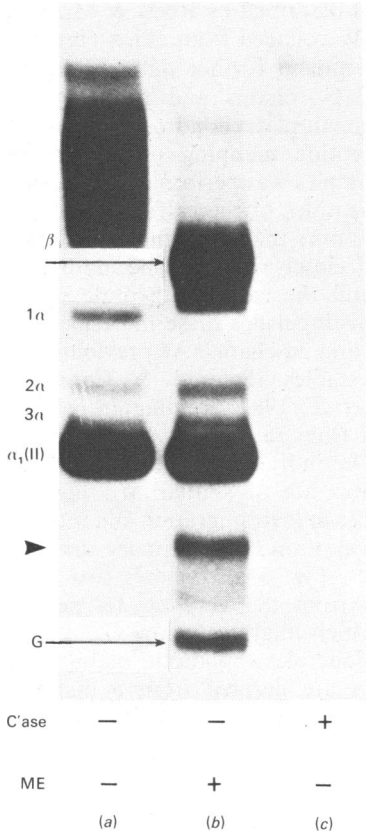

Fig. 1. *SDS/polyacrylamide-gel-electrophoresis fluorograph of 1.0M-NaCl extracts before and after reduction and after collagenase digestion*

Freeze-dried samples of intact or collagenase-digested 1.0M-NaCl extracts were denatured in the presence or in the absence of 5% (v/v) 2-mercaptoethanol and 2% SDS as described in the Experimental section and were electrophoresed in 3.5–10%-polyacrylamide exponential-gradient gels as described by Benya (1981) and Benya & Schaffer (1982). Lanes: (*a*), electrophoresis without reduction; (*b*), electrophoresis after reduction with 2-mercaptoethanol (ME); (*c*), electrophoresis under non-reducing conditions of samples digested with purified bacterial collagenase (C'ase).

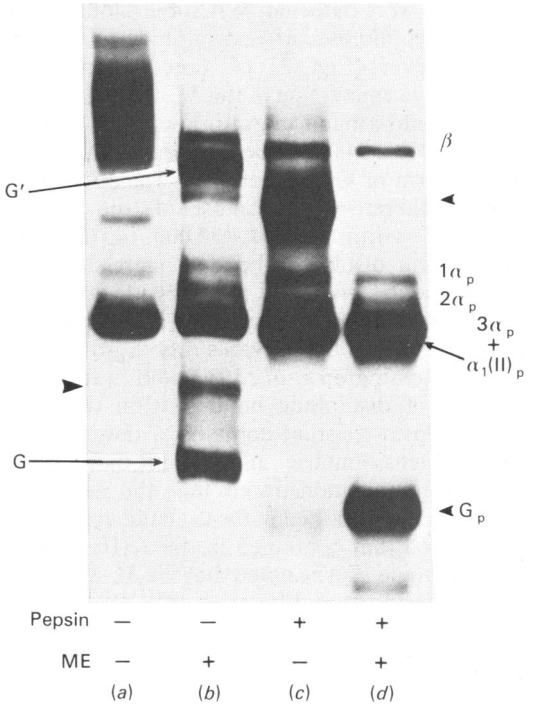

Fig. 2. *SDS/polyacrylamide-gel-electrophoresis fluorograph of pepsin-digested 1.0M-NaCl extracts before and after reduction*

Freeze-dried samples of intact or pepsin-digested 1.0M-NaCl extracts were denatured and electrophoresed as described in Fig. 1. Lanes: (*a*), electrophoresis without reduction of intact extract before pepsin digestion; (*b*), as lane (*a*) after reduction; (*c*), electrophoresis of pepsin-digested extract without reduction; (*d*), as lane (*c*) after reduction. The subscript p denotes the chains resulting after digestion with pepsin.

clature we have chosen to use the terms employed by Gibson *et al.* (1982, 1983) and by us in a previous publication (Remington *et al.*, 1983), until further characterization permits a more accurate denomination. The 180 000-M_r species (upper arrow) has similar electrophoretic behaviour to the higher-M_r form of G collagen termed G′, and the 60 000-M_r species (lower arrow) shows a similar electrophoretic pattern to the short-chain, or G, collagen. The third chain (M_r 85 000) seen in the reduced samples was also seen in the rabbit samples and appears to be related to the HMW collagen described by Reese & Mayne (1981), as discussed previously (Remington *et al.*, 1983).

To characterize further the collagens present in the 1.0M-NaCl extracts, these were submitted to limited pepsin digestion (Fig. 2). When pepsin-digested extracts were electrophoresed under non-reducing conditions (Fig. 2; cf. lanes *a* and *c*), it was found that most of the radioactivity migrating diffusely above β-chains showed a more rapid migration and appeared as a more discrete band between β- and α-chains, with an apparent M_r of 135 000–140 000 (small arrowhead). The more rapid migration of these chains indicated the removal of pepsin-sensitive domains from the high-M_r components. In these samples, only a negligible amount of small-M_r bands migrating in the position of G chains (lane *b*, bottom arrow) or

G_p chains were detected. When the same samples were electrophoresed after disulphide-bond reduction however (Fig. 2; cf. lanes *c* and *d*), a quantitative conversion of the M_r-135 000–140 000 material into a band migrating with an apparent M_r of 45 000–50 000, corresponding to the pepsin-treated form of G collagen (G_p) was observed. It appears therefore that most of the material contained within the M_r-135 000–140 000 band represents a disulphide-bonded aggregate which after reduction gives rise to the M_r-45 000–50 000 G_p subunits. This pattern is significantly different from that seen in the previous rabbit growth-plate studies (Remington *et al.*, 1983) and indicates the presence of disulphide bonds within the triple-helical pepsin-resistant domains of these collagen chains. Densitometric analysis of these fluorographs again demonstrated that the amount of radioactivity contained in the G_p band approached or exceeded that contained in the α_1(II) band. In Fig. 2 it should also be noted that the M_r-85 000 collagen molecule migrating after reduction immediately below α_1(II)-chains (large arrowhead, lane *b*) appears to be converted by pepsin digestion into a low-M_r pepsin-resistant chain migrating below the G_p chains (cf. lanes *b* and *d*), a finding consistent with that described by Reese & Mayne (1981) for the HMW collagen from chick cartilage.

To document further the similarities between the calf G_p chains and those synthesized by rabbit growth-plate chondrocytes, two-dimensional CNBr-peptide mapping of pepsin-digested reduced samples was performed. Comparison of the CNBr peptides of the calf and rabbit G_p chains (Fig. 3) shows that these molecules, although not identical, closely resemble one another. In both the rabbit and the calf, the peptide pattern of G_p clearly distinguishes these molecules from α_1(II), 1α-, 2α- and 3α-chains. As previously reported in several studies (Schmid & Conrad, 1982*a*,*b*; Gibson *et al.*, 1982; Remington *et al.*, 1983) the peptides from G_p chains are all in the low-M_r range. The individual peptides of the rabbit and calf chains are of similar M_r; however, minor differences in their migration and intensity suggest species variation in the primary structure of these molecules. Fig. 3 also shows two of the CNBr peptides from the very-low-M_r pepsin-resistant chain, which migrates below G_p. These peptides have unique electrophoretic mobility, indicating that they are derived from a distinct collagen chain, most likely the HMW collagen.

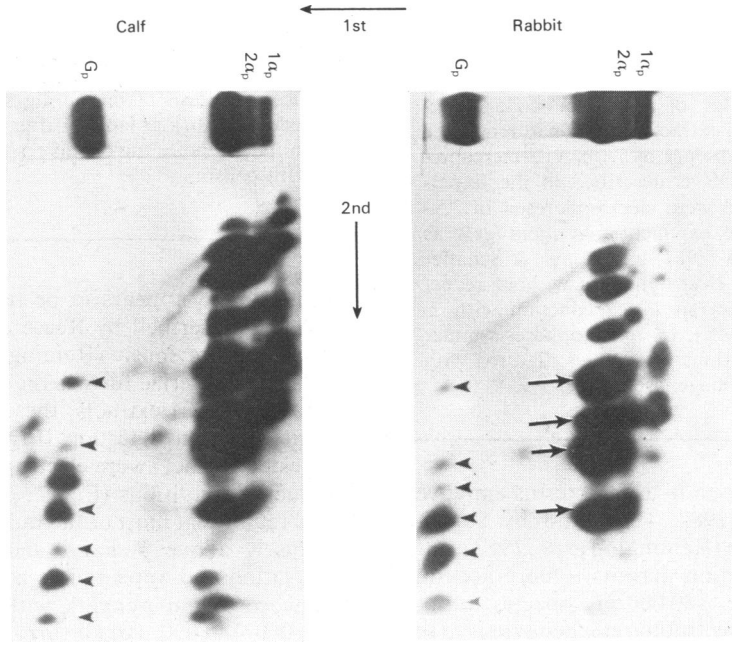

Fig. 3. *Fluorograph of two-dimensional CNBr-peptide mapping of calf and rabbit G_p collagen chains*
Two-dimensional CNBr-peptide mapping was performed exactly as described previously (Remington *et al.*, 1983). First-dimension samples were pepsin-digested reduced extracts of hypertrophic-zone cartilage from calf and rabbit. Direction of electrophoresis is indicated by arrows. The peptide patterns of the G_p chains from these two species are shown by small arrowheads. Main CNBr peptides from α_1(II)-chain are shown by arrows.

Discussion

A significant finding of this study was the demonstration that the hypertrophic zone of calf growth-plate cartilage devotes a large portion of its biosynthetic commitment to the synthesis of a short-chain collagen. The amount of this collagen species biosynthesized *in vitro* approached or exceeded that of type II collagen. This molecule appears to be similar to the short chain (SC) collagen described by Schmid & Conrad (1982*a*,*b*) and to the G collagen described by Gibson *et al.* (1982, 1983) in chick chondrocytes grown in three-dimensional gels and by us in rabbit growth-plate cartilage (Remington *et al.*, 1983). Evidence obtained from electrophoretic studies before and after reduction indicates that this molecule is normally present in the form of a high-M_r aggregate, which after disulphide-bond reduction dissociates into two species, one migrating with an apparent M_r of approx. 180 000 (G′) and the other migrating with an apparent M_r of 60 000 (G). The dissociation of the high-M_r material into G′ and G chains after disulphide-bond reduction is similar to that found in samples from rabbit growth plate (Remington *et al.*, 1983). However, a remarkable difference between these two tissues was detected in the present studies. Pepsin digestion of the high-M_r collagen from rabbit growth plate yields individual G_p chains. In contrast, when the high-M_r species from calf growth plate is digested with pepsin, the entirety of G_p subunits remain associ-

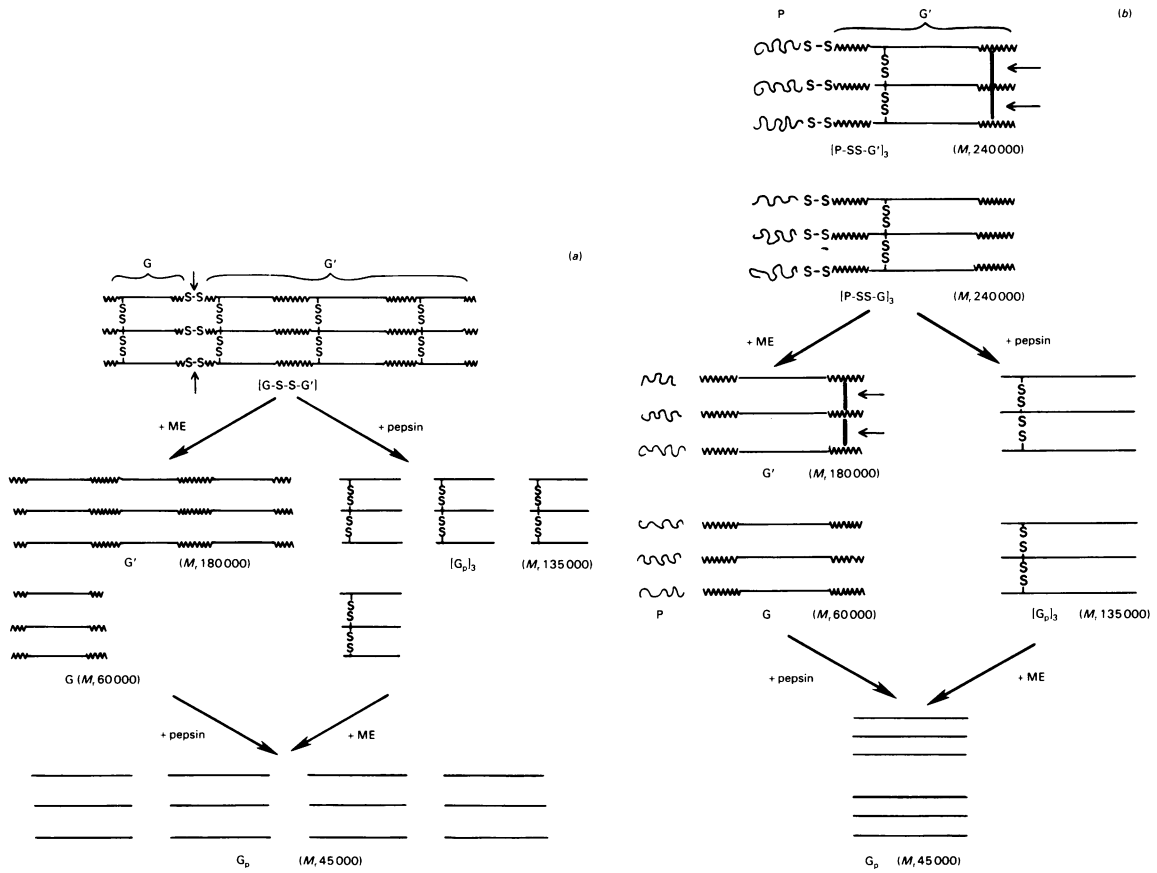

Fig. 4. *Diagrams of two theoretical models of the molecular arrangement of G collagen biosynthesized by calf growth-plate hypertrophic cartilage*

In the model shown in (*a*), each chain comprises alternating collagenous (——) and non-collagenous (〰) domains held together by inter-chain disulphide bonds (S–S). Additional intra-chain disulphide bonds are shown by small arrows. In the model shown in (*b*), each chain comprises a procollagen-like globular domain (P) joined by disulphide bonds (S–S) to the rest of the chain, which contains collagenous (——) and non-collagenous regions (〰). Some molecules contain only inter-chain disulphide bonds, whereas others contain inter-chain disulphide and non-disulphide (2-mercaptoethanol-resistant) bonds (arrows). The effects of disulphide-bond reduction with 2-mercaptoethanol (ME) and of pepsin digestion (subscript p) are shown for each model.

ated by disulphide bonds into a species with an apparent M_r of 135 000–140 000. Subsequent reduction results in quantitative conversion of the chains contained in this band into G_p chains. These findings demonstrate that, in the calf, the G collagen chains are held together by disulphide bonds, which are located in regions of the molecule that are resistant to pepsin digestion. The synthesis of molecules containing disulphide bonds within the pepsin-resistant domains of the calf G collagen appears to be a unique property of the calf tissues and may explain the findings that much of the growth-plate collagen is resistant to pepsin extraction (Remington *et al.*, 1980). On the basis of the electrophoretic behaviour under reducing and non-reducing conditions and on the results of limited proteolytic cleavage, we have constructed two theoretical models to explain the possible molecular arrangement of the short-chain collagen biosynthesized by hypertrophic-zone calf growth-plate cartilage. As shown in Fig. 4(*a*), one possibility is that the large molecular aggregate migrating above β-chains contains molecules each comprising three chains with alternating collagenous and non-collagenous domains. Assuming that the calf G and G_p molecules have similar M_r values to those described by Schmid & Linsenmayer (1983) for the short-chain (SC) collagen synthesized by chick-embryo tibiotarsus, we propose that each chain contains four M_r-60 000 subunits (G chains). Each of these subunits comprises a M_r-12 000 non-collagenous domain and a M_r-45 000 pepsin-resistant (G_p chains) collagenous domain. The collagenous domains of these subunits form a triple helix, which is stabilized by interchain disulphide bonds between the pepsin-resistant helical domains. In addition, a set of disulphide bonds is localized in the non-collagenous pepsin-sensitive domain of one of the subunits, joining it to the remaining three subunits (arrows). Disulphide-bond reduction of the large aggregate will result in its dissociation into a M_r-180 000 component (G′) comprising three intact subunits and a M_r-60 000 component (G′) corresponding to the remaining subunit. Subsequent pepsin digestion results in removal of the non-collagenous domains and yields the expected G_p chains. On the other hand, pepsin digestion of the large aggregate will result in cleavage of the non-collagenous domains and yield three individual G_p chains each of M_r-45 000, held together by the interchain disulphide bonds into a trimer with an approximate M_r of 135 000. Subsequent reduction of these disulphide-bonded G_p trimers will again yield the expected individual G_p chains. In the alternative model (Fig. 4*b*), the high-M_r species is an M_r-80 000 procollagen-like chain containing a globular region of M_r approx. 20 000 (P) joined by disulphide bonds to the rest of the chain (G). Three of these chains are associated in a triple helix, which is stabilized by disulphide bonds localized between the collagenous domains of the G chains. In addition, a portion of these molecules contains 2-mercaptoethanol-resistant cross-links in the pepsin-sensitive, non-collagenous, domains of the G chains (arrows). Reduction of these molecules would result in removal of the globular region (P) and the appearance of G′ chains held together by the 2-mercaptoethanol-resistant cross-links and G chains which do not contain these crosslinks. Subsequent pepsin digestion would yield the expected G_p chains. When pepsin digestion of the high-M_r species is performed before disulphide-bond reduction, the disulphide-bonded G_p trimers are obtained, which after subsequent reduction yield the individual G_p chains. Although this is a simpler model, it assumes the formation of 2-mercaptoethanol-resistant cross-links between the non-collagenous domains of a portion of the G chains (to yield G′ trimers).

The significance of initiation of abundant G-collagen synthesis by hypertrophic chondrocytes of calf growth-plate cartilage and the role of the disulphide bonds localized within the triple-helical pepsin-resistant domains will only be appreciated by further characterization of this molecule and of its role in matrix structure.

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