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The origin of human cartilage proteoglycan link-protein heterogeneity and fragmentation during aging

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Human articular-cartilage link proteins are resolved into three components by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, indicative of three different structures. The action of the proteinase clostripain yields a single link-protein component with electrophoretic properties analogous to those of the smallest (most mobile) native link protein, suggesting that this link protein may be derived naturally from one or both of the larger molecules by proteolytic cleavage *in situ*. Upon chemical deglycosylation of native link protein two components are resolved, suggesting that two of the link proteins differ only in their degree and/or type of oligosaccharide substitution. This pattern is compatible with a proteolytic origin for the smallest link protein. During aging further proteolytic fragmentation occurs, though it is only apparent on reduction of disulphide bonds. This fragmentation occurs at identical sites in all three native link proteins, indicating the existence of a large region common to all the link proteins, which appears to consist predominantly of the *C*-terminal half of the molecules. These observations are compatible with the variation in oligosaccharide and proteolytic heterogeneity occurring at the *N*-terminus of the link proteins.

INTRODUCTION

Link proteins are glycoproteins (M_r 40000–50000) that stabilize the interaction of proteoglycan subunits with hyaluronic acid in the formation of proteoglycan aggregates (Hardingham, 1979; Tang *et al.*, 1979; Franzén *et al.*, 1981). Such aggregates are thought to endow articular cartilage with its shock-absorbing qualities. In all species studied, link proteins exhibit various degrees of electrophoretic heterogeneity. On isoelectric focusing human cartilage (Roughley *et al.*, 1982), bovine cartilage and Swarm rat chondrosarcoma (Poole *et al.*, 1984) link proteins all exhibit a cluster of bands with pI values in the range 6.0–7.0. When analysed by SDS/polyacrylamide-gel electrophoresis, Swarm rat chondrosarcoma link protein appears as a single component (Oegema *et al.*, 1975), whereas bovine articular-cartilage link proteins appear as two major components (Baker & Caterson, 1977, 1979; Bonnet *et al.*, 1978; Tang *et al.*, 1979). In previous work from this laboratory (Roughley *et al.*, 1982; Mort *et al.*, 1983) both neonatal and adult human cartilage link proteins were shown to occur as three major components on SDS/polyacrylamide-gel electrophoresis, although other workers have reported only two components for human link proteins (Pal *et al.*, 1978; Bayliss & Ali, 1978; Pearson & Mason, 1979; Ryu *et al.*, 1982; Glant, 1982). In addition, further heterogeneity in adult human cartilage link proteins was observed with increasing age of the individual, where a significant proportion of the molecules were found to be cleaved yielding a series of fragments M_r 26000–30000 (Mort *et al.*, 1983). This fragmentation was only observed under reducing conditions, suggesting that the clipped molecules are maintained in a pseudo-native conformation by disulphide bridges.

The aim of the present work is to account for the various degrees of human cartilage link-protein heterogeneity. Contributions to this could arise from three sources. The most fundamental source of this heterogeneity would be that link proteins are the biosynthetic products of more than one gene. There is at present, however, no evidence to support such a proposal. A second source of heterogeneity is in post-translational glycosylation, which modifies proteins with respect to both molecular mass and isoelectric point. It has been shown that the larger-M_r component of bovine link proteins is more glycosylated than is the smaller component (Baker & Caterson, 1979), and that the largest component of neonatal human link proteins is more heterogeneous on isoelectric focusing than are the other forms (Roughley *et al.*, 1982), suggesting a greater degree of glycosylation. Recent studies (Caterson *et al.*, 1985*a*, *b*) indicate that the heterogeneity of bovine nasal-cartilage and Swarm rat chondrosarcoma link proteins on SDS/polyacrylamide-gel electrophoresis is in part due to the presence of high-mannose oligosaccharides. Further heterogeneity can be introduced post-translationally by limited proteolytic cleavage, and this has been suggested as the origin of the fastest-migrating human link-protein component (Roughley *et al.*, 1982).

In the present paper human link-protein heterogeneity has been examined by a variety of techniques, and the apparent discrepancy between the number of human link-protein bands found on analysis by SDS/polyacrylamide-gel electrophoresis has been investigated. A review of the data shows that the main difference underlying the discrepancy appeared to be in the

Abbreviations used: SDS, sodium dodecyl sulphate; TFMS, trifluoromethanesulphonic acid.

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gel-electrophoretic method used. Thus we have investigated the difference in resolution of human link proteins as analysed by continuous compared with discontinuous polyacrylamide-gel-electrophoresis systems. In addition, the origin of the fragmentation in adult human link proteins has been investigated. Because of the problems encountered in preparing proteoglycan aggregate, and hence link protein, from adult human articular cartilage (Roughley *et al.*, 1984), much of this work was performed with monospecific antibodies to identify link proteins after electrophoresis and immunoblotting of total cartilage protein preparations. However, this technique has the advantage of permitting the analysis of total rather than just functional link protein. The results of these studies combined with other literature data have allowed us to formulate a scheme that accounts for the various degrees of heterogeneity in human link proteins.

MATERIALS AND METHODS

Materials

Trifluoromethanesulphonic acid (TFMS) and anisole were from Aldrich Chemical Co. (through Terochem, Toronto, Ont., Canada), and guanidinium chloride was from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Materials for polyacrylamide-gel electrophoresis were from Bio-Rad Laboratories (Toronto, Ont., Canada), except for Zetabind transfer membranes, which were from AMF (Meridien, CT, U.S.A.).

Cartilage proteins

Samples of adult and neonatal human articular cartilage, bovine articular cartilage and Swarm rat chondrosarcoma were extracted in 4 M-guanidinium chloride/0.1 M-sodium acetate buffer, pH 6.0, in the presence of proteinase inhibitors (Roughley & White, 1980). Cartilage extracts were subjected to CsCl-density-gradient centrifugation, with a starting density of 1.50 g/ml, under dissociative conditions to separate cartilage proteins from proteoglycans. Cartilage proteins were isolated from the upper part of the gradient (density less than 1.45 g/ml), dialysed exhaustively against water and then freeze-dried.

Neonatal human cartilage link protein and its clostripain-derived product were purified from proteoglycan aggregates as described previously (Roughley *et al.*, 1982).

Antibodies

The monospecific rabbit antiserum raised against bovine nasal-cartilage link protein has been previously described (Poole *et al.*, 1980). This serum has been shown to cross-react with human and Swarm rat chondrosarcoma link proteins (Mort *et al.*, 1983). Mouse monoclonal antibodies 9/30/8-A-4 and 9/30/8-A-5 were produced by immunization with Swarm rat chondrosarcoma link protein. The characterization of the specificity of the 9/30/8-A-4 and 9/30/8-A-5 monoclonal antibodies has been described (Caterson *et al.*, 1985*a*,*b*) and they were shown to be IgG2b and IgG2a immunoglobulins respectively. Both of the monoclonal antibodies recognize epitopes present in the *C*-terminal half of the molecule, which are located on a highly conserved region of the link-protein polypeptide. The epitopes are present in all link-protein subspecies isolated from rat, human, bovine and chicken hyaline cartilages. Antibody-rich ascitic fluid was iodinated by the chloramine-T method (Greenwood *et al.*, 1963).

Comparison of electrophoretic methods

For comparison of the discontinuous and continuous polyacrylamide-gel-electrophoresis techniques, the procedures of Laemmli (1970) and of Weber & Osborn (1969) were used. Pure neonatal human link protein and its clostripain-digestion products were analysed on 10% polyacrylamide slab gels under non-reducing conditions. The gels were stained with Coomassie Brilliant Blue R-250 (Fairbanks *et al.*, 1971).

For comparison of electophoretic methods in two dimensions, pure neonatal human link protein was first analysed by the Weber & Osborn (1969) procedure. A gel strip was cut out and equilibrated in 0.0625 M-Tris/HCl buffer, pH 6.8, containing 2.3% (w/v) SDS and 10% (v/v) glycerol for 1 h, then layered on top of a standard discontinuous gel and embedded in 1% agarose made in the equilibrating solution. After electrophoresis, the gel was stained by the silver method (Bio-Rad) for highest sensitivity.

Chemical deglycosylation

Link-protein samples were chemically deglycosylated by the method described by Edge *et al.* (1981). Freeze-dried cartilage protein fractions were dissolved in TFMS/anisole (2:1, v/v) at 10 mg/ml with stirring under N_2 in a Reacti-Vial (Pierce Chemical Co., Rockland, IL, U.S.A.) at 0 °C or 20 °C for various times. The reaction products were recovered by precipitation in 10% n-hexane in diethyl ether at −78 °C. The precipitates were washed in the same solvent at −78 °C, then dissolved in 4 M-guanidinium chloride/50 mM-Tris/HCl buffer, pH 7.5. For gel electrophoresis these solutions were dialysed into 0.125 M-Tris/HCl buffer, pH 6.8, containing 0.1% SDS. As a control for the acidic conditions used in the chemical deglycosylation procedure, a neonatal human cartilage protein preparation was dissolved in 70% (v/v) formic acid (10 mg/ml) and then left at 20 °C for 10 h, and the proteins were recovered by ether/hexane precipitation as above. Control and deglycosylated samples were analysed by the discontinuous method of Laemmli (1970) under non-reducing conditions, followed by electrophoretic transfer of the resolved proteins to nitrocellulose (Towbin *et al.*, 1979). Link proteins were located by an indirect method described in detail previously (Roughley *et al.*, 1982), by using a rabbit antiserum to bovine nasal-cartilage link protein followed by a peroxidase-labelled pig antiserum to rabbit IgG and finally peroxidase reaction.

Analysis of human link-protein fragmentation

Cartilage proteins were dissolved at 1 mg/ml in SDS sample buffer {0.125 M-Tris/HCl buffer, pH 6.8, containing 2% (w/v) SDS, 10% (v/v) glycerol and 0.001% Bromophenol Blue [and also 5% (v/v) 2-mercaptoethanol when reducing conditions were required]} by heating in a boiling-water bath for 3 min. Proteins were resolved by using the discontinuous system (10% gels) and then transferred to Zetabind membrane. The transfer buffer was 15.6 mM-Tris/120 mM-glycine, pH 8.0. After transfer the sheet was quenched for 16 h at 45 °C in 1% (w/v) bovine haemoglobin in phosphate-buffered saline (10 mM-sodium potassium phosphate buffer, pH 7.2, containing

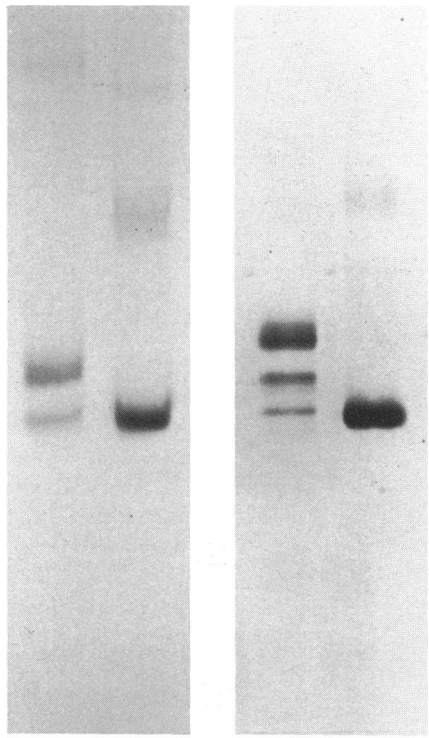

Fig. 1. Comparison of electrophoresis conditions for the analysis of human link proteins

Samples of purified neonatal human link protein [either intact (lanes A and C) or clostripain-derived (lanes B and D)] were analysed by SDS/polyacrylamide-gel electrophoresis (non-reducing conditions) under the conditions of Weber & Osborn (1969) (lanes A and B) and Laemmli (1970) (lanes C and D). Proteins were stained with Coomassie Brilliant Blue R-250.

0.145 M-NaCl and 0.05% NaN_3). The sheet was then incubated overnight at room temperature in quenching solution containing ^{125}I-labelled monoclonal antibody to link protein. After washing of the membrane with several changes of phosphate-buffered saline containing 0.1% (v/v) Tween 20, link proteins were located by autoradiography with Kodak XAR film.

For two-dimensional electrophoresis under non-reducing conditions in the first dimension followed by reducing conditions in the second dimension, samples of adult cartilage proteins were treated as above. Gel strips obtained from the first dimension were equilibrated in three changes of sample buffer containing 5% (v/v) 2-mercaptoethanol, then layered on to a second gel and embedded in 1% agarose in sample buffer containing 2-mercaptoethanol. Link-protein components were located as indicated above.

RESULTS

Human link-protein heterogeneity analysed by different SDS/polyacrylamide-gel electrophoresis methods

Our previous studies indicate that human link proteins separate into three components (Roughley *et al.*, 1982) and that the proportions of these components change with age (Mort *et al.*, 1983), whereas others have reported the presence of only two components (Pal *et al.*, 1978; Bayliss & Ali, 1978; Pearson & Mason, 1979; Ryu *et al.*, 1982; Glant, 1982). Our analyses were carried out with the discontinuous stacking system of Ornstein (1964) as adapted for SDS/polyacrylamide gels by Laemmli (1970), whereas others used the continuous gel system described by Weber & Osborn (1969). A comparison of the resolution of pure link protein from neonatal human cartilage by both methods was therefore undertaken. A clear difference in the pattern of link-protein components was seen, depending on the method used. The continuous system gave two major bands (Fig. 1, lane A), though the faster-moving component exhibited a distinct leading edge. In contrast, the discontinuous system gave three major bands (Fig. 1, lane C).

In order to find out how the bands seen in the two systems relate to each other, a two-dimensional gel was run in which link protein was separated first by the continuous system and then by the discontinuous system (Fig. 2). The resulting pattern is a series of three spots. The least-mobile component in both systems corresponds, but its diagonal appearance indicates further heterogeneity. Previous analysis has shown that this is at least in part due to differential sialic acid substitution (Roughley *et al.*, 1982). In contrast, the more-mobile major component in the first dimension was resolved into two components in the second dimension. Interestingly, the fastest-migrating spot is partially resolved by two-dimensional electrophoresis, suggesting further heterogeneity in this component.

When neonatal human proteoglycan aggregate is subjected to proteolytic digestion by clostripain the three-banded pattern of native link protein is converted into a single band co-migrating with the smallest native component (Fig. 1, lane D). It was suggested that the third component of human link protein may be a product of

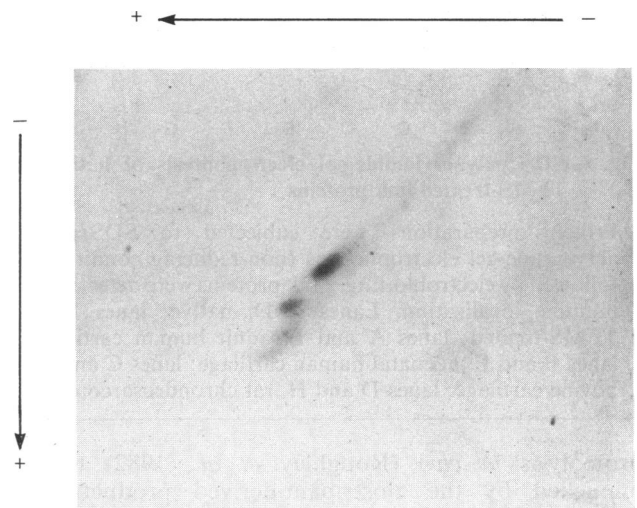

Fig. 2. Analysis of human link protein by electrophoresis in two dimensions

Purified neonatal human link protein was analysed by SDS/polyacrylamide-gel electrophoresis (non-reducing conditions) under the conditions of Weber & Osborn (1969) in the first dimension (right to left) and those of Laemmli (1970) in the second dimension (top to bottom). Proteins were detected by silver staining.

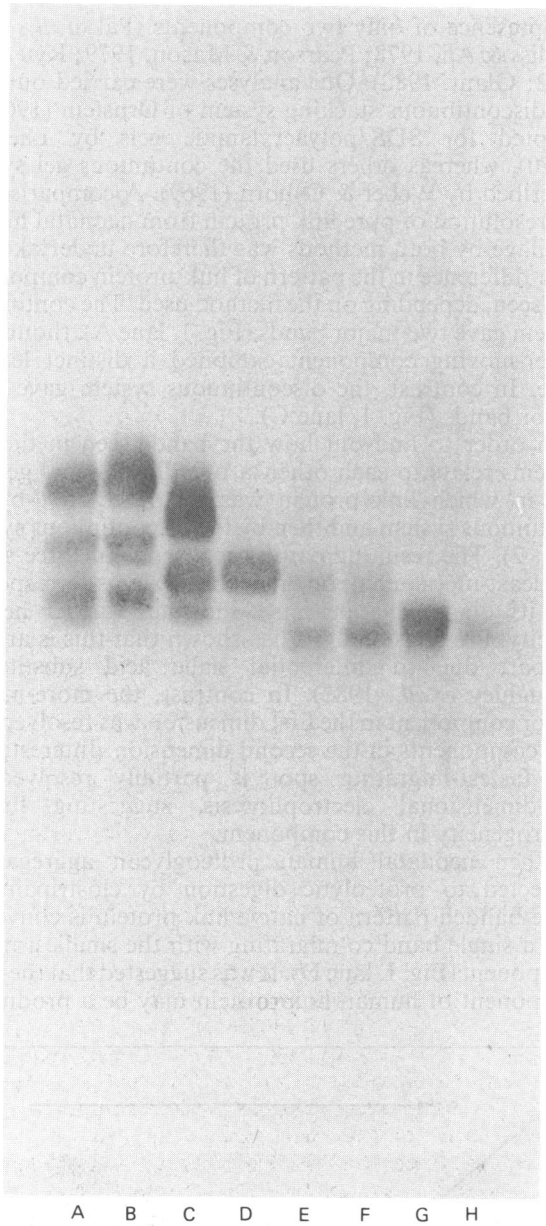

Fig. 3. SDS/polyacrylamide-gel electrophoresis of native and TFMS-treated link proteins

Protein preparations were subjected to SDS/polyacrylamide-gel electrophoresis (non-reducing conditions) followed by electroblotting. Link proteins were detected by immune localization. Lanes A–D, native; lanes E–H, TFMS-treated; lanes A and E, adult human cartilage; lanes B and F, neonatal human cartilage; lanes C and G, bovine cartilage; lanes D and H, rat chrondrosarcoma.

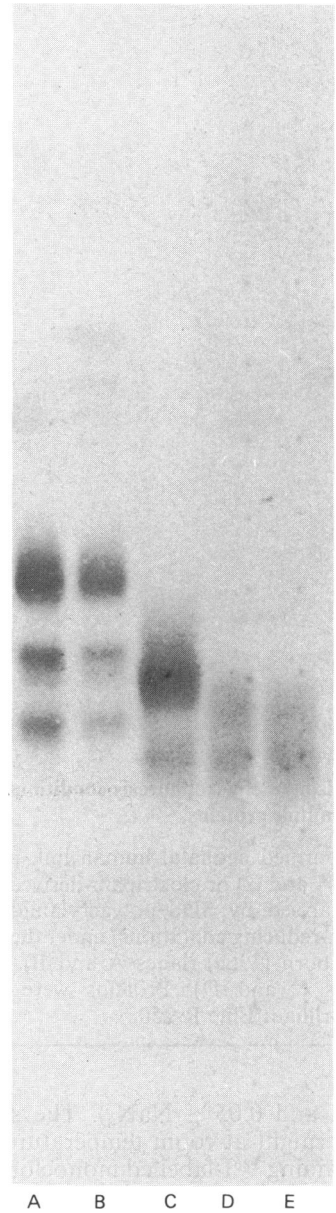

Fig. 4. Effect of TFMS treatment on human link protein

Neonatal human cartilage proteins were subjected to SDS/polyacrylamide-gel electrophoresis (non-reducing conditions) followed by electroblotting. Link proteins were detected by immune localization. Lane A, untreated; lane B, formic acid-treated; lane C, TFMS-treated for 2 h at 0 °C; lane D, TFMS-treated for 2 h at 20 °C; lane E, TFMS-treated for 4 h at 20 °C.

proteolysis *in vivo* (Roughley *et al.*, 1982) that is mimicked by the clostripain-derived product. This conclusion is supported by the observation that, when analysed by the continuous electrophoretic system, clostripain-derived link protein also co-migrated with the second native link-protein component (Fig. 1, lane B).

Electrophoretic heterogeneity of native link proteins from various species

Link proteins present in cartilage protein preparations were analysed by SDS/polyacrylamide-gel electrophoresis under non-reducing conditions with the discontinuous method of Laemmli (1970), then localized on immunoblots. Both adult and neonatal human preparations appeared as three major bands (Fig. 3, A and B), whereas the bovine preparation appeared as two major bands and a more-mobile minor band (Fig. 3, lane C). It may be noted that the bovine molecules did not co-migrate with their human counterparts. As shown previously (Baker & Caterson, 1977; Tang *et al.*, 1979; Mort *et al.*, 1983), the various components are less well resolved on reducing gels, and so the differences in mobilities are less apparent. The rat chondrosarcoma preparation gave a single major

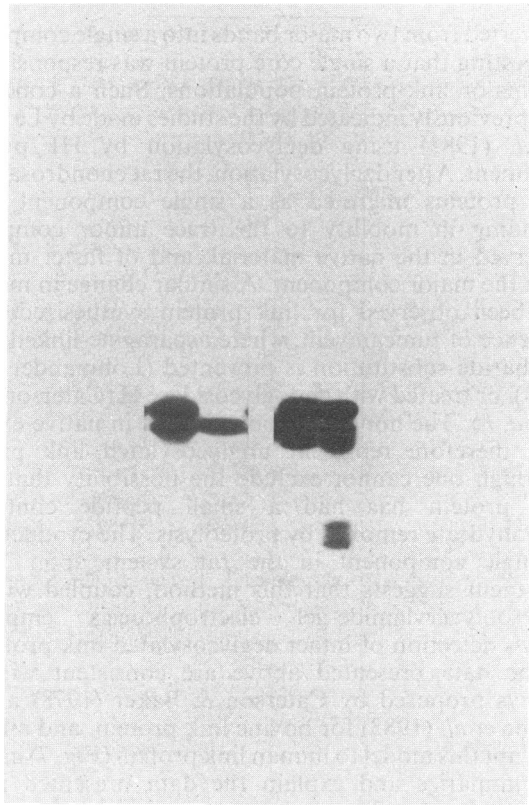

Fig. 5. Immune localization of link-protein components with the use of monoclonal antibody

Cartilage proteins were subjected to SDS/polyacrylamide-gel electrophoresis (reducing conditions) followed by electroblotting. Link proteins were then detected with the use of the monoclonal antibody 9/30/8-A-4. Lane A, bovine articular cartilage; lane B, rat chrondrosarcoma; lane C, neonatal human articular cartilage; lane D, adult human articular cartilage.

band and a trace of a faster-migrating minor component (Fig. 3, lane D). The mobilities of the rat moieties were similar to those of the more-mobile bovine molecules. Thus, though there may be functional analogy between the link proteins from different species, there is not necessarily structural identity. Glycosylation may be a major contributor to these differences, as implied by the data of Périn *et al.* (1978).

Effect of chemical deglycosylation on link-protein heterogeneity

Newborn human cartilage protein preparations were subjected to chemical deglycosylation by using the trifluoromethanesulphonic acid (TFMS) method of Edge *et al.* (1981), which produces cleavages at *O*-linked glycosidic bonds in polysaccharides and oligosaccharides. It does not cleave the *O*-glycosidic and *N*-glycosidic bonds that join oligosaccharides to protein, i.e. GalNAc–Ser(Thr) and GlcNAc–Asn bonds. The resulting deglycosylated link proteins were analysed by SDS/polyacrylamide-gel electrophoresis followed by electroblotting and detection by immune localization. The effects of TFMS treatment were time- and temperature-dependent, converting the three original components into two faster-moving species (Fig. 4). Whereas 2 h at 0 °C gave incomplete modification of the link proteins, the same time at 20 °C appeared to give a limiting product. From further work it was judged that reaction for 1 h at 20 °C was optimal for link-protein deglycosylation. As a control for non-specific cleavage due to the acidic conditions that the cartilage proteins are subjected to during TFMS treatment, samples were also incubated at 20 °C in 70% formic acid (Périn *et al.*, 1980). With the gel-electrophoresis system used in these studies there was no detectable change in the link-protein pattern after acid treatment.

With the use of TFMS treatment for 1 h at 20 °C as a standard condition, the products of chemical deglycosylation of link proteins from various species were compared. The link protein from the Swarm rat chondrosarcoma remained as a single major band, but of increased mobility compared with that observed with the native molecules (Fig. 3, lane H). Bovine articular-cartilage link protein was converted from two major bands into a single band (Fig. 3, lane G), which had a mobility similar to that of the minor band in the native preparation and the deglycosylated form of rat chondrosarcoma link protein. Both adult (Fig. 3, lane E) and neonatal (Fig. 3, lane F) human link proteins gave two bands after deglycosylation. The fastest-moving band was of similar mobility to that produced from bovine and rat link proteins, with the slower-migrating band being less mobile than the smallest native human component. This implies that, in contrast with the bovine and rat link proteins, two distinct protein sizes may contribute to the heterogeneity observed in the human system.

Origin of adult human link-protein fragmentation

In a previous study it was demonstrated that with increasing age of the individual a significant percentage of human cartilage link protein exists in a fragmented state (Mort *et al.*, 1983). With the use of a polyclonal antiserum to link protein, SDS/polyacrylamide-gel electrophoresis of cartilage proteins revealed a cluster of fragments in the M_r range 26000–30000, suggesting various specific cleavages of the molecule to yield fragments of almost half of the original size. When a monoclonal antibody to link protein (9/30/8-A-4), which by definition should only recognize a unique region of the protein, was used, the same set of fragments was detected (Fig. 5, lane D), indicating that all the fragments contained the same region of the molecule. As expected, all the link-protein bands observed in bovine, rat and human species reacted with this antibody (Fig. 5, lanes A–C). The same patterns were also observed with a different monoclonal antibody (9/30/8-A-5).

The origin of the fragments was further investigated by using electrophoresis in two dimensions, in order to identify the products derived from each of the individual link-protein components. Since the cleaved molecules are held in a pseudo-native configuration by disulphide bridges, the three link-protein components can be separated in the first dimension under non-reducing conditions. The fragments of these components can then be detected by second-dimension electrophoresis under reducing conditions (Fig. 6). It is clear that fragments are produced from all three native link-protein components, and that the relative abundance of the fragments generated from each link protein corresponds to that of the parent molecules. Furthermore, the same fragmentation pattern is observed from each component,

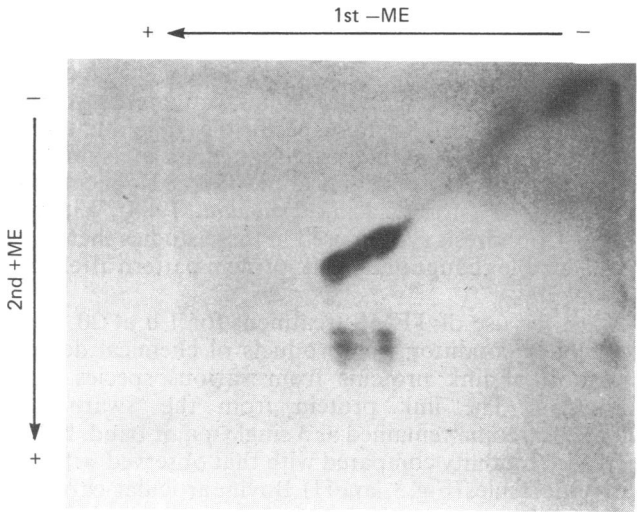

Fig. 6. Fragmentation of individual link-protein components

Adult human cartilage proteins were subjected to SDS/polyacrylamide-gel electrophoresis under non-reducing conditions (right to left) and then under reducing conditions (top to bottom). The gel was electroblotted and link-protein components were detected with the use of monoclonal antibody 9/30/8-A-4.

implying the existence of a conserved region of M_r 30000 for each link-protein component.

DISCUSSION

The results of comparison of the SDS/polyacrylamide-gel electrophoresis methods for analysis of human link protein demonstrate that the more-mobile component in the Weber & Osborn (1969) system resolves into two components in the Laemmli (1970) system, though the reason for this separation is not apparent. Such an observation would resolve the apparent discrepancy in the heterogeneity of human link protein described in the literature. It is also noteworthy that the clostripain-derived link protein co-migrates with the smallest native link protein in both electrophoretic systems. Such an observation is compatible with this native link protein being derived by proteolysis *in vivo* of the larger link proteins.

After deglycosylation, the human link proteins were converted from three components into two components in SDS/polyacrylamide-gel electrophoresis. Although the less mobile of the two components does decrease in intensity with prolonged exposure to TFMS, there is no concomitant increase in the abundance of the more-mobile component, suggesting that the more-mobile band is not derived from the less-mobile band. The reason for this decrease in intensity is not at present known, but could be accounted for by an inability to solubilize the molecule after complete deglycosylation or by a loss of immune reactivity. It is therefore not unreasonable to conclude that two different protein structures may exist in the human link protein. This would be consistent with the proposal that the smallest human link protein is derived from the larger components by proteolytic modification.

After deglycosylation, the bovine link proteins were converted from two major bands into a single component, suggesting that a single core protein was responsible for the major link-protein populations. Such a conclusion was previously indicated by the studies made by Le Glédic *et al.* (1983) using deglycosylation by HF/pyridine treatment. After deglycosylation, the rat chondrosarcoma link proteins migrated as a single component corresponding in mobility to the trace minor component observed in the native material, and of faster mobility than the major component. A similar change in mobility has been observed for link protein synthesized in the presence of tunicamycin, where asparagine-linked oligosaccharide substitution is prevented (Lohmander *et al.*, 1983), or treated with endoglycosidase H (Caterson *et al.*, 1985*a*, *b*). The minor component seen in native extracts may therefore represent unglycosylated link protein, although one cannot exclude the possibility that some link protein has had a small peptide containing carbohydrate removed by proteolysis. The production of a single component in the rat system upon TFMS treatment suggests that this method, coupled with the SDS/polyacrylamide-gel electrophoresis employed, allows detection of intact deglycosylated link protein.

The data presented above are consistent with the models proposed by Caterson & Baker (1978) and Le Glédic *et al.* (1983) for bovine link protein, and allow us to adapt this model to human link protein (Fig. 7) in order to summarize and explain the data presented in the present paper. These data are consistent with the synthesis of a single protein core that undergoes different degrees of glycosylation, giving rise to the two higher-M_r native components resolved by SDS/polyacrylamide-gel electrophoresis. The more-extensive glycosylation of the largest form gives rise to the more-extensive set of isoforms seen on isoelectric focusing (Roughley *et al.*, 1982). Such link proteins may also possess differences in the type of oligosaccharide chains (either complex or high-mannose). Although high-mannose oligosaccharides have been reported in bovine and rat link proteins (Caterson *et al.*, 1985*a*, *b*), the human would appear to contain only the complex type, as in the latter case endoglycosidase H treatment produced no change in electrophoretic mobility of the three link-protein components (J. S. Mort & P. J. Roughley, unpublished work). At this stage one cannot make any definitive comment on either the number or the structure of the oligosaccharide chains on the link proteins; however, it does appear that in the human even the smallest native link protein contains some carbohydrate (Roughley *et al.*, 1982). This native component of lowest M_r would then correspond to a product of proteolytic degradation *in vivo* of the two higher-M_r components, which becomes more abundant as the individual ages.

The observation that each human link-protein species yields identical fragments during aging indicates that a major portion of the link-protein structure is conserved among the three native components, and because of the specificity of the monoclonal antibody (Caterson *et al.*, 1985*a*, *b*) this region is towards the *C*-terminus. This is consistent with the model suggested for bovine link protein (Le Glédic *et al.*, 1983), where glycosylation occurs predominantly towards the *N*-terminal region of the molecules. Fragmentation of adult human link protein can therefore be most simply ascribed to the existence of a short proteolytically susceptible peptide region, which, because of the fragment sizes, must be

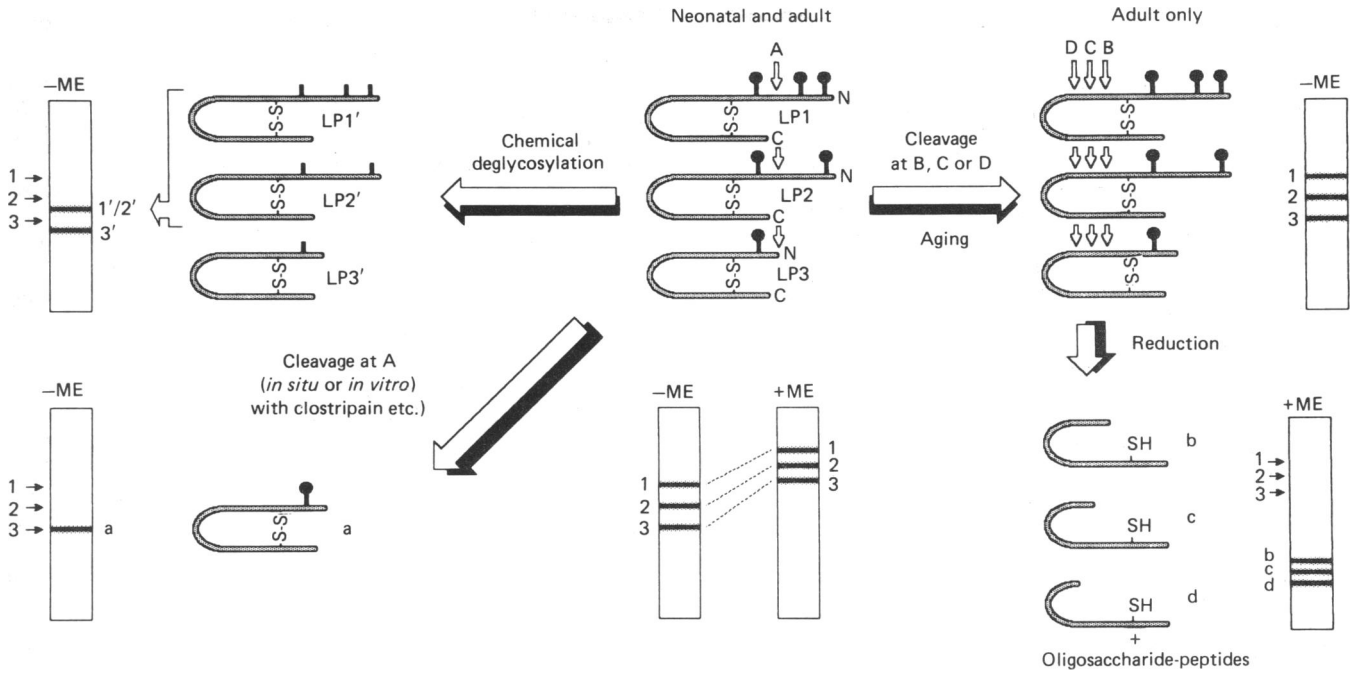

Fig. 7. Scheme summarizing the accumulated data on human cartilage link protein

Native neonatal link protein is composed of three components (LP1, LP2 and LP3), which are glycosylated (—●) to various degrees in the *N*-terminal region. The extent of glycosylation is LP1 > LP2 > LP3, though the absolute number of chains in each component is still speculative. Each molecule is held in its native configuration by disulphide bonds (-S-S-), giving rise to different mobilities under reducing compared with non-reducing conditions of electrophoresis. The number and position of these bonds is also speculative. LP3 represents a proteolytic modification *in situ* of LP1 and LP2 occurring near the *N*-terminus at site A. An analogous product is obtained *in vitro* by the action of clostripain. Chemical deglycosylation of the native link proteins produces two components, the larger (1′/2′) derived from LP1 and LP2 and the smaller (3′) from LP3. During aging additional proteolytic fragmentation occurs at sites B, C and D in each of the three native link proteins. This gives rise to three common fragments (b, c and d), which can be detected immunologically only under reducing conditions. The complementary oligosaccharide-bearing peptides are apparently unreactive to the monoclonal and polyclonal antibodies studied. An idealized representation of each link-protein species is illustrated, together with a diagrammatic representation of its migration on SDS/polyacrylamide-gel electrophoresis (−ME, non-reducing conditions; +ME, reducing conditions).

located close to the centre of the molecule, and, in a given link protein, undergoes a single proteolytic cleavage at one of several possible sites. An alternative explanation of the fragmentation pattern is that a cleavage occurs at a single site in the central region and, in addition, at one of multiple cleavage sites near the *C*-terminus. This, however, is less likely, as it requires a greater complexity of disulphide bonds and multiple proteolytic cleavages in a single link protein. Finally, since the present studies on the characterization of these fragments rely on their detection by immune localization, it seems clear that the part of the molecules in which variation in carbohydrate substitution occurs does not bear the epitopes for the polyclonal and monoclonal antibodies investigated so far.

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REFERENCES

Baker, J. R. & Caterson, B. (1977) Biochem. Biophys. Res. Commun. **77**, 1–10
Baker, J. R. & Caterson, B. (1979) J. Biol. Chem. **254**, 2387–2393
Bayliss, M. T. & Ali, S. Y. (1978) Biochem. J. **176**, 683–693
Bonnet, F., Périn, J.-P. & Jollès, P. (1978) Biochim. Biophys. Acta **532**, 242–248
Caterson, B. & Baker, J. R. (1978) Biochem. Biophys. Res. Commun. **80**, 496–503
Caterson, B., Baker, J. R., Christner, J. E. & Couchman, J. R. (1985*a*) Fed. Proc. Fed. Am. Soc. Exp. Biol. **44**, 386–393
Caterson, B., Baker, J. R., Christner, J. E., Lee, Y. & Lentz, M. (1985*b*) J. Biol. Chem., **260**, 11348–11356
Edge, A. S. B., Faltynek, C. R., Hol, L., Reichert, L. E. & Weber, P. (1981) Anal. Biochem. **118**, 131–137
Fairbanks, G., Steck, T. L & Wallach, D. F. H. (1971) Biochemistry **10**, 2606–2616
Franzén, A., Björnsson, S. & Heinegård, D. (1981) Biochem. J. **197**, 669–674
Glant, T. (1982) Biochem. Biophys. Res. Commun. **106**, 158–163
Greenwood, F. C., Hunter, W. H. & Glover, J. S. (1963) Biochem. J. **89**, 114–123
Hardingham, T. E. (1979) Biochem. J. **177**, 237–247

Laemmli, U. K. (1970) Nature (London) **227**, 680–685
Le Glédic, S., Périn, J.-P., Bonnet, F. & Jollès, P. (1983) J. Biol. Chem. **258**, 14759–14761
Lohmander, L. S., Fellini, S. A., Kimura, J. H., Stevens, R. L. & Hascall, V. C. (1983) J. Biol. Chem. **258**, 12280–12286
Mort, J. S., Poole, A. R. & Roughley, P. J. (1983) Biochem. J. **214**, 269–272
Oegema, T. R., Hascall, V. C. & Dziewiatkowski, D. D. (1975) J. Biol. Chem. **250**, 6151–6159
Ornstein, L. (1964) Ann. N.Y. Acad. Sci. **121**, 321–349
Pal, S., Strider, W., Margolis, R., Gallo, G., Lee-Huang, S. & Rosenberg, L. (1978) J. Biol. Chem. **253**, 1279–1289
Pearson, J. P. & Mason, R. M. (1979) Biochim. Biophys. Acta **583**, 512–526
Périn, J.-P., Bonnet, F. & Jollès, P. (1978) Mol. Cell. Biochem. **21**, 71–82
Périn, J.-P., Bonnet, F., Pizon, V., Jollès, J. & Jollès, P. (1980) FEBS Lett. **119**, 333–336

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Poole, A. R., Reiner, A., Tang, L.-H. & Rosenberg, L. (1980) J. Biol. Chem. **255**, 9295–9305
Poole, A. R., Reiner, A., Mort, J. S., Tang, L.-H., Choi, H. U., Rosenberg, L. C., Caputo, C. B., Kimura, J. H. & Hascall, V. C. (1984) J. Biol. Chem. **259**, 14849–14856
Roughley, P. J. & White, R. J. (1980) J. Biol. Chem. **255**, 217–224
Roughley, P. J., Poole, A. R. & Mort, J. S. (1982) J. Biol. Chem. **257**, 11908–11914
Roughley, P. J., White, R. J., Poole, A. R. & Mort, J. S. (1984) Biochem. J. **221**, 637–644
Ryu, J., Towle, C. A. & Treadwell, B. V. (1982) Ann. Rheum. Dis. **41**, 164–167
Tang, L.-H., Rosenberg, L., Reiner, A. & Poole, A. R. (1979) J. Biol. Chem. **254**, 10523–10531
Towbin, H., Staehelin, T. & Gordon, J. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 4350–4354
Weber, K. & Osborn, M. (1969) J. Biol. Chem. **244**, 4406–4412