Fibronectin Lacking the ED-B Domain Is a Major Structural Component of Tracheal Cartilage*

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Fibronectin is a highly conserved dimeric glycoprotein found in high concentrations in plasma and widely distributed in low concentrations in the extracellular matrix of tissues. The protein is the product of a single gene, but multiple splicing variants are expressed that show tissue specificity. Three exons (IIIA, IIIB, and V) can be alternatively spliced to produce different fibronectin isoforms. We report here that fibronectin is a remarkably abundant component of the extracellular matrix of bovine tracheal cartilage, increasing with age to more than 20% of the tissue, dry weight. This matrix form of fibronectin is inextractable by 4 M guanidine HCl, indicating that it is a covalently cross-linked structural component. By protein sequence analysis, the main molecular form of fibronectin in bovine tracheal cartilage was shown to lack the ED-B domain encoded by exon IIIB.

Fibronectin is a widely distributed glycoprotein abundant in plasma and in small amounts in most extracellular matrices. It functions as an adhesion molecule with a key role suspected in various cellular processes including migration, morphogenesis, differentiation, and tissue repair (reviewed in Refs. 1 and 2). Evidence of altered fibronectin expression has also been reported for a range of tissues and pathologic conditions that include osteoarthritis (3–6) and rheumatoid arthritis (7–9).

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In attempting to define the mechanism of CMP cross-linking in tracheal cartilage, we found that fibronectin is also a major structural component of the extracellular matrix, reaching 20% or more of the tissue, dry weight, in older animals. From sequence analysis of fibronectin fragments extracted proteolytically from the tissue, we also show that the predominant fibronectin isoform in bovine tracheal cartilage lacks the ED-B domain.

EXPERIMENTAL PROCEDURES

Source and Preparation of Tissue—Bovine tracheae from cattle of three age groups (calf, 2-year, and 5-year animals) were obtained at slaughter and immediately frozen and stored at −20 °C prior to analysis. Soft tissue and perichondrium were carefully removed, and the individual tracheal rings were minced on ice, ground to a fine powder under liquid nitrogen (Spex mill), and then extracted with 4 M guanidine HCl, 0.05 M Tris-HCl, pH 7.5, at 4 °C for 24 h.

CNBr and Trypsin Digestion—Cyanogen bromide (CNBr) and trypsin digests were carried out on portions of the washed residue. Conditions for trypsin digestion were 1% trypsin (w/w) in 0.2 M NH₄HCO₃, at 37 °C for 24 h. Supernatant and washings were combined, lyophilized, and weighed, and the trypsin-insoluble residual tissue was weighed. CNBr digestion was carried out in 70% (w/v) formic acid under argon at room temperature for 24 h with a CNBr concentration of 2.5 mg CNBr/mg of tissue, dry weight (26). To separate collagenous peptides from noncollagenous peptides, the CNBr digest of bovine tracheal cartilage (20 mg) was extracted 5 times with 1 ml of 1% (v/v) trifluoroacetic acid at 60 °C for 2 min. The trifluoroacetic acid-soluble material was separated by microcentrifugation (Eppendorf). The pellet and pooled supernatants were lyophilized separately and weighed.

SDS-PAGE and Amino-terminal Sequence Analysis—Both the trifluoroacetic acid-soluble and -insoluble components were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (27) and also transblotted onto polyvinylidene difluoride membrane for microsequence analysis (28). Edman amino-terminal sequence analysis of phenylthiohydantoindervatives was performed on a gas-phase protein microsequencer (Porton 2090E) equipped with on-line high performance liquid chromatography (HPLC) analysis using the manufacturer’s standard program.

Peptide Chromatography—A 47-kDa CNBr peptide of fibronectin was purified by molecular-sieve HPLC. Two columns (Toso-Haas G3000SW, 7.5 mm × 90 cm) were coupled in series and eluted with 2 M guanidine HCl, 0.05 M Tris-HCl, pH 6.8, monitoring for absorbance at 220 nm (29). The isolated peptide was desalted by dialysis and digested with endoproteinases Asp-N (sequencing grade, Boehringer Mannheim).

1 The abbreviations used are: CMP, cartilage matrix protein; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography.

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The digest was fractionated by reverse-phase HPLC (30). Individual peptide yields were estimated from the integrated absorbance (220 nm) profile and phenylthiohydantoin-derivative recoveries on sequence analysis.

RESULTS AND DISCUSSION

The pool of acid-insoluble material from CNBr-digested tracheal cartilage, which proved to consist primarily of non-collagenous components, was reduced to determine the yield of acid-insoluble fraction and the yield of non-collagenous matrix proteins by two different methods shown to select for non-collagenous components. Thus, the recovered dry weights of the acid-insoluble fraction of a CNBr digest of tissue and of a trypsin-soluble fraction from another tissue aliquot were expressed as % of total digest weights.

| Animal age | CNBr digest acid-insoluble fraction | Trypsin digest soluble fraction |
|------------|------------------------------------|--------------------------------|
| 4 months   | 6%                                 | 11%                            |
| 2 years    | 37%                                | 26%                            |
| 5 years    | 57%                                | 59%                            |

FIG. 1. SDS-PAGE of the CNBr digest of bovine tracheal cartilage. The acid-soluble (lane 1) and acid-insoluble (lane 2) fractions were run on a 12.5% gel. The acid-soluble pool shows the typical CNBr peptide pattern of type II collagen (31). The acid-insoluble pattern is quite distinct with the major CNBr fragments being shown to be derived from bovine fibronectin. The results of amino-terminal sequence analysis of individual transblotted bands are shown.

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The pool of acid-insoluble material from CNBr-digested tracheal cartilage, which proved to consist primarily of non-collagenous protein fragments, increased with age for the three age groups analyzed to 57% of the digest (% of total digest weights) from a 5-year-old cow (Table I). The trypsin-soluble pool of tracheal cartilage, which was extracted without heat-denaturing the collagen in order to assess the content of non-collagenous matrix proteins by another method, also increased with age to 59% of the 4M guanidine-insoluble dry weight. In contrast, articular cartilage from the 5-year animal yielded only 12% of its 4M guanidine-insoluble dry weight as trypsin-soluble material when treated similarly.

Fig. 1 compares the SDS-PAGE profiles of the trifluoroacetic acid-soluble and trifluoroacetic acid-insoluble fractions from CNBr-digested 5-year bovine tracheal cartilage. The trifluoroacetic acid-soluble fraction showed the typical profile of type II collagen CNBr peptides (Fig. 1, lane 1) (31). The identity of each peptide band was confirmed by protein microsequencing after blotting to polyvinylidene difluoride membrane (sequence data not shown). The trifluoroacetic acid-insoluble material gave an electrophoretic profile totally different from that of the acid-soluble fraction. Protein microsequence analysis identified all the major protein bands from 5-year tracheal cartilage as CNBr peptides of bovine fibronectin (31). Microsequence analysis from SDS-PAGE transblots of the trypsin extract of tissue showed that all major protein bands were tryptic peptides of either bovine fibronectin or bovine CMP (data not shown).

Based on the recovered dry weights of the various tissue fractions and the yields of protein bands on electrophoresis estimated by their staining intensities and sequencing results, fibronectin accounted for about 20% of the total dry weight of

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**TABLE I**

Age-related increase in the yield of non-collagenous matrix proteins from bovine tracheal cartilage

Yields of matrix proteins from tissue previously extracted by 4 m guanidine HCl, pH 7.5, were estimated by two different methods shown to select for non-collagenous components. Thus, the recovered dry weights of the acid-insoluble fraction of a CNBr digest of tissue and of a trypsin-soluble fraction from another tissue aliquot were expressed as % of total digest weights.

| Animal age | CNBr digest acid-insoluble fraction | Trypsin digest soluble fraction |
|------------|------------------------------------|--------------------------------|
| 4 months   | 6%                                 | 11%                            |
| 2 years    | 37%                                | 26%                            |
| 5 years    | 57%                                | 59%                            |
tracheal cartilage from the 5-year animal. This conservative estimate takes into account the material initially extracted by the 4 M guanidine HCl, which for the 5-year animal was less than 10% of the tissue, dry weight, as determined by dialyzing, freeze-drying, and weighing the extract.

The 47-kDa CNBr peptide that would contain the ED-B domain (if present) was purified by molecular-sieve HPLC (Fig. 2) and then digested with endoproteinase Asp-N. On the basis of the digest’s elution profile on reverse-phase HPLC, individual Asp-N peptides were selected for microsequence analysis (Fig. 3). Amino-terminal sequence results on each peak are shown in Table II. Of particular interest is the peptide giving the DTIIPAVPPPT sequence (Table II and peak D5 in Fig. 3). Compared to the published cDNA-predicted sequence (11), this peptide clearly does not contain an ED-B domain, indicating that tracheal cartilage fibronectin lacks ED-B (Fig. 4). The presence or absence of the other variable domain, ED-A, could not be established from these analyses. The findings are in agreement with a recent report demonstrating that fibronectin mRNA from canine tracheal cartilage was 100% ED-B-negative (32).

Fibronectin is present in normal articular cartilage in low concentration (33). The splicing pattern of fibronectin mRNA from chick cartilage is reported to change during chondrogenesis (22). In mesenchymal tissue of the developing limb of the embryo, fibronectin mRNA includes both ED-A and ED-B, but only ED-B is present later, in chick cartilage. In contrast, fibronectin mRNAs from chick muscle and tendon contain ED-A and lack ED-B, leading the authors to speculate that cartilage expresses an unusual form of fibronectin (22). Here, we clearly show at the protein level that the predominant fibronectin splicing variant in bovine tracheal cartilage lacks the ED-B domain. This may reflect the expression of a splicing variant that is peculiar to respiratory tract cartilages or that is indicative of a plasma origin.

The surprising finding is that fibronectin increases with age to account for 20% of the dry weight of tracheal cartilage in the most mature animal. At this level, fibronectin rivals type II collagen in content, and it may be the most prominent structural protein of the tissue. In combination, fibronectin and the 148-kDa cartilage matrix protein certainly exceed the mass of collagen in the older tissue. The results are consistent with previous observations on cultures of tracheal cartilage that synthesized fibronectin and deposited mostly in the matrix in contrast with articular cartilage explants, which released mostly to the medium (34). The findings are also comparable to the behavior of CMP, which was shown to increase in content in bovine tracheal cartilage with age in parallel with a decrease in the protein’s extractability in 4 M guanidine HCl (35). It would appear, therefore, that both fibronectin and CMP become major structural components of adult respiratory tract cartilages. This is in sharp distinction to articular cartilage, which contains very small amounts of fibronectin and CMP.

The cross-linking mechanism responsible for the inextractability of fibronectin and CMP in mature tracheal cartilage is uncertain. However, it is well documented that transglutaminase is responsible for the covalent cross-linking of plasma fibronectin to fibrin during blood-clotting (36). A key site of transglutaminase action in plasma fibronectin has been identified as the glutamine at the third residue from the amino terminus (37). Transglutaminase will also cross-link plasma fibronectin to native type I or type III collagens in vitro (38). It seems reasonable to suspect, therefore, that fibronectin becomes cross-linked in tracheal cartilage to collagen fibrils and/or other matrix proteins through transglutaminase-mediated reactions. CMP may accumulate and become cross-linked by the same mechanism. The biological significance is unknown, but profound effects on the material properties of the tissue of such a large pool of interfibrillar protein can be expected.

![Fig. 4. Amino acid sequence of the domain of bovine fibronectin (11) corresponding to the 47-kDa CNBr peptide.](image)

(See Fig. 1, lane 2, upper band.) Endoproteinase Asp-N cleavage sites are indicated by arrows. The Asp-N peptide expected to contain the ED-B domain is underlined. The sequence of peptide D5 (Fig. 3 and Table II) is that predicted for the plasma form of fibronectin, which lacks the ED-B domain.

**Table II**

| Peak | Sequence |
|------|----------|
| D1  | 919EEVVH |
| D2  | 922DEKESVP |
| D3  | 927DITGVRITTTP |
| D4  | 928DAPTNQFINE |
| D5  | 929DTIIPAVPPPT |
| D6  | 934WTPPESPV |
| D7  | 938DAPIVKVKV |
| D8  | 940ENLSPLGE |
| D9  | 949DITGVRITTTP |
| D10 | 950DTGVL |
| D11 | 957DITGVV |
| D12 | 961EVTL |
| D13 | 967DAPTNLQFINE |
| D14 | 968DTIIPAVPPPT |
| D15 | 970DAPIVKVKV |
| D16 | 975WTPPESPV |

![Diagram](image)

ED-B
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REFERENCES

1. Hynes, R. O. (1990) Fibronectins, pp. 113–364, Springer-Verlag New York Inc., New York
2. Ruoslahti, E. (1988) Annu. Rev. Biochem. 57, 375–413
3. Wurster, N. B., and Lust, G. (1982) Biochem. Biophys. Res. Commun. 109, 1094–1101
4. Hemmingsberg, G. A., Meyers, R., and Xie, D. (1992) J. Biol. Chem. 267, 3597–3604
5. Miller, D. R., Mankin, H. J., Shoji, H., and D’Ambrosia, R. D. (1984) Connect. Tissue Res. 12, 267–275
6. Chevalier, X. (1993) Semin. Arthritis Rheum. 22, 307–318
7. Vartio, T., Vaheri, A., Von Essen, R., Isomaki, H., and Stenman, S. (1981) Eur. J. Clin. Invest. 11, 207–212
8. Scott, D. L., Wainwright, A. C., Walton, K. W., and Williamson, N. (1981) Ann. Rheum. Dis. 40, 142–153
9. Carsons, S., Lavietes, B. B., and Diamond, H. S. (1989) in Fibronectin (Mosher, D. F., ed) pp. 327–361, Academic Press, San Diego, CA
10. Petersen, T. E., Skorstengaard, K., and Vibe-Pedersen, K. (1989 in Fibronectin (Mosher, D. F., ed) pp. 1–24, Academic Press, San Diego, CA
11. Kornblihtt, A. R., Umezawa, K., Vibe-Pedersen, K., and Baralle, F. E. (1985) EMBO J. 4, 1755–1759
12. Hynes, R. O. (1985) Annu. Rev. Cell Biol. 1, 67–90
13. Tamkun, J. W., Schwarzauer, J. E., and Hynes, R. O. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 5140–5144
14. Schwarzauer, J. E., Tamkun, J. W., Lemiachka, I. R., and Hynes, R. O. (1983) Cell 33, 421–431
15. Hynes, R. O., Yamada, Y., Sullivan, M., DeCrombrugge, B., Pastan, I., and Yamada, K. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 46–50
16. Schwarzauer, J. E., Patel, R. S., Fonda, D., and Hynes, R. O. (1987) EMBO J. 6, 2573–2580
17. Kornblihtt, A. R., Vibe-Pedersen, K., and Baralle, F. E. (1984) EMBO J. 3, 221–226
18. Kornblihtt, A. R., Vibe-Pedersen, K., and Baralle, F. E. (1984) Nucleic Acids Res. 12, 5863–5868
19. Vibe-Pedersen, K., Kornblihtt, A. R., and Baralle, F. E. (1984) EMBO J. 3, 3511–3516
20. Norton, P. A., and Hynes, R. O. (1987) Mol. Cell. Biol. 7, 4297–4307
21. Bennett, V. D., Pallante, K. M., and Adams, S. L. (1991) J. Biol. Chem. 266, 5918–5924
22. Heinegård, D. K., and Pimentel, E. R. (1992) in Articular Cartilage and Osteoarthritis (Kuetten, K. E., Schleyerbach, R., Peyron, J. G., and Hascall, V. C., eds) pp. 95–111, Raven Press, Ltd., New York
23. Paulsson, M., and Heinegård, D. (1981) Biochem. J. 197, 367–375
24. Paulsson, M., and Heinegård, D. (1982) Biochem. J. 207, 297–313
25. Eyre, D. R., and Muir, H. (1975) Biochem. J. 151, 596–602
26. Laemmli, U. K. (1970) Nature 227, 680–685
27. Towbin, H., Staelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4350–4354
28. Bogaat, R., Wilkin, D., Wilcox, W. R., Lachman, R., Cohn, D. H., and Eyre, D. R. (1994) Am. J. Hum. Genet. 55, 1128–1136
29. Eyre, D. R. (1987) Methods Enzymol. 144, 115–139
30. Wu, J. J., and Eyre, D. R. (1984) Biochemistry 23, 1850–1857
31. Zhang, D., Burton-Wurster, N., and Lust, G. (1995) J. Biol. Chem. 270, 1817–1822
32. Burton-Wurster, N., and Lust, G. (1984) Biochim. Biophys. Acta 800, 52–58
33. Larsson, T. (1989) Cartilage Matrix Biology: Studies on Factors of Relevance for Tissue Homeostasis. Doctoral dissertation, University of Lund
34. Paulsson, M., Inerot, S., and Heinegård, D. (1984) Biochem. J. 221, 623–630
35. Tamaki, T., and Aoki, N. (1981) Biochem. Biophys. Acta 661, 280–286
36. Mosher, D. F., Schad, P. E., and Kleinman, H. K. (1979) J. Clin. Invest. 64, 761–767
37. McDonagh, R. P., McDonagh, J., Petersen, T. E., Thogersen, H. C., Skorstengaard, K., Sottrup-Jensen, L., Magnusson, S., Dell, A., and Morris, H. R. (1981) FEBS Lett. 127, 174–178