The cartilage-specific $(V+C)^-$ fibronectin isoform exists primarily in homodimeric and monomeric configurations

Nancy BURTON-WURSTER*[1], Rina GENDELMAN*, Hao CHEN†, Da-Nian GU†, Jonathan W. TETREAULT†, George LUST*, Jean E. SCHWARZBAUER‡ and James N. MACLEOD†

*James A. Baker Institute for Animal Health, Department of Microbiology and Immunology, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853, U.S.A., †James A. Baker Institute for Animal Health, Department of Biomedical Sciences, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853, U.S.A., and ‡Department of Molecular Biology, Princeton University, Princeton, NJ 08544, U.S.A.

Fibronectin is an extracellular-matrix glycoprotein encoded by a single gene, but with significant protein heterogeneity introduced through alternative RNA splicing and post-translational modifications. The $(V+C)^-$ splice variant, in which nucleotides encoding protein segments III-15 and I-10 are deleted along with the entire variable region, is unique in that expression is restricted to cartilaginous tissues. All known fibronectin splice variants retain the two C-terminal cysteine residues essential for dimerization, but cellular and/or structural constraints appear to influence homo- and heterodimerization patterns. Dimerization patterns of the $(V+C)^-$ isoform were studied under native conditions within canine articular cartilage and experimentally in COS-7, NIH-3T3 and CHO-K1 cell cultures. In all systems, $(V+C)^-$ fibronectin secretion was predominantly in a homodimeric configuration. Lower levels of $(V+C)^-$ monomers were also present. Heterodimers of $(V+C)^-$ with V^+,C^+ (V120) isoforms were not detected. Heterodimers of $(V+C)^-$ with V^-,C^+ (V0) subunits were detected only at low levels. Functional properties may differ significantly among monomers, homodimers and heterodimers. The unique dimerization pattern of $(V+C)^-$ fibronectin is consistent with this isoform having specialized functional properties *in situ* that are important for either the structural organization and biomechanical properties of cartilage matrix or regulation of a chondrocytic phenotype.

Key words: chondrocyte, dimerization, splice variant.

INTRODUCTION

Fibronectin is an extracellular-matrix glycoprotein encoded by a single gene. Alternative RNA splicing had, until recently, been reported at only three sites, extra type-III domain A (ED-A or EIIIA), extra type-III domain B (ED-B or EIIIB) and the variable or V region (sometimes called IIICS). We have discovered that as many as 50–80% of the fibronectin transcripts in

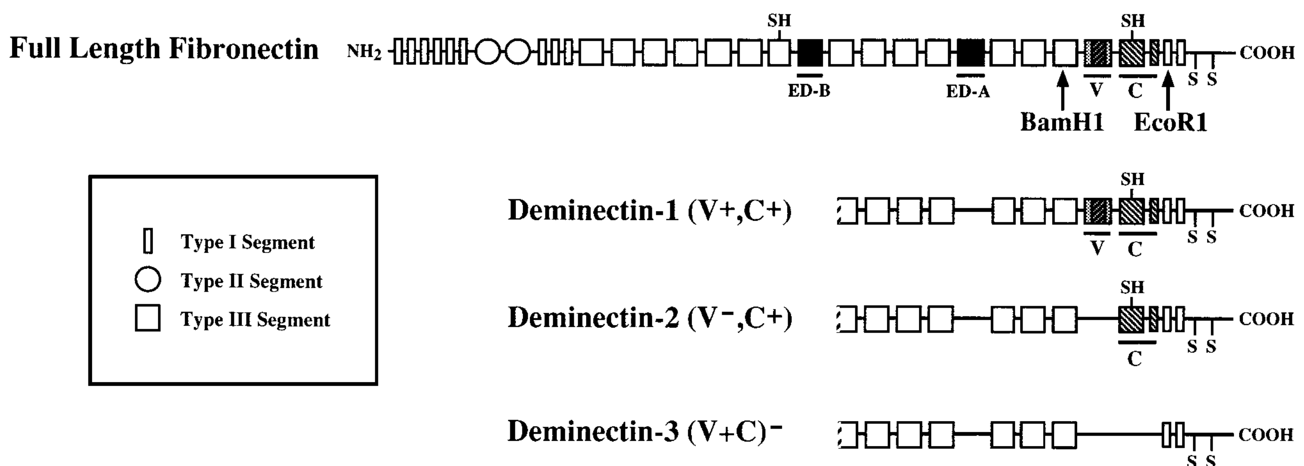

Figure 1 Schematic representation of fibronectin protein isoforms and the derived truncated DN constructs

Monomeric fibronectin protein structure and three fibronectin isoforms in cartilage predicted by RNA-splicing patterns in the V and C regions are indicated. $(V+C)^-$ symbolizes the RNA splice variant in which nucleotides encoding the III-15 and I-10 domains are deleted together with the complete V region. From 50 to 80% of steady-state fibronectin transcripts in canine and equine adult articular cartilage have the (V+C) region deleted. In adult cartilage, approx. 25% of fibronectin transcripts retain ED-B. In contrast, ED-A is deleted. Expression of the $(V+C)^-$ splice variant appears to be regulated independently of retention or deletion of ED-B. Two cysteine residues near the C-terminus, necessary for dimerization, are indicated by S. DNs contain the C-terminal half of fibronectin, starting within protein segment III-8. Construction of DN1 and DN2 cDNAs have been described previously [3,9]. DN3 was prepared by exchanging the *Bam*HI/*Eco*R1 fragment from DN1 with the corresponding fragment from the $(V+C)^-$ splice variant. For expression studies, the three DN constructs coupled to fibronectin's signal-sequence segment (bases −20–95 relative to the ATG codon) were cloned into the eukaryotic expression vector pcDNA1.

Abbreviations used: V region, variable region; C region, cartilage region; FBS, fetal bovine serum; ITS+, insulin, transferrin and selenium plus linoleic acid and BSA; DN, deminectin.

[1] To whom correspondence should be addressed (e-mail niw1@cornell.edu).

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chondrocytes from adult articular cartilage have a novel structure that results from a previously unreported RNA splicing pattern [1]. This splice variant, which we have designated $(V+C)^-$, deletes not only the V region but also nucleotides that would normally encode the fifteenth type-III repeat (III-15) and the tenth type-I repeat (I-10; Figure 1). The III-15 and I-10 segments are referred to collectively as the C region (for cartilage). This mRNA structure readily explains the existence of the small cartilage fibronectin protein isoform, which is not recognized by two different monoclonal antibodies to epitopes within the III-15 segment [2]. The tissue-specific expression pattern of the $(V+C)^-$ fibronectin isoform suggests that it has an important function in cartilaginous tissues [1].

Through two highly conserved disulphide linkages at the C-terminus, different fibronectin isoforms have the potential to form homodimers or heterodimers. The secreted products, however, do not always reflect the laws of random assortment, since homodimers lacking a V region are unstable and fail to be secreted by some cells [3]. Additional deletion of the C region might further alter dimerization potential and stability, but nothing is yet known about the dimerization patterns of $(V+C)^-$ fibronectin. In this study, we have examined dimerization patterns of the $(V+C)^-$ isoform under native conditions within canine articular cartilage and also in COS-7, NIH-3T3 and CHO-K1 cell cultures using isoform-specific truncated deminectins (DNs).

MATERIALS AND METHODS

Materials

L-[^{35}S]Methionine (1200 Ci/mmol) was purchased from Amersham (Arlington Heights, IL, U.S.A.). Monoclonal antibodies 1H9 (αHep 2) and $13G_3B_7$ (αIII-15) have been characterized and described previously [2]. In brief, monoclonal antibody 1H9 recognizes an epitope within segments III-12 to III-14 (the Hep 2 region), whereas monoclonal antibody $13G_3B_7$ recognizes an epitope in the III-15 segment. Monoclonal antibody IC-3 is specific for rat fibronectin and has been characterized and described previously [4]. Lipofectin reagent for transient-transfection assays was obtained from Gibco-BRL Life Technologies (Grand Island, NY, U.S.A.). COS-7, NIH-3T3 and CHO-K1 cells were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). Peroxidase-linked goat anti-mouse IgG was purchased from Cappel Biomedicals (Westchester, PA, U.S.A.). Dulbecco's modified Eagle's medium, Ham's F-12 medium, Gey's balanced salt solutions, Dulbecco's PBS and fetal bovine serum (FBS) were purchased from Gibco. A serum-free medium supplement containing insulin, transferrin and selenium plus linoleic acid and BSA (ITS$^{+™}$ culture supplement) was purchased from Collaborative Research (Bedford, MA, U.S.A.). Collagenase type CLS1 from *Clostridium histolyticum* was purchased from Worthington Biochemicals (Freehold, NJ, U.S.A.).

Source of explanted cartilage and culture conditions

The source of cartilage was Labrador Retriever dogs from a colony maintained at the James A. Baker Institute for Animal Health. Articular cartilage was obtained at necropsy from femoral and humeral heads of three adult animals. It was washed three times with Gey's balanced salt solution and either used immediately or frozen at −20 °C. For explant cultures, cartilage shavings were cultured in Ham's F-12 supplemented with ITS$^+$ and calcium (3.3 mM final concentration) and metabolically labelled with [^{35}S]methionine as described previously [5,6]. For monolayer cultures, chondrocytes were isolated by digestion with collagenase [7] and maintained in Ham's F-12 supplemented with 10% (v/v) FBS (fibronectin-depleted). Both explant and monolayer cultures were incubated at 37 °C in 5% CO_2/95% air. At harvest, culture medium was removed to a cocktail of protease inhibitors (0.3 mM benzamidine/20 mM EDTA/10 mM *N*-ethylmaleimide/0.4 mM PMSF, final concentrations). Cartilage and conditioned medium were then frozen separately at −20 °C until further analyses.

Extraction, purification and identification of cartilage fibronectin

Fibronectin was extracted from cartilage with 4 M urea in 0.05 M phosphate buffer, pH 7.2, containing the cocktail of protease inhibitors (0.1 ml of buffer per 10 mg of wet-weight cartilage, extracted three times over a 2-day period). Fibronectins were purified from extract and conditioned medium by gelatin-affinity chromatography [8]. Individual isoforms were differentiated by size and immunoreactivity by Western-blot analyses as described below, using the monoclonal antibody to the heparin-binding region common to all known splice variants (1H9) and the monoclonal antibody to the alternatively spliced III-15 segment ($13G_3B_7$) to assess the presence or absence of the C region.

Construction of $(V+C)^-$ deminectin 3 (DN3) and transfection experiments

Truncated rat fibronectin constructs termed DNs that extend from within segment III-8 to the C-terminus (Figure 1) were used in cell-culture expression assays. The V^+,C^+ deminectin 1 (DN1) and the V^-,C^+ deminectin 2 (DN2) have been described previously [3,9]. To obtain DN3, we first isolated total RNA from rat articular cartilage and amplified fibronectin cDNA by reverse-transcriptase PCR using primers within exons encoding III-12 and I-12. An internal *Bam*HI/*Eco*R1 fragment extending from III-14 to I-11 (Figure 1) was isolated from the amplified $(V+C)^-$ band and substituted for the corresponding fragment in DN1 [3,9], which had previously been cloned into the eukaryotic expression vector, pcDNA1 (InVitrogen, Carlsbad, CA, U.S.A.). Direct DNA sequence analysis of the DN3 construct confirmed its identity and the absence of any PCR or cloning artifacts. Each DN construct, both singly and in combination with the other isoforms, were transiently transfected into COS-7 and NIH-3T3 cells using cationic liposomes (Lipofectin) following the protocol recommended by the manufacturer (Gibco-BRL). At 24 h post-transfection, the cells were re-fed with Dulbecco's modified Eagle's medium/10% FBS. Conditioned media were collected 24 h later (48 h post-transfection), stabilized with protease inhibitors, clarified by centrifugation (175 ***g***), and stored at −20 °C until analysed for secreted DN protein. Homodimers, heterodimers and monomers of recombinant proteins in the conditioned medium were separated under non-reducing conditions by SDS/PAGE, blotted on to nitrocellulose, probed with the rat fibronectin-specific monoclonal antibody IC-3, and detected by chemiluninescence as described below. Only recombinant rat DNs were detected on the Western blots. Endogenous cell fibronectins were not recognized by monoclonal antibody IC-3 unless they were heterodimerized with the recombinant rat DN.

Stable expression of DN3 was established in CHO-K1 cells by co-transfection with pRSVneo at a 10:1 molar ratio using calcium phosphate co-precipitation [10,11]. Following transfection, G418 (400 μg/ml) was added to the culture medium to eliminate non-transformants. Individual cell clones were identified, isolated and expanded in Ham's F-12 supplemented with 10% FBS. For co-expression experiments in these CHO-K1 clones, DN1 was transiently transfected using cationic liposomes.

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Conditioned culture medium and cell-associated lysates were collected 24 or 48 h later as indicated. After removal of the culture medium, the cell/cell matrix layer was rinsed twice with chilled PBS and extracted sequentially with 1 % Nonidet P40 (v/v, in 150 mM NaCl/50 mM Tris/HCl, pH 7.5) and 1 % SDS (w/v, in sterile distilled water). The cocktail of protease inhibitors described above was also added to both detergent solutions. To a 962-mm² culture well (6-well dish), 1 ml of chilled Nonidet P40 was added and the culture surface scraped with a rubber policeman. The lysate was transferred to a microfuge tube and centrifuged at 9000 ***g*** for 15 min. The resulting supernatant was defined as the Nonidet P40-soluble fraction. Pelleted material in the microfuge tube and the original cell-culture well were then each re-extracted with 0.5 ml of SDS at room temperature. These two 0.5-ml SDS fractions were pooled and centrifuged at 9000 ***g*** for 15 min. The resulting supernatant was defined as the SDS-soluble fraction.

Electrophoresis and Western-blot analyses

Samples were prepared for electrophoresis by heating at 90 °C for 10 min in the presence of 0.2 % (w/v) SDS, and in the presence or absence of 2 % (v/v) 2-mercaptoethanol. Fibronectins and DNs were separated according to size on SDS/polyacrylamide gels in Tris/glycine buffer at pH 8.6 [12]. After electrophoresis, gels were either stained with Coomassie Brilliant Blue R-250 and dried, or transferred on to nitrocellulose membranes [13] and probed with the appropriate monoclonal antibodies followed by a peroxidase-linked anti-mouse IgG antibody. Peroxidase activity was identified either by colour using substrate solutions (Kirkegard and Perry Laboratories, Gaithersburg, MD), or with chemiluminescence (ECL® Western-blotting detection system, Amersham). Gels and blots containing radioactive samples were scanned in a Fujix Bio-Imaging Analyser and quantified using MacBas software (Fuji Medical Systems USA, Stamford, CT, U.S.A.).

Two-dimensional electrophoresis

Identical samples containing purified [^{35}S]fibronectin were run in parallel in the first, unreduced, dimension. Strips containing resolved dimers and monomers were cut from the gel and kept frozen. Another gel was poured while one of the frozen samples was defrosted and soaked in Tris/glycine buffer (pH 8.6) containing 2 % 2-mercaptoethanol at 96 °C for 10 min. The strip, on a piece of filter paper, was placed carefully to abut a new resolving gel, and a stacking gel was poured around the strip to avoid air spaces and create a tight seal. After resolution under reducing conditions, the second-dimension stacking gel was removed and the duplicate strip from the first dimension gel was placed in the same position, dried together with the resolving gel, and analysed using the Fujix Bio-Imaging Analyser.

RESULTS

Dimerization patterns of native-cartilage fibronectins

Most fibronectin isoforms are secreted as dimers, but the dimerization capacity of the cartilage-specific (V + C)⁻ fibronectin isoform was not known. Therefore, to study dimerization of fibronectins in cartilage, native-cartilage fibronectins were purified by gelatin-affinity chromatography and analysed by gel electrophoresis and immunoblotting with two monoclonal antibodies that can be used to differentiate C⁺ and C⁻ isoforms [2]. Two distinct populations of dimers plus some monomeric fibronectin were resolved by SDS/PAGE and detected by monoclonal antibody 1H9, which recognizes an epitope common to all fibronectin isoforms (αHep 2; Figure 2, lane 4). The mobility of the larger dimer group was close to that of a plasma fibronectin dimer (approx. 450 kDa, results not shown). The molecular mass of the smaller dimer group (approx. 400 kDa) and the monomeric fibronectin (approx. 200–220 kDa), as well as their failure to react with monoclonal antibody $13G_3B_7$ (epitope in the III-15 segment; αIII-15), indicated their identity as the (V + C)⁻ fibronectin isoform (Figure 2, lane 3).

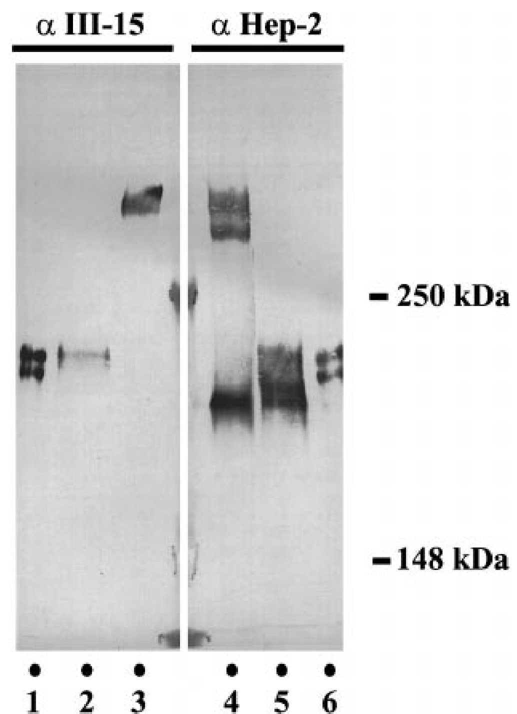

Figure 2 Homodimers and monomers of (V + C)⁻ fibronectin are present in extracts of articular cartilage

Cartilage fibronectins, purified from extracts of cartilage, were separated by SDS/PAGE (4 % gel), transferred on to nitrocellulose, and probed with monoclonal antibodies $13G_3B_7$ (αIII-15; lanes 1–3) and 1H9 (αHep 2; lanes 4–6). Sample identities are: lanes 1 and 6, plasma fibronectin, reduced; lanes 2 and 5, cartilage fibronectin, reduced; and lanes 3 and 4, cartilage fibronectin, non-reduced. Monoclonal antibody αHep 2 recognized all isoforms of cartilage fibronectin both non-reduced (lane 4) and reduced (lane 5), as well as purified plasma fibronectin (lane 6). Monoclonal antibody αIII-15 does not bind specifically to the (V + C)⁻ isoform and, therefore, recognized only the large dimer of cartilage fibronectin (lane 3) and the higher-molecular-mass monomers of reduced cartilage fibronectin (lane 2), but recognized all isoforms of plasma fibronectin (lane 1).

To quantify the amount of (V + C)⁻ homodimers and monomers synthesized by cartilage in explant culture, cartilage was cultured in the presence of [^{35}S]methionine for 18 h and labelled fibronectin purified separately by gelatin-affinity chromatography from both a urea extract of the cartilage matrix and from the explant-conditioned medium. In a separate experiment, chondrocytes were enzymically isolated from cartilage and cultured in monolayer for two passages before the addition of [^{35}S]methionine. It is known that chondrocytes in monolayer culture rapidly lose expression of the (V + C)⁻ isoform [1,14], so that [^{35}S]fibronectin obtained from the culture medium of the second passage chondrocytes served as a negative control. The purified [^{35}S]fibronectins were analysed by SDS/PAGE and quantified using a PhosphorImager. A typical autoradiogram is shown in Figure 3 and quantitative data are summarized in Table

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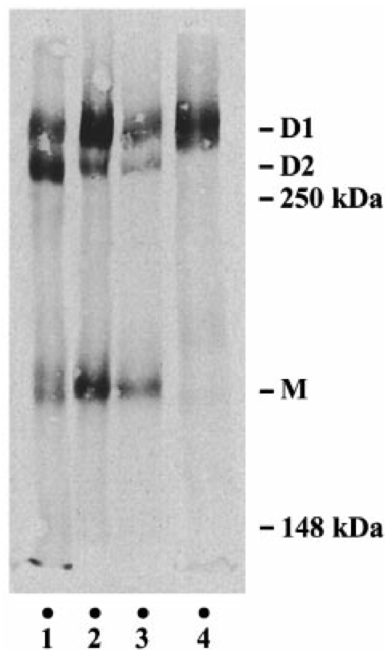

Figure 3 (V + C)⁻ fibronectin dimers and monomers are synthesized and secreted by cartilage in explant culture but not by chondrocytes passaged in monolayer cultures

[^{35}S]Fibronectins, purified from urea extracts of the cartilage matrix (lane 1), from conditioned media of cartilage in explant culture (lanes 2 and 3), or from second-passage chondrocytes in monolayer culture (lane 4), were separated by SDS/PAGE (4% gel, non-reduced) and visualized by autoradiography. The $(V+C)^-$ fibronectin dimers (D2) and monomers (M) synthesized only by cartilage were substantially reduced in medium conditioned by monolayer cultures of passaged chondrocytes (lane 4). $V^{+/-}$,C^+ fibronectin dimers (D1) were detected in all samples.

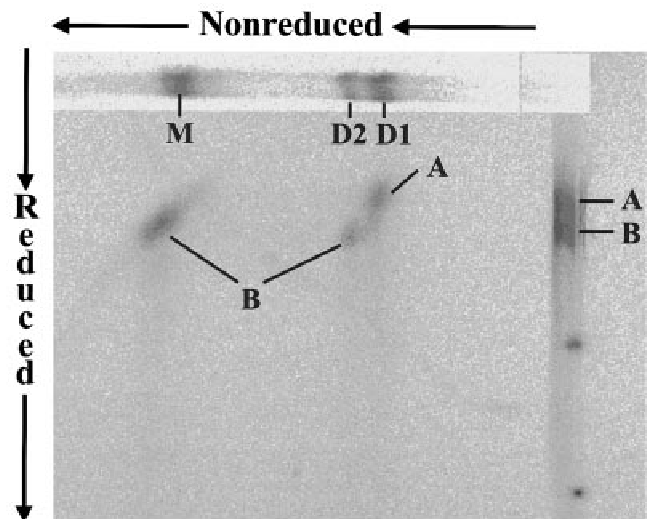

Figure 4 The putative 400-kDa $(V+C)^-$ fibronectin homodimer dissociates into monomers consistent with $(V+C)^-$ fibronectin subunits

[^{35}S]Fibronectin purified from cartilage-explant culture medium was analysed by two-dimensional SDS/PAGE. Species consistent with the 450-kDa dimer (D1), the 400-kDa dimer (D2) and the 200–220-kDa monomer (M) shown in Figure 3 were resolved in the first dimension (3.5% gel, non-reduced). SDS/PAGE (5% gel) in the second dimension under reducing conditions confirmed that the 400-kDa species was indeed a dimer that dissociated into monomers of 200–220 kDa (B). The larger 450-kDa dimer population dissociated primarily (95%) into monomers at 225–240 kDa (A).

1, where the distribution of radioactivity among presumptive $V^{+/-}$,C^+ dimers (D1), $(V+C)^-$ homodimers (D2) and $(V+C)^-$ monomers (M) has been tabulated. Quantitative analysis of the small dimer and monomer indicated that the $(V+C)^-$ fibronectin isoform represents at least 55% of the total cartilage [^{35}S]fibronectin in adult canine articular cartilage. Both homodimer and monomer were released to the culture medium; however, the ratio of $(V+C)^-$ fibronectin monomer to homodimer was 5:1 in the conditioned medium but only 1:1 in the cartilage matrix. In contrast, as expected, no more than 5% of [^{35}S]fibronectin in medium conditioned by second-passage monolayer cultures of articular chondrocytes was the $(V+C)^-$ isoform.

To determine the subunit composition of the dimers, [^{35}S]fibronectin purified from the urea extract of the cartilage matrix was analysed by two-dimensional SDS/PAGE. The smaller, approx. 400-kDa fibronectin dimer (Figure 4, D2) dissociated into monomers consistent with $(V+C)^-$ fibronectins (Figure 4, B). The larger, approx. 450-kDa fibronectin dimer population (Figure 4, D1), dissociated into monomers consistent with $V^{+/-}$,C^+ fibronectins (Figure 4, A).

Dimerization patterns of recombinant DNs

The formation and secretion of $(V+C)^-$ fibronectin homodimers by chondrocytes in articular cartilage was surprising since it was shown previously that V^-,C^+ (V0) homodimers are not secreted at significant levels [3,15]. Furthermore, most V^-,C^+ isoforms in plasma are found as heterodimers with V^+,C^+ isoforms [16] and not as homodimers. Therefore, we wanted to know if our

Table 1 Distribution of [^{35}S]fibronectin dimers and monomers

[^{35}S]Fibronectins were purified and analysed by SDS/PAGE as described in Figure 3. Cartilage samples were obtained from hips and shoulders of two different dogs, with the number of replicates indicated in parentheses. Gels were either dried directly or first subjected to Western-blot analysis to confirm identification of $(V+C)^-$ bands by differential staining with monclonal antibodies αHep2 and αIII-15. [^{35}S]Fibronectins on the dried gel or blot were then quantified by PhosphorImager. Replicate samples showed no differences in distribution whether quantified from a dried gel or from a blot. The $(V+C)^-$ monomer to $(V+C)^-$ homodimer ratio was higher in explant-conditioned media (5:1) compared with explant matrix (1:1). The $(V+C)^-$ fibronectin isoform constituted less than 5% of the total [^{35}S]fibronectin synthesized by monolayer cultures of passaged chondrocytes. Data represent the percentage of total fibronectin and are presented as means ± S.D.

	D1 $V^{+/-}$,C^+ dimers	D2 $(V+C)^-$ homodimers	M $(V+C)^-$ monomers
Explant matrix	49 ± 9% (4)	24 ± 8% (4)	27 ± 4% (4)
Explant medium	48 ± 15% (8)	8 ± 4% (8)	44 ± 18% (8)
Second-passage chondrocyte medium	95%	0%	5%

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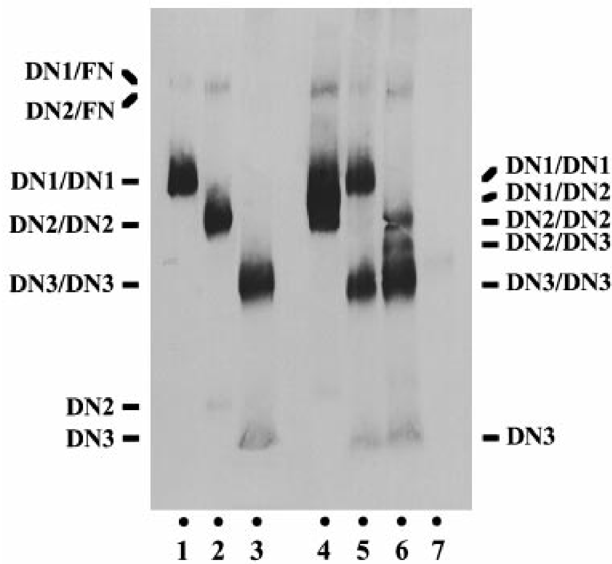

Figure 5 $(V+C)^-$ DN3 constructs are secreted in COS-7 cells predominantly as homodimers even when co-transfected with V^+,C^+ (DN1) and V^-,C^+ (DN2) constructs

Culture media were collected from COS-7 cells transiently transfected with DN1 (lane 1), DN2 (lane 2), DN3 (lane 3), DN1 plus DN2 (lane 4), DN1 plus DN3 (lane 5) and DN2 plus DN3 (lane 6), and mock transfected (lane 7). DN secretion patterns were analysed by SDS/PAGE (4 – 10% gradient, non-reduced) and Western-blot analysis using a monoclonal antibody specific for rat fibronectin (FN). All three recombinant DNs were secreted by COS-7 cells as homodimers (lanes 1–3). As expected, co-transfection with DN1 and DN2 resulted in a high level of DN1/DN2 heterodimers (lane 4). In contrast, no DN1/DN3 heterodimers were formed in cells co-transfected with DN1 and DN3 (lane 5). A small number of DN2/DN3 heterodimers were detected, but most of the DN3 isoform remained in a homodimeric configuration when cells were co-transfected with DN2 and DN3 (lane 6). In all cells transfected with DN3, some DN3 monomers were observed (lanes 3, 5 and 6).

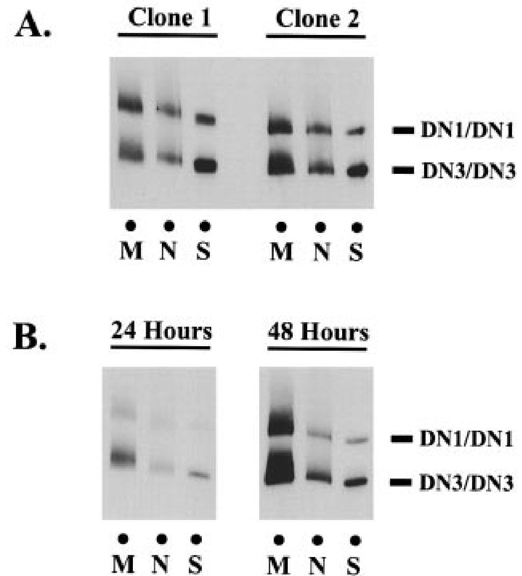

Figure 6 $(V+C)^-$ DN3 is efficiently secreted as a homodimer in stably transfected CHO-K1 cells even when transiently co-transfected with V^+,C^+ (DN1)

(**A**) Two independent CHO-K1 clones with stable expression of DN3 were transiently co-transfected with DN1 as described in the Materials and methods section. After 48 h, the culture medium (M) was removed and the cell layer extracted sequentially with Nonidet P40 (N) to extract intracellular and membrane-bound proteins and SDS (S) to extract proteins incorporated into the extracellular matrix. Aliquots of each sample were then resolved using SDS/PAGE (4–10% gradient, non-reduced) and analysed by immunoblotting using a monoclonal antibody specific for rat fibronectin. DN1 and DN3 were present as homodimers in all fractions. There was no evidence of DN1/DN3 heterodimerization, even in the cell lysates (N). To enhance this illustration, sample loading volumes were adjusted to roughly normalize band intensities between individual fractions. (**B**) Time-course analysis of a CHO-K1 clone with co-expression of DN1 and DN3. Duplicate cultures were established with the stable DN3 clone 1 following transient co-transfection with DN1. After 24 h, and after 48 h, medium was removed and the cell layer extracted sequentially with Nonidet P40 and SDS as for (**A**). Samples were again resolved by SDS/PAGE. No sample-volume adjustments were made, enabling relative comparisons between the different fractions at the two time points.

conclusions about $(V+C)^-$ fibronectin dimerization patterns in cartilage would be corroborated when critical and careful analyses of dimer formation were performed in a simplified experimental system using a defined number of subunits. Accordingly, we utilized the three DN constructs described in Figure 1. Each construct, both singly and in combination, was transiently transfected into COS-7 and NIH-3T3 cells.

All three recombinant DN proteins (see Figure 1), DN1 (V^+,C^+), DN2 (V^-,C^+) and DN3 [$(V+C)^-$], were secreted as homodimers when transfected alone (Figure 5, lanes 1–3). In co-transfection experiments, DN1/DN2 heterodimers predominated, as expected (Figure 5, lane 4). In marked contrast, no DN1/DN3 heterodimers were found in conditioned culture medium from cells co-transfected with DN1 and DN3 (Figure 5, lane 5). Instead, DN1/DN1 and DN3/DN3 homodimers were clearly evident. A small number of DN2/DN3 heterodimers was detected, but DN3/DN3 homodimers also predominated in culture medium from cells co-transfected with DN2 and DN3 (Figure 5, lane 6). Note also, the presence of some DN3 monomers (Figure 5, lanes 3, 5 and 6). The same DN3 dimerization and secretion patterns were observed in transiently transfected NIH-3T3 cells (results not shown).

One potential explanation for the DN3 dimerization pattern observed in conditioned medium is that heterodimers may actually form within the cell, but are not efficiently secreted. To evaluate this possibility, heterodimerization patterns in conditioned medium were compared in parallel with Nonidet P40 and SDS-soluble cell-associated lysates. For these experiments, CHO-K1 clones with stable expression of DN3 were generated and then transiently co-transfected with DN1. In each fraction, only DN1 and DN3 homodimers were observed. There was no evidence of DN1/DN3 heterodimers, even in the cell lysates (Figure 6A). To evaluate secretion efficiency, the experiment was repeated with samples collected at 24 and 48 h (Figure 6B). Substantial accumulation of DN1 and DN3 homodimers occurred in the medium during this time frame. A lower-magnitude increase in the Nonidet P40 and SDS fractions was observed, which probably reflects increased cell numbers at 48 h and possibly the kinetics of transient gene expression. Importantly, the relative ratio between the two DN homodimers remained fairly constant at both time points in all three fractions, suggesting roughly equivalent rates of processing and secretion. Combined, these results do not support a problem with secretion, but indicate instead that $(V+C)^-$ DN does not heterodimerize efficiently with the V^+,C^+ isoform.

DISCUSSION

In this study, we demonstrated that the cartilage-specific $(V+C)^-$ fibronectin isoform is formed and secreted as a homodimer.

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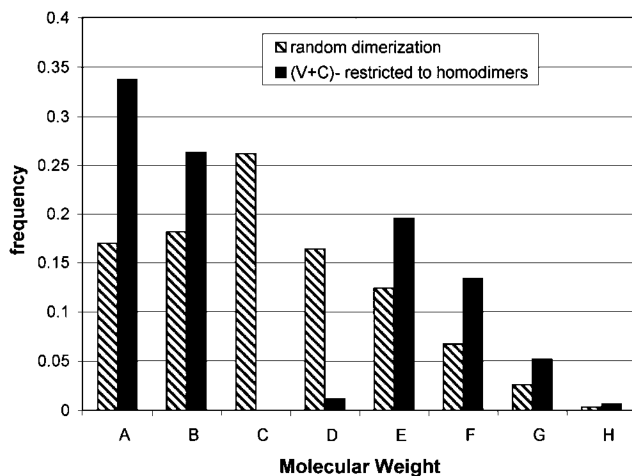

Figure 7 Theoretical distribution of fibronectin dimers in canine articular cartilage

For the analyses of the frequency distributions of fibronectin-dimer molecular masses in articular cartilage, we assumed the following eight isoforms and monomer molecular masses based on a molecular mass for plasma fibronectin of about 463 000 Da: B^-,V^+,C^+, 237 825 Da; B^-,V^-,C^+, 225 225 Da; B^-,V^{95},C^+, 235 200 Da; $B^-,(V+C)^-$, 210 420 Da; B^+,V^+,C^+, 247 275 Da; B^+,V^-,C^+, 234 675 Da; B^+,V^{95},C^+, 244 650 Da; and $B^+,(V+C)^-$, 219 870 Da. We then calculated a molecular-mass distribution of the 36 possible dimers based on random assortment of the predicted isoform ratios from steady-state mRNA data [14] (hatched bars). However, if $(V+C)^-$ fibronectin isoforms can form homodimers but are unable to form stable heterodimers, two distinct dimer populations are predicted (solid bars). This bimodal profile is consistent with the pattern actually observed in cartilage (compare Figures 2–4). The rationale for these calculations is developed more fully in the Discussion section. A, 420–429 kDa; B, 430–439 kDa; C, 440–449 kDa; D, 450–459 kDa; E, 460–469 kDa; F, 470–479 kDa; G, 480–489 kDa; and H, 490–499 kDa.

Monomeric $(V+C)^-$ fibronectin was also present in the cartilage matrix and cartilage-explant-conditioned medium, but heterodimers with $V^{+/-},C^+$ isoforms were not detected. Quantitative estimates of $(V+C)^-$ fibronectin protein in canine articular cartilage reported here are consistent with a value of 55 % steady-state $(V+C)^-$ mRNA from ribonuclease-protection-assay analyses of articular cartilage in adult dogs [14].

Fibronectin is assembled into a disulphide-bonded dimer in the endoplasmic reticulum and transported subsequently through the Golgi and secreted [17]. Previous experiments using DN constructs expressed in SVT2 cells (simian virus 40-transformed 3T3 fibroblasts) demonstrated that efficient secretion of the dimer is dependent on the presence of the V region in at least one subunit of the dimer [3]. Indeed this observation readily explained the failure to find V0 (V^-,C^+) homodimers in rat plasma fibronectins. Thus it was unexpected to find that $(V+C)^-$ homodimers, which also lack the V region on both subunits, were readily formed and secreted in native cartilage. It seemed prudent, therefore, to confirm this result from cartilage in a simplified and defined experimental system. When DN constructs were transfected, alone and in combination, into the COS-7, NIH-3T3 and CHO-K1 cells, DN3 homodimers were secreted in the medium and present in cell-associated detergent lysates. Thus the preferential formation and secretion of the $(V+C)^-$ isoform in a homodimeric configuration was confirmed.

The presence of $(V+C)^-$ fibronectin monomers in cartilage was also unexpected. The possibility cannot be excluded that all $(V+C)^-$ fibronectin is secreted in a homodimeric configuration with a portion subsequently cleaved at the C-terminus (a site known to be very susceptible to proteolytic cleavage) to produce an apparent monomer. The higher ratio of monomer to homodimer in the explant-conditioned medium compared with the cartilage matrix might be consistent with proteolytic cleavage if the fibronectin is increasingly susceptible to cleavage as it passes from the matrix into the culture medium. However, at least some DN3 monomers, but not DN1 monomers, were also detected in the media and cell-associated fractions of all three transfected cell lines. To explain this result by dimer degradation would require postulating that $(V+C)^-$ fibronectin is more susceptible to proteolytic cleavage than the $V^{+/-},C^+$ isoforms. On the other hand, Sottile and Mosher [18] showed that a mutant fibronectin with a deletion of the I-10 to I-12 segments was secreted by COS cells with monomers exceeding dimers by two to one. Therefore, it is possible that the lack of the I-10 segment in $(V+C)^-$ fibronectin is responsible for the secretion of a portion of this isoform as a monomer.

Heterodimerization of DN2 with DN1 and with endogenous fibronectin (Figure 5, lane 4) was consistent with previous studies [3]. In contrast, DN3 heterodimerized poorly with DN2 (Figure 5, lane 6) and not at all with DN1 (Figure 5, lane 5, and Figure 6). The absence of DN1/DN3 heterodimers not only in conditioned medium, but also in cell-associated fractions of CHO-K1 cells with stable expression of DN3 and transient co-expression of DN1 (Figure 6), suggests a problem with heterodimer formation rather than secretion. The secretion of DN2 homodimers (Figure 5, lane 2), however, is not consistent with published findings in SVT2 cells [3]. One possibility is that requirements for secretion may be less stringent in cells that have been subjected to transient-transfection procedures compared with cells selected for stable expression. This may explain why COS-7 cells appear more permissive to DN2 homodimer secretion. In contrast, DN3 homodimers were formed by both transiently transfected COS-7 and NIH-3T3 cells, as well as stably transfected CHO-K1 cells. More significantly, $(V+C)^-$ fibronectin dimers were present at high levels in cartilage matrix, demonstrating that homodimers of this isoform are secreted by chondrocytes within cartilage *in vivo*.

The appearance of two distinct populations of dimers in articular cartilage can be predicted theoretically if we limit dimerization of $(V+C)^-$ fibronectin to the formation of homodimers, but not heterodimers. The population of fibronectin isoforms in native cartilage is complex. It should be noted that the dimers D1 and D2, shown in Figures 2–4, are in fact a population of dimers, but no attempt was made in this study to resolve all native-cartilage fibronectin isoforms that arise as a result of alternative splicing and post-translational modifications. Nevertheless, ribonuclease protection assays [14,19] and Northern-blot analyses [1] of mRNA permit us to predict a minimum of eight isoforms of fibronectin in canine cartilage: B^+,V^+,C^+; B^-,V^+,C^+; B^+,V^{95},C^+; B^-,V^{95},C^+; B^+,V^-,C^+; B^-,V^-,C^+; $B^+,(V+C)^-$; and $B^-,(V+C)^-$ (where B is the alternatively spliced ED-B region, and V^{95} is the splice variant in which the first 25 amino acids of the V region are absent). Virtually all cartilage fibronectin isoforms are $[\text{ED-A}]^-$ [19]. Theoretically, these eight isoforms have the potential to form 36 different homodimeric and heterodimeric combinations. Ribonuclease protection assays with probes extending into the region coding for the ED-B segment [19] and into the C region [14] reveal the following distributions for fibronectin mRNAs in adult canine articular cartilage: 25 % ED-B(+)/75 % ED-B(−); 55 % $(V+C)^-$/45 % $V^{+/-},C^+$. Ribonuclease protection assays in the V region of $V^{+/-},C^+$ transcripts [14] justifies an estimate of 18 % V^+/65 % V^{95}/18 % V^-. Based on random assortment of the fibronectin isoforms predicted by these mRNA ratios, a frequency-distribution curve with a single peak was calculated from the range

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of molecular masses for the 36 theoretical dimer combinations (Figure 7, hatched bars). In contrast, a two-peak pattern is predicted if structural constraints limit $(V+C)^-$ isoforms to homodimerization (Figure 7, solid bars). Thus the electrophoretic patterns in Figures 2–4, which reveal two distinct populations of fibronectin dimers in cartilage, are most consistent with theoretical predictions if the $(V+C)^-$ isoform is restricted to the formation of homodimers. A role for III-15 and/or I-10 in V-dependent dimer formation would explain the different dimerization patterns observed with $(V+C)^-$ and C^+ isoforms.

Experiments that assess the functional properties of $(V+C)^-$ fibronectin in comparison with other isoforms have not been reported. However, the highly tissue-restricted expression pattern, together with the rapid loss of $(V+C)^-$ fibronectin when chondrocytes are removed from their normal matrix, clearly suggests an important role in either the structural organization and biomechanical properties of cartilage matrix or in the regulation of chondrocytic phenotype [20]. A dimerization profile in which homodimers and monomers predominate markedly over heterodimers also differs from other fibronectin splice variants, supporting further the prediction that this cartilage-specific isoform will have specialized functional properties *in situ*.

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