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Selective binding of anchorin CII (annexin V) to type II and X collagen and to chondrocalcin (C-propeptide of type II collagen)

Implications for anchoring function between matrix vesicles and matrix proteins

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Anchorin CII is a collagen binding protein of the annexin family associated with plasma membranes of chondrocytes, osteoblasts, and many other cells. As a major constituent of cartilage-derived matrix vesicles it has been shown to bind to native type II and X collagen. In accordance with this observation, here we show the localization of anchorin CII in the extracellular matrix of calcifying cartilage in the fetal human growth plate, and that it was restricted to the chondrocyte surface in proliferating and resting cartilage. Furthermore, we present evidence, using a slot blot assay, that anchorin CII not only binds to native type II and X collagen, but also to chondrocalcin, the carboxy-terminal extension of type II procollagen, in a calcium-independent manner. Pepsin digestion of type II collagen results in loss of anchorin CII binding, confirming our previous notion that the telopeptide region of type II collagen carries anchorin CII binding sites.

Anchorin CII (annexin V); Collagen binding; Chondrocalcin binding; Cartilage calcification

1. INTRODUCTION

Anchorin CII is a collagen binding protein of M_r 34,000 originally isolated from chicken chondrocyte membranes by affinity chromatography on type II collagen [1,2]. It is a member of the annexin family which are cell membrane-associated proteins in the M_r range 34,000–38,000 or 67,000 with an affinity to calcium and phospholipids [3]. Immunostaining and cell surface iodination studies localized anchorin CII to the surface of chondrocyte membranes and microvilli [2,4]. Its function remained obscure for several years, but recent findings suggest that it may be involved in the calcification process of cartilage and bone: (i) it is highly expressed in calcifying chicken growth plate cartilage and in bone (Hofmann et al., in preparation), (ii) it was shown to be a major constituent of matrix vesicles [5–7], which are cell-derived microstructures found in the extracellular matrix of calcifying cartilage and bone where they initiate mineral deposition [8–10], and (iii) X-ray analysis of crystallized annexin V, the human analogue to chicken anchorin CII, revealed a calcium channel structure [11].

Chromatography of matrix vesicle proteins confirmed the binding of anchorin CII and a 67 kDa annexin to native type II collagen, but also revealed binding to type X collagen and, to a lesser extent, to type I collagen [12]. These observations support the concept that anchorin CII may be responsible for the anchorage of matrix vesicles to the collagenous matrix of calcifying, hypertrophic cartilage. Here we report on further studies specifying the collagen binding sites for anchorin CII: we show that anchorin CII not only binds to type II and X collagen in a calcium-independent manner, but also to chondrocalcin, the carboxy-terminal extension of type II procollagen [13]. The binding to native type II collagen is greatly diminished after mild pepsin treatment, suggesting that anchorin CII binds to the telopeptide region.

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2. MATERIALS AND METHODS

2.1. *Proteins and antibodies*

Anchorin CII was isolated from fetal chicken sternal cartilage and purified to homogeneity as described previously [1–3]. Anti-anchorin CII IgG was prepared from rabbit antisera by chromatography on Protein A Superose (Pharmacia) as described, and tested for specificity by ELISA, immunoblotting and immunoprecipitation [2].

Type X collagen and chondrocalcin (C-propeptide of type II collagen) were isolated from fetal bovine growth plate cartilage and purified as described previously [14]. The purity of the proteins is documented in Fig. 1.

Type II collagen was isolated from fetal epiphyseal human cartilage by neutral salt extraction and purified by precipitation with 0.8 M NaCl in 0.5 M acetic acid followed by DEAE-cellulose chromatogra-

phy as described previously [15]. Type I collagen was isolated from human skin after pepsin digestion and purified by acidic salt precipitation as described in [16].

2.2. *Immunofluorescence staining*

This was done on frozen sections of fetal human growth plate cartilage (6–8 μm) as described previously [17].

2.3. *Binding assays of anchorin CII to different matrix molecules*

1–50 μg proteins were applied to nitrocellulose filters (Schleicher and Schuell) using a slot blot apparatus. The nitrocellulose sheets were then incubated in a solution containing 0.1 M NaCl and 50 mM Tris-HCl, pH 7.5, for 2 h with three changes of buffer, and then blocked with low-fat milk protein. After washing, the filters were incubated with purified anchorin CII (5 μg/ml) in 0.1 M NaCl, 50 mM Tris-HCl, pH 7.5, for 24 h at 4°C. In order to assess the Ca^{2+} dependence of the binding, either 10 mM $CaCl_2$ or 5 mM EDTA were added to the buffer. After blocking with low-fat milk protein, bound anchorin CII was immunostained with a rabbit anti-anchorin CII IgG, followed by peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad, Richmond, CA, USA) in a 1:1,000 dilution as second antibody and α-chloronaphthol as colour substrate. The optical density of the colour reaction was determined using a Hoefner densitometer.

2.4. *Protein determination*

Protein concentrations of samples were determined according to Bradford [18] with the microassay procedure described by Bio-Rad.

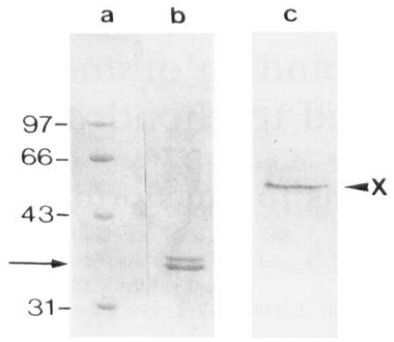

Fig. 1. SDS gel electrophoresis under reducing conditions of chondrocalcin (b) and type X collagen (c) extracted and purified from fetal bovine cartilage [18]. Chondrocalcin (b) often appears as a double band; the band at 70 kDa represents completely reduced dimers of the C-propeptide of type II procollagen. (a) Molecular weight marker proteins stained by Coomassie blue.

3. RESULTS

3.1. *Immunolocalization of anchorin CII in fetal human growth plate cartilage*

Immunofluorescence examination of a fetal distal

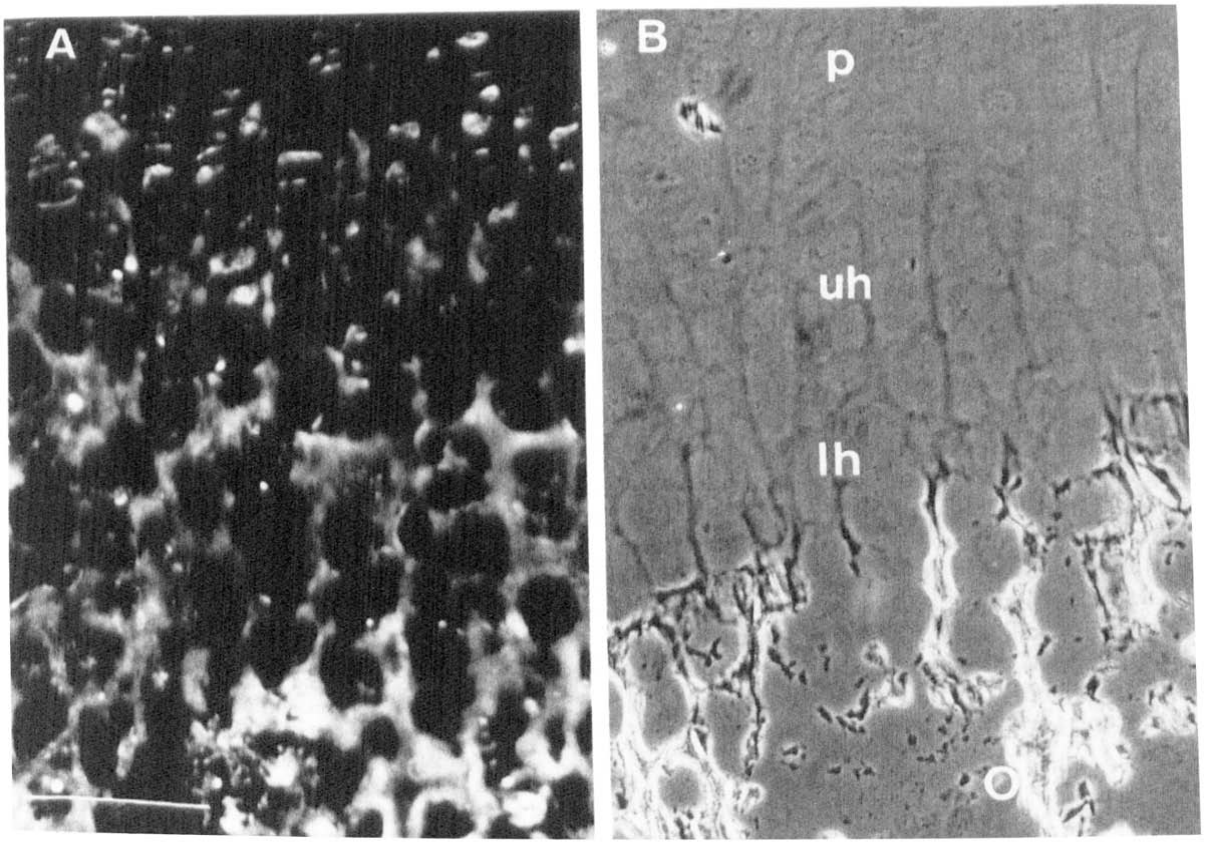

Fig. 2. Immunolocalization of anchorin CII in longitudinal sections of a fetal human growth plate. After fixation and treatment with hyaluronidase, frozen sections were stained with a polyclonal antibody against anchorin CII followed by Texas red-labeled donkey anti-rabbit IgG. Panel A reveals an extensive staining of the matrix only in the calcifying cartilage, while in the proliferative zone, anchorin CII was localized only on the surface of chondrocytes. (B) Phase contrast microscopy. p, proliferative zone; uh, upper hypertrophic zone; lh, lower hypertrophic zone. Bar = 100 μm.

femoral growth plate revealed an extensive staining of the matrix with anti-anchorin CII IgG in the hypertrophic, calcifying cartilage and in the newly formed bone trabeculae. In the proliferative zone anchorin CII was restricted to the chondrocyte surface. Staining of the matrix started to become visible in the upper hypertrophic zone, however, the most extensive matrix labeling was observed in the zone of calcification (Fig. 2).

3.2. *Binding of anchorin CII to different matrix molecules*

The binding of anchorin CII to various collagens and other proteins was tested in an overlay assay. The proteins were dotted, in amounts from 1 to 50 μg, onto nitrocellulose filters and incubated with a solution of anchorin CII in the presence of Ca^{2+} or EDTA. Bound anchorin was detected by a rabbit antiserum against anchorin CII. Addition of phosphatidylserine did not change the binding properties (data not shown).

In the presence of calcium, anchorin CII showed a strong binding to chondrocalcin, but no binding to calmodulin or serum albumin (Fig. 3A). Anchorin CII also bound to native bovine type X collagen and to neutral salt-extracted native type II collagen. After pepsin digestion of type II collagen at 4°C the binding to anchorin CII was eliminated, indicating that the binding sites are localized within the telopeptide regions of type II collagen. In the presence of calcium there was no significant binding of anchorin CII to pepsin-digested type I collagen (Fig. 3B).

In the absence of calcium, i.e. in the presence of EDTA, the binding of anchorin CII to chondrocalcin (Fig. 4A) and to native, type II and X collagen (Fig. 4B) did not change significantly. Also the affinity to pepsin-

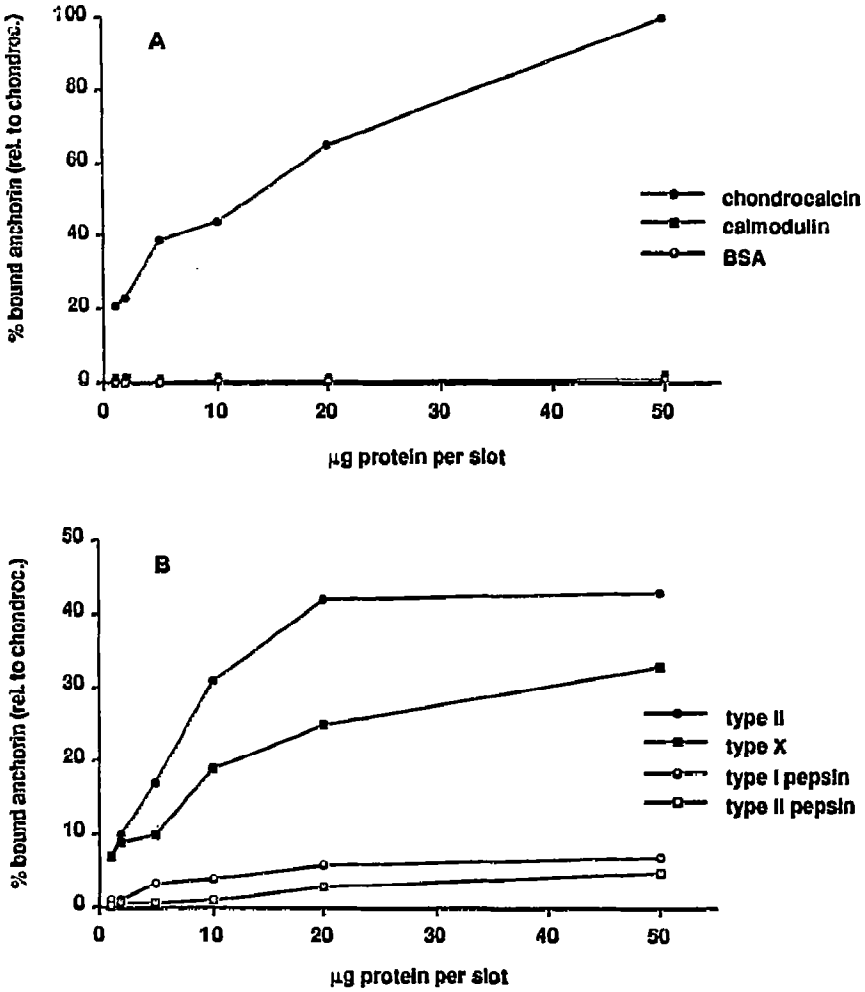

Fig. 3. Anchorin CII binding to different matrix molecules in the presence of calcium. Various amounts, in the range between 1 and 50 μg, of chondrocalcin, calmodulin and BSA (A), human type II (native, not pepsin-digested), human type I (pepsin digested) and bovine type X collagen (native, not pepsin-digested) (B) were applied to nitrocellulose filters and incubated with purified anchorin CII (5 μg/ml) in a solution containing 10 mM $CaCl_2$, and immunostained with a rabbit anti-anchorin CII IgG fraction as described in Materials and Methods. Stained filters were analyzed by densitometry; the data are expressed as integrated optical density for each band. The optical density obtained for anchorin CII binding to 50 μg chondrocalcin was set as 100%.

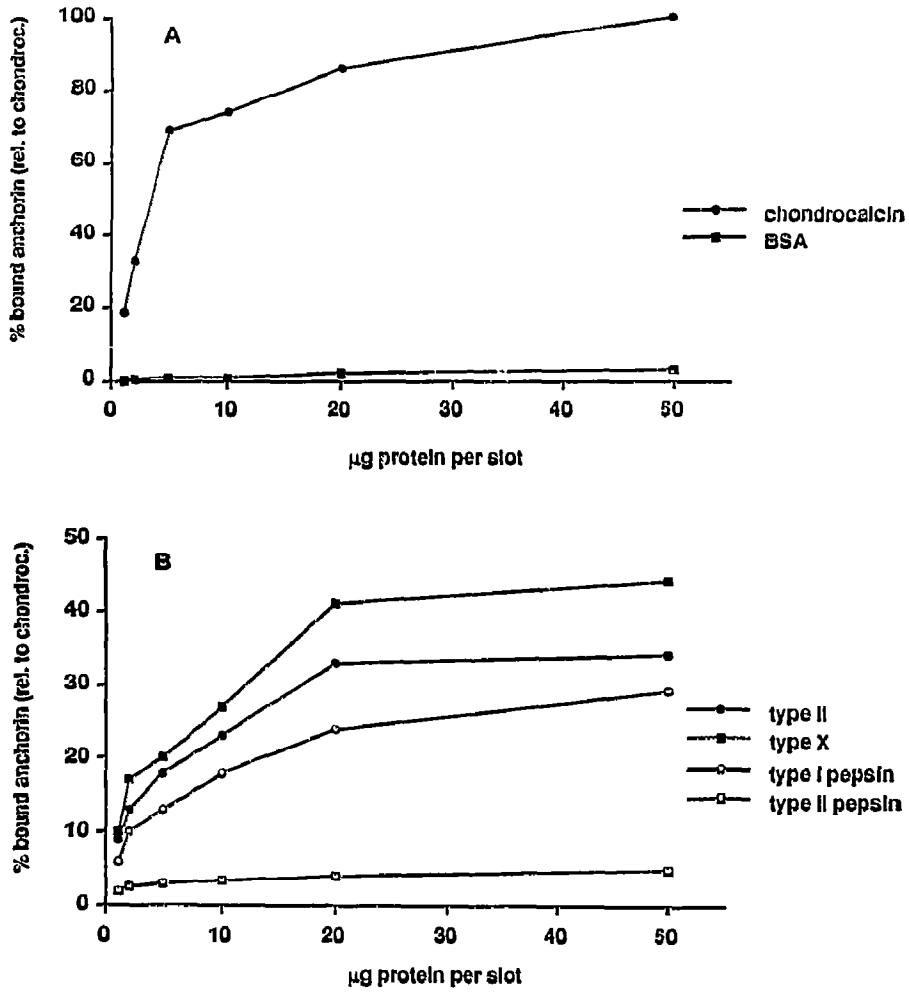

Fig. 4. Anchorin CII binding to different matrix molecules in the absence of calcium. The binding studies were done as described in Fig. 3 except that the incubation buffer contained 5 mM EDTA instead of $CaCl_2$. (A) Binding of anchorin CII to chondrocalcin and BSA. (B) Binding to native bovine type X, and type II collagen, and to human pepsin-digested type II and I collagens.

digested type II collagen was not altered, however, the binding to pepsin-digested type I collagen increased in the absence of calcium (Fig. 4B).

4. DISCUSSION

Matrix vesicles are cell-derived microstructures found in the interstitial matrix of calcifying cartilage and bone [8–10,19]. They are rich in inorganic phosphate, Ca^{2+} and alkaline phosphatase and thus provide a microenvironment and nucleation site for calcium phosphate crystallization [19]. As major calcium and phospholipid binding constituents of matrix vesicles, several proteins of the annexin family were identified [5,6]: anchorin CII (annexin V), annexin II (calpactin I), and annexin VI [7,12,20].

Matrix vesicles associate with the major macromolecules of hypertrophic cartilage, including link protein, the hyaluronic acid binding region of aggrecan, and type X and II collagen [21]. Anchorin CII and a 67 kDa annexin were shown to be the major collagen binding components of matrix vesicles [12], indicating that these annexins are responsible for the anchorage of the vesicles in the cartilage matrix. The binding conditions of matrix vesicle-derived anchorin CII to type II collagen [12] were similar to those reported previously for the binding of chondrocyte-derived anchorin CII [1]: in both studies anchorin CII eluted from type II collagen columns at salt concentrations above 100 mM NaCl; in both cases anchorin CII did not bind to denatured type II collagen.

In previous studies we have provided evidence that the telopeptide region of type II collagen might be involved in the binding to anchorin CII: removal of the telopeptides of salt-extracted chicken type II collagen

decreased the binding to anchorin CII liposomes by 60% [22]. Here we show that pepsin digestion of native human type II collagen results in almost complete loss of anchorin CII binding. Whether the amino- or carboxy-terminal telopeptides, or both, carry anchorin CII binding sites remains to be shown. In contrast, pepsin-treated type I collagen retains an affinity for anchorin CII, suggesting different binding sites, however, for both collagens it is not clear whether complete removal of telopeptides with pepsin was achieved. The fact that denatured α1(II) chains do not bind anchorin CII (see also [12]) indicates that only telopeptides in a native configuration (probably β-pleated sheet) are able to bind anchorin CII.

Surprisingly, anchorin CII binds with even higher affinity to chondrocalcin, the C-terminal extension of type II procollagen. Chondrocalcin has a rather long half-life in cartilage after its proteolytic cleavage from type II procollagen, and can be extracted from fetal cartilage in significant quantities [13,23]. Owing to its affinity for hydroxyapatite, it accumulates in calcifying cartilage and fetal bone, where it has been located immunohistologically [24]. Although chondrocalcin binds calcium with high affinity [13,25], its binding to anchorin CII does not require calcium. The binding of type X and II collagen to anchorin CII is also not affected by Ca^{2+}, nor by the presence of EDTA. Accordingly, the anchorin CII–collagen interaction is not affected by phosphatidylserine, which enhances binding of Ca^{2+} to anchorin CII.

The affinity of anchorin CII for type X collagen and chondrocalcin, both components enriched in hypertrophic calcifying cartilage [24,26,27], is consistent with the immunofluorescence localization of anchorin CII in the calcifying zone of growth plate cartilage (see also [7]).

In conclusion, anchorin CII seems to have two important, perhaps related functions in the calcification of tissues: (i) as a calcium channel [11,28] located in the membrane of matrix vesicles it might be involved in creating a Ca^{2+}-rich microenvironment inside the matrix vesicles, necessary for crystallization; (ii) as a collagen binding membrane component it anchors matrix vesicles to the matrix of calcifying cartilage.

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