CMLS, Cell. mol. life sci. 53 (1997) 539–545
1420-682X/97/060539-07 $ 1.50 + 0.20/0

CMLS Cellular and Molecular Life Sciences

Annexin V interactions with collagen

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Abstract. Annexin V was originally identified as a collagen-binding protein called anchorin CII and was isolated from chondrocyte membranes by affinity chromatography on native type II collagen. The binding of annexin V to native collagen type II is stable at physiological ionic strength when annexin V is reconstituted in liposomes. The binding to native collagen types II and X, and to some extent to type I as well, was confirmed using recombinant annexin V. A physiological role for annexin V interactions with extracellular collagen is consistent with the localization of annexin V on the outer cell surface of chondrocytes, microvilli of hypertrophic chondrocytes, fibroblasts and osteoblasts. A breakthrough in our understanding of the function of annexin V was made with the discovery of its calcium channel activity. At least one of several putative functions of annexin V became obvious from studies on matrix vesicles derived from calcifying cartilage. It was found that calcium uptake by matrix vesicles depend on collagen type II and type X binding to annexin V in the vesicles and was lost when collagens were digested with collagenase; calcium influx was reconstituted after adding back native collagen II or V. These findings indicate that annexin V plays a major role in matrix vesicle-initiated cartilage calcification as a collagen-regulated calcium channel.

Key words. Cartilage; collagen binding; annexin V; calcium flux.

Anchorin CII, a collagen-binding protein of the annexin family

In searching for a collagen receptor on chondrocytes, Mollenhauer et al. [1] isolated a protein called anchorin CII from the membranes of chick cartilage cells by affinity chromatography on type II collagen [1, 2]. The protein of Mr 34 kD was partially hydrophobic and bound to ^{125}I-labelled native type II collagen when reconstituted to liposomes. Besides type II collagen, it also bound with lower affinity to collagen types I, IX, XI and V, but not to denatured type II collagen, and with much lower affinity to pepsin-digested type II collagen [2]. A similar protein was also found in the membranes of fibroblasts and bone cells. Annexin V is expressed predominantly in hypertrophic chondrocytes, in bone cells and liver cells, but is also found in other tissues and at very early embryonic stages [3].

Fab fragments of an anti-anchorin CII serum were able to block adhesion of chondrocytes to collagen substrates by 50% as well as the binding of ^{125}I-labelled collagen to chondrocytes [4], indicating that anchorin CII might be involved in chondrocyte-collagen interactions. Preliminary evidence was presented for tissue-specific differences of annexins from chondrocytes, bone cells, fibroblasts or other tissues [5]. The analysis of the annexin V gene, however, provided no evidence for more than one annexin V gene, splice variants or isoforms [6, 7].

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The concept of anchorin CII as a collagen-receptor of chondrocyte membranes was consistent with its localization on the chondrocyte surface by immunofluorescence [2]. This was questioned, however, when the analysis of the protein structure [8] revealed homology to calpactin I and lipocortin II, and thus documented the absence of a hydrophobic signal peptide and transmembrane sequences. Like other annexins, chick anchorin CII consists of four repeat units of about 70 amino acid residues, each containing a consensus sequence of 17 residues [9]. Crystallization and X-ray analysis of human annexin V [10, 11, 12], rat annexin V [13] and chick annexin V [14] revealed a four-domain structure with each domain consisting of five α-helices wound in a right-handed superhelix. A pore in the centre of the molecule presumably serves as Ca^{++} channel. Domain I, II and IV each contain a calcium binding site on the convex side of the molecule [12], while in repeat III a tryptophan residue serves as lipophilic anchor in the lipid bilayer of the plasma membrane [15]. The sequence of chick annexin V is 80% identical to human annexin V [16]. The short, eight residue N-terminal peptide differs significantly from that of other annexins, but its function is not yet clear.

Secretion and cell surface localization of Annexin V

Most annexins, like calpactin I (=annexin II), had originally been located only at the inner face of the plasma membrane [17, 18], while lipocortin I (annexin I)

was found in the culture medium of macrophages [19]. By metabolic labelling of chick chondrocytes and fibroblasts with ^{35}S-methionine, a part of the newly synthesized annexin V was found to be secreted or shed into the culture medium of fibroblasts after 30 min, and by chondrocytes after 20 hours [9]. Since the majority of the annexin was retained in the cytoplasmic and membrane fraction of the cells, secretion or shedding is certainly not the normal fate of annexin V in proliferating, intact cells. However, annexin V was unequivocally localized on the surface of chick chondrocytes by immunofluorescence [2], by immunoelectronmicroscopy and by cell surface iodination [9]. It was most prominent on the surface and on microvilli of hypertrophic chondrocytes (fig. 1). This location is consistent with the recent finding of annexin V on the surface of apoptotic cells [20, 21] where it binds to phosphatidylserine that is exposed to the outer cell surface after membrane flipover [22]. Similarly, annexin V was located on the cell surface of fibroblasts by immunofluorescence and cell surface iodination (M. Borchert, T. Krieg, unpublished). Later, other annexins were also shown to be secreted [23] and were located on the surface of tumour cells [24, 25].

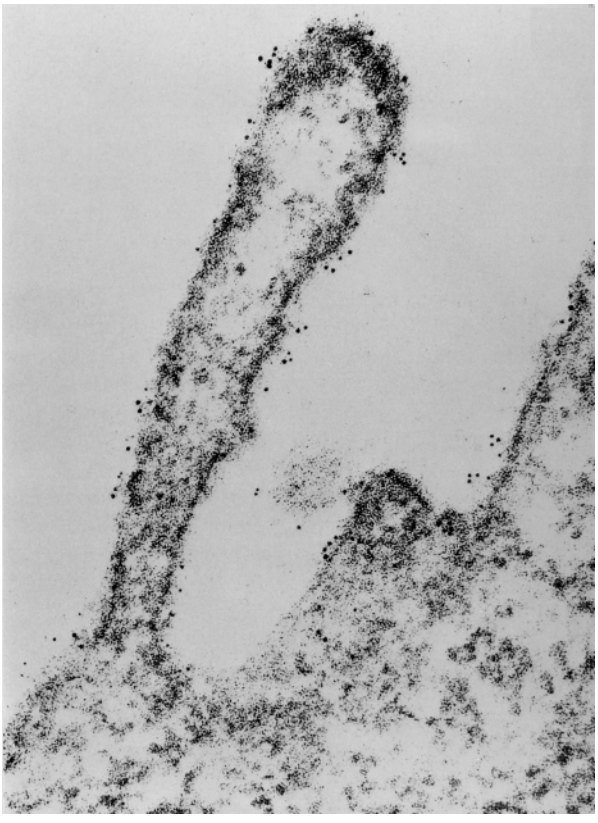

Figure 1. Immunoelectronmicroscopical localization of annexin V on the surface of chondrocytes by immunogold labelling. Hypertrophic chick chondrocytes were cultured for 24 h and incubated with rabbit anti-annexin V IgG, followed by protein A gold particle complexes. Annexin V is prominent on the surface of microvilli, but also present on other sites of the cell surface. Magnification: 94,000×. By courtesy of F. Ruggiero, Lyon: taken from: Pfaeffle et al. [9].

Exposure of phosphatidylserine, which is normally restricted to the inner face of the plasma membrane, to the outer face, probably mediated by the recently described phosphatidyl translocase [22], marks cells for phagocytosis by macrophages and other inflammatory cells [26, 27, 28]. Thus, an important function of annexins on the cell surface may be masking of phosphatidylserine on the cell surface, protecting apoptotic cells from phagocytosis. FITC- labeled annexin V is now used to label apoptotic cells specifically [29].

Affinity and specificity of annexin V interactions with collagens

In vitro, chick annexin V binds to native type II collagen, and with lower affinity also to pepsin-digested type II collagen, but not to denatured type II collagen [1, 2]. Annexin V also binds other cartilage collagens IX and XI, and to a lesser extent type V collagen as well. Interestingly, it also has a high affinity to the C-propeptide of type II procollagen [30]. The binding of annexin to native type II collagen was shown by affinity chromatography as well as by incubation of ^{125}-labelled type II collagen with annexin V, reconstituted to liposomes, followed by sucrose density gradient centrifugation [1]. In both systems the annexin V: collagen II binding was stable at physiological salt concentrations (e.g. 0.1 M NaCl, 50 mM Tris) in the absence of detergent. In the presence of detergent such as 0.1% Triton X-100, however, annexin dissociated from collagen at low salt concentrations (25–50 mM NaCl). In solid phase binding assays, e.g. after coupling collagens to nitrocellulose in a slot blot assay, annexin V was also found to bind to native, but not to denatured type II collagen, and with lower affinity to native type I collagen [30, 31, 32]. The binding of annexin V to type II collagens occurs both in the presence of 5 mM Ca^{++} [30] or in the presence of 5 mM EGTA in 0.1 M NaCl, 50 mM Tris [5, 32], in other words, in a calcium-independent manner. Böhm et al. [5] reported different affinities of annexin V for type I collagen when used as inhibitor in a liposome binding assay to type II collagen, depending on whether annexin V was isolated with EDTA from muscle or cartilage membranes. Since cartilage- and muscle derived annexin V are identical in their sequence, conformational or microenvironmental, tissue-specific differences between annexins were postulated [5].

The observation that annexin V binds to native but not to denatured type II collagen suggested that the binding site of annexin V is located in the triple helix of type II collagen. On the other hand, annexin V binding to type II collagen is lost partially after pepsin digestion, and completely after pronase digestion [1, 2], both enzymes known to remove the N- and C-terminal telopeptides. After cleavage of native II collagen with collagenase only

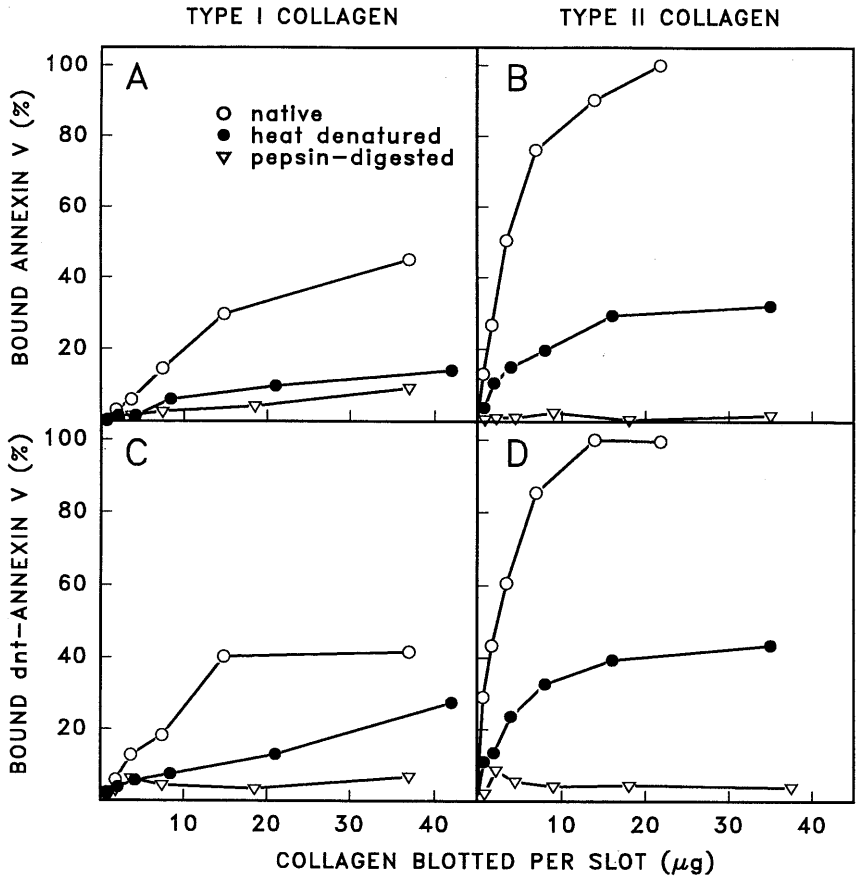

Figure 2. Binding of recombinant chicken annexin V to collagens. Wild type annexin V (A, B) and mutagenized annexin V from which the N-terminal eight amino acid residues were removed (dnt-annexin) were expressed in *E. coli* and purified by precipitation with phosphatidylserine [32]. Native, heat-denatured and pepsin-digested chicken types I and II collagen were blotted onto nitrocellulose filters and incubated with purified full length annexin V (A, B) or dnt-annexin V at 7.5 mg /ml in 50 mM Tris, pH 7.4, 50 mM NaCl, 5 mM EGTA. Collagen molecules were obtained by neutral salt extraction (native, open dots), or by pepsin digestion (open triangles). Denaturation (closed dots) was done at 50 °C for 30 min. Bound annexin V was stained with a rabbit antibody against chicken annexin V followed by immunoperoxidase reaction. Data are expressed as integrated optical density, setting the maximal value as 100 percent. (Taken from: Turnay et al. [32]).

the N-terminal fragment binds to annexin V [M. Pfäffle, PhD thesis 1988, Munich]. These findings implicated that annexin V binds to the N-terminal telopeptide of type II collagen only in its native conformation, which is a β-pleated sheet [33]. In addition, however, annexin V also binds the C-terminal propeptide of type II procollagen [30]. The collagen II binding site on the annexin V molecule is as yet unknown. Binding studies with recombinant wild-type chick annexin V and a mutant in which the N-terminal eight amino acid residues were absent showed identical affinities to native collagen II, indicating that the collagen binding site is not located at the N-terminal of annexin V, but must be located within the four repeat units of annexin V [32]. Interestingly, also recombinant annexin V also revealed a higher affinity to type II than to type I collagen, while it did not bind at all to pepsin-treated types I and II collagen (fig. 2).

Interaction of annexin V with type X collagen

Type X collagen is a short chain, network-forming collagen found predominantly in hypertrophic cartilage of the growth plates of fetal and juvenile long bone and ribs [34, 35]. It is a homotrimeric molecule with a 105 nm triple helical part flanked by a small N- and large C-terminal globular domains. The C-terminal NC-1 domains show a strong tendency for self-assembly through hydrophobic forces, thus allowing the assembly of type X collagen into a hexagonal meshwork [36, 37]. As a major constituent of hypertrophic cartilage, types X and II collagen were shown to bind to matrix vesicles (MV). These are membrane vesicles budding off from the tip of microvilli on the surface of hypertrophic chondrocytes into the extracellular space [38, 39]. They have been shown to initiate mineral deposition in calcifying cartilage [40] by accumulating high levels of intravesicular calcium ions which recruit phosphate from phospholipids and phosphonucleotides of the vesicles to form nucleation crystals of octa calcium phosphate [41]. This transforms into hydroxyapatite crystals that grow out of the vesicles and nucleate mineral deposition in the extracellular matrix [42].

In an attempt to identify calcium-binding proteins of chick cartilage matrix vesicles, Genge et al. [43, 44] isolated Mr 33 kD, 36 kD and a 68 kD calpactin-like molecules, which were later identified as annexin V (anchorin CII), annexin II and annexin VI [45]. In the presence of calcium all these annexins bind preferentially to phosphatidylserine, a phospholipid highly enriched in MV. Wu and coauthors [45] observed a close association of MV with types II and X collagen when MV were isolated from hypertrophic chick cartilage by homogenization, mild enzymatic digestion and sucrose density gradient centrifugation. In order to identify the collagen-binding proteins, matrix vesicles were isolated using collagenase to remove adherent collagens, and detergent-extracted proteins were chromatographed on affinity columns containing native types I, II or X collagen coupled to Sepharose [45]. In the presence of 0.1% Triton X-100 a 33 kD protein – annexin V – eluted at fairly low ionic strength (25–50 mM NaCl) from collagen type II or I affinity columns, confirming previous observations that the presence of detergent significantly lowers the binding affinity of annexin V to collagen II [1]. Together with annexin V, under these conditions also 36 kD and 67 kD annexins (annexins II and VI) were also retained on the collagen affinity matrix [45]. In another system, direct binding of annexin V to type X collagen was shown by blotting increasing amounts of bovine type X collagen onto nitrocellulose and incubation with chick annexin V; bound annexin was detected by anti annexin V antibodies [30]. Binding of annexin to type X collagen was saturable and followed a similar dose dependence curve as type II collagen or the C-terminal propeptide of type II procollagen.

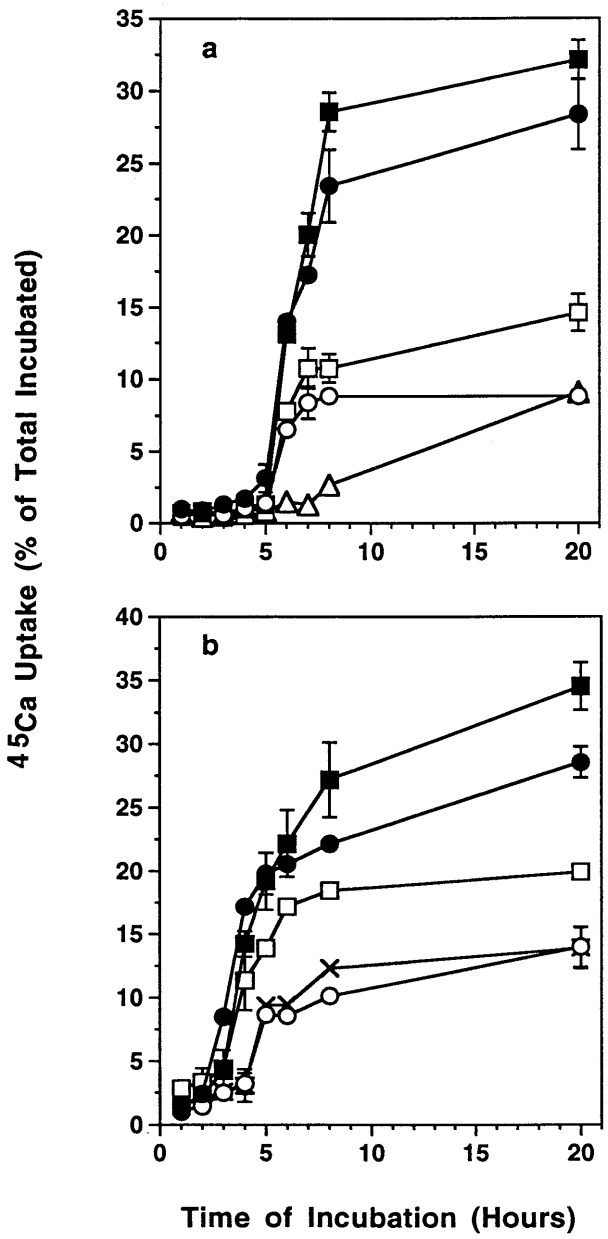

Figure 3. Effect of type II (a) or type X (b) collagen on $^{45}Ca^{++}$ uptake by collagenase treated matrix vesicles. Matrix vesicles were treated with 1 M NaCl and collagenase to remove collagens or left untreated and $^{45}Ca^{++}$ uptake was measured in 20 µl aliquots after or without incubation with various forms of collagen type II (a) or X (b). (a) ● untreated matrix vesicles (MV); ○ collagenase and 1 M NaCl-treated MV; ■ collagenase-treated MV incubated with native type II collagen; □ collagenase-treated MV plus pepsin-digested type II collagen; △ collagenase-treated MVF plus denatured type II collagen. (b) ● untreated MV; ○ collagenase-treated MV; X collagenase-treated MV plus albumin; ■ collagenase-treated MV plus native type X collagen; □ collagenase-treated MV plus pepsin-treated type X collagen. By courtesy of T. Kirsch, taken from Kirsch and Wuthier [31].

Role of collagen II and X in the regulation of Ca^{++}-uptake by matrix vesicles

Several lines of evidence indicated that annexin V, which acts as a Ca^{++} ion channel after reconstitution into lipid bilayers or liposomes [46, 47, 48] may also serve as a Ca^{++} channel in MV. Not only is Ca^{++} uptake by MV protease – sensitive [49], it is also inhibited by Zn^{++}, which inhibits the Ca^{++}-channel activity of reconstituted annexin V [41]. In view of the interaction of annexin V with extracellular type II and X collagen, Kirsch and Wuthier [31] tested the possibility that Ca^{++} uptake by MV through annexin V might be regulated by collagen binding. MV were prepared by collagenase digestion in the presence of hyaluronidase, a treatment which removed all membrane-associated collagen. MV prepared in such a way significantly lost the ability to take up $^{45}Ca^{++}$, in comparison to MV prepared in a way which retained surface-bound collagen, e.g. without enzymatic digestion. Interestingly, digestion of MV with collagenase in the presence of trypsin, without hyaluronidase, did not completely remove MV-bound collagens [50]. When collagen-stripped MV were incubated with native type II or

native type X collagen, the $^{45}Ca^{++}$-uptake was regained, approaching the levels of undigested MV (fig. 3) [31]. When MV were digested with collagenase plus chymotrypsin, which removes annexin V from the membrane, $^{45}Ca^{++}$-uptake can no longer be regained by adding collagen II or X. Studies with various protein fractions of MV showed that, in addition to collagens, proteins of the nucleational core complex are essential for Ca^{++} uptake by matrix vesicles [50].

These studies indicate that Ca^{++} uptake by MV may be regulated by collagen II and X. Whether the Ca^{++} ion flux through annexin V channels is in fact regulated by collagen II and X remains to be confirmed by using reconstituted annexin V liposomes.

Further evidence for a role of type X collagen in MV-induced cartilage calcification arose from the analysis of a transgenic mouse with a null mutation in both alleles in the type X collagen gene [51]. Ultrastructural analysis of the growth plate cartilage revealed that MV were reduced in the hypertrophic zone, lacking type X collagen, but appeared in the resting zone. This finding suggests that in normal hypertrophic cartilage MV shedding from hypertrophic chondrocytes are arrested by the type X collagen network through annexin V.

Extracellular protein binding by other annexins

Besides its function as a collagen receptor, annexin V has been described as a potential extracellular docking site for hepatitis B virus [52]. The specific ligand protein was defined as the small hepatitis B envelope protein.

Endothelial cells specifically bind components of the fibrinolytic system including plasminogen (PLG) and tissue plasminogen activator (t-PA) as one of the PLG activators. An endothelial cell surface protein has been purified by a t-PA affinity column and identified as annexin II (anx II). In mammalian expression systems anx II has been demonstrated binding to both PLG and t-PA [53]. Anti-anx II antiserum inhibited ^{125}I-tPA binding to endothelial cells as did the treatment of endothelial cells with antisense oligonucleotides directed against anx II mRNA.

Anx II has also been identified in glioma and endothelial cells at the cell-surface as high affinity receptor for the extracellular matrix protein tenascin [54]. As with the discovery of ACII, an affinity column of native tenascin was loaded with cell membrane extracts. Integrin subunits were pre-eluted with the GRGDSP hexapeptide. High ionic strength was required to elute the bound anx II. Despite these striking similarities to the findings with annexin V/type II collagen, no further evidence has been presented thus far for the physiological role of the interaction between annexin II and tenascin. These studies showed that like annexin V, annexin II also may also function as a cell surface receptor for extracellular components, including extracellular matrix proteins.

Conclusions

There is ample experimental evidence for specific interactions between annexin V and native type II and type X collagen. High levels of annexin V in cells of calcifying tissues, such as hypertrophic chondrocytes and osteoblasts and in MV, and the fact that Ca^{++} uptake by MV is regulated by types II and X collagen, points to at least one major function of annexin V: a role in cartilage calcification. Whether other collagen types also regulate the Ca^{++}- channel activity of annexins in other tissues remains to be investigated.

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