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Collagen Binding Activity of Recombinant and N-Terminally Modified Annexin V (Anchorin CII)

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Abstract We have cloned the full coding cDNA sequence of chicken annexin V and of a mutant lacking 8 amino acid residues of the N-terminal tail for prokaryotic expression. Both proteins were synthesized in *Escherichia coli* upon induction with isopropyl thio-β-D-galactoside, and were purified following two different protocols: one based on the ability of these proteins to interact reversibly with liposomes in the presence of calcium, and the other based on two sequential ion-exchange chromatographic steps. Spectroscopical analysis of recombinant annexin V revealed that binding of calcium did not change the circular dichroism spectra indicating no significant changes on the secondary structure; however, a conformational change affecting the exposition to the solvent of the tryptophan residue 187 was detected by analysis of fluorescence emission spectra. Recombinant annexin V binds with high affinity to collagen types II and X, and with lower affinity to collagen type I in a calcium-independent manner. Heat denaturing of collagen decreases this interaction while pepsin-treatment of collagen almost completely abolishes annexin V binding. Mutated annexin V interacts with collagen in a similar way as the nonmutated recombinant protein, indicating that the N-terminal tail of annexin V is not essential for collagen binding. © 1995 Wiley-Liss, Inc.

Key words: collagen binding protein, calcium binding protein, phospholipid binding protein, endonexin II, lipocortin V, protein purification

Annexins [for nomenclature, see Crumpton and Dedman, 1990] are a highly conserved family of major cellular proteins found not only in mammalian and avian species, but also in fish, higher plants, and several invertebrates [Crompton et al., 1988; Klee 1988; Burgoyne and Geisow, 1989; Römisch and Paques, 1991]. They are cell membrane-associated proteins in either a lower (32,000–38,000) or higher (67,000) M_r, with affinity to calcium and phospholipids. Their core structure is highly conserved consisting of four (or eight, in annexin VI) repeats of about 70 amino acid residues, each including a consensus sequence (endonexin fold) of 17 residues involved in Ca^{2+} binding [Thiel et al., 1991; Huber et al., 1992]. On the other hand, no significant similarities are found in the amino terminal domain of annexins, and it is speculated that this domain is responsible for the different functions of annexins [Crompton et al., 1988; Drust and Creutz, 1988; Burgoyne and Geisow, 1989; Barton et al., 1991]. Multiple functions have been proposed for these proteins, including inhibition of phospholipase A_2 and blood coagulation, as well as a possible role in signal transduction, cell growth, and differentiation [Morgan and Fernández, 1991]. However, no clear biological role for any of this proteins has been established.

Chicken annexin V was initially isolated as "anchorin CII," a collagen-binding protein from chondrocyte membranes by affinity chromatography on type II collagen–Sepharose [Mollenhauer and von der Mark, 1983; Mollenhauer et al., 1984]. Amino acid sequence analysis revealed an 80% homology with human annexin V [Iwasaki et al., 1987; Fernández et al., 1990], even though the apparent molecular mass, pI, and chromatographic behavior more closely resemble mammalian annexin IV than mammalian annexin V [Boustead et al., 1993]. X-ray crystal structure analyses of human [Huber et al., 1990a,b, 1992; Sopkova et al., 1993], chicken [Bewley et al., 1993], and rat annexin V [Concha

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et al., 1993] show that annexin V displays structural features of channel-forming integral membrane proteins. Although annexin V is only partially integrated into the membrane, a voltage-dependent calcium channel activity has been reported [Rojas et al., 1990; Berendes et al., 1993]. Although present in the cytoplasm and in the nucleus [Koster et al., 1993], it has also been localized on the chondrocyte surface by immunofluorescence, immunogold labeling, and cell surface iodination [Mollenhauer et al., 1984; Pfäffle et al., 1988; von der Mark et al., 1991]. Even though its function remains unclear, several lines of evidence suggest a role for this protein in bone and cartilage calcification, e.g., the high expression of annexin V in calcifying chicken growth plate cartilage and in bone [Hofmann et al., 1992] and its presence in the membrane of matrix vesicles, which initiate mineral deposition in cartilage [Genge et al., 1989, 1990, 1992; Wu et al., 1993]. Annexin V and VI are major components of these matrix vesicles and both annexins bind to type II and type X collagen, the major collagens of hypertrophic cartilage [Wu et al., 1991; Kirsch and Pfäffle, 1992]. Interestingly, the calcium uptake of matrix vesicles seems to be regulated by the interaction with collagen molecules, significantly decreasing after collagenase digestion of the vesicles [Kirsch and Wuthier, 1994]. Thus, the interaction of annexin V with collagen may not only play a role in cell–matrix interactions, but also in the regulation of cartilage and bone mineralization.

We report here on the cloning, expression, purification and characterization of chicken annexin V and a mutant (dnt-annexin V) lacking the most significant amino acid residues in the amino terminal domain. The potential molecular mechanisms of interaction between collagen and annexin V are discussed.

MATERIALS AND METHODS

Construction of Chicken Annexin V Clone

The full coding sequence of annexin V was obtained from four different overlapping cDNA clones covering the cDNA regions −18–342, 200–859, 859–1159, and 1026–1386 (numbers refer to the initiator ATG). The two central clones were isolated from a λgt11 chick cartilage cDNA library [Fernández et al., 1988; Hofmann et al., 1992]. The upstream clone was obtained by reverse transcription of total chick chondrocyte RNA with an oligo dT-primer followed by a PCR reaction using specific primers (ac2up and ac2dw; Table I). The downstream clone, which covers the 3′ end and most of the 13th exon, was obtained from a genomic DNA clone [Pfannmüller et al., 1993].

The clones were joined through internal restriction sites, and the construct was cloned between the *Bam*HI and *Hind*III sites of pUC19. The resulting plasmid pACII.1 was identified by restriction mapping and DNA sequence analysis [Sanger et al., 1977]. An *Nco*I restriction site was introduced at the start codon by replacing a *Bam*HI/*Acc*I–fragment of pACII.1 by synthetic oligonucleotides [nco1 and nco2 (Table I)]. A *Nco*I/*Hind*III fragment stretching from the new strat codon into the 3′ untranslated region was cloned in the prokaryotic expression vector pTrc99A (Pharmacia LKB, Uppsala, Sweden), which contains the hybrid *trc* promoter [Amann

TABLE I. Synthetic Oligonucleotide Sequences*

Oligomer sequence		
ac2up	5′-CGGATCCCGGCGAACCGCGGAGATGGC-3′	*Bam*HI + (−15–5)
ac2dw	5′-GGAATTCAACACTTTCTCATTGGTTCC-3′	*Eco*RI + comp(310–329)
nco1	5′-GATCCCGGCGAACCGGGGACCATGGCGAAGT-3′	
	|||||||||||||||||||||||||||||	
nco2	3′-GGCCGCTTGGCCCCTGGTACCGCTTCATA-5′	
dnt1	5′-GATCCCGGCGAAGCGAGTACCATGGCAG-3′	
	|||||||||||||||||||||||||||||	
dnt2	3′-GGCCGCTTCGCTCATGGTACCG-5′	

*Oligonucleotides were synthesized in an automatic DNA synthesizer (Pharmacia gene assembler plus). Oligomer ac2up has the same sequence as the mRNA, while oligomer ac2dw is complementary (comp) to the mRNA. In both cases restriction endonuclease sites were included. dnt1/dnt2 and nco1/nco2 oligomer pairs are complementary. Protruding ends allow their recloning into suitable vectors (*Nco*I restriction sites containing the initiator ATG are underlined). Nucleotides are numbered from the initiator ATG.

et al., 1988]. The resulting plasmid, pACII.E, was again identified by restriction mapping and DNA sequence analysis.

Construction of dnt–annexin V Clone

The recombinant plasmid pACII.1 was digested with *Bam*HI and *Bsm*I and the liberated fragment (nucleotides −21 to +31) was substituted by synthetic oligonucleotides [dnt1 and dnt2 (Table I)]. This synthetic fragment lacks the codons for amino acids 3–10 (Fig. 1) and includes appropriate protruding ends and an *Nco*I site at the start position. The resulting shorter mutant *Nco*I/*Hind*III fragment was cloned into pTrc99A, as described for the full-length annexin V cDNA expression clone, resulting in pdntACII.E.

Production of Recombinant Chicken Annexin V and its dnt–mutant

Cultures of the *Escherichia coli* DH5α strain carrying either pACII.E, pdntACII.E or vector alone were grown at 37°C in Luria broth containing 100 μg/ml ampicillin until the absorbance at 500 nm reached 0.5–0.7. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 2.5 mM. Cells were collected 3 h after induction by centrifugation at 3,000 rpm for 15 min. Protein production was checked by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), followed by Western blot analysis using polyclonal antibodies against chicken cartilage annexin V raised in rabbits in our laboratory.

Purification of Recombinant Annexin V and its dnt–mutant

Pelleted *E. coli* cultures containing either pACII.E, pdntACII.E, or the expression vector, were sonicated on ice in 50 mM Tris, pH 7.8, 50 mM NaCl, 2 mM $MgCl_2$ (buffer A) containing 1 mM EGTA and 1 mM phenylmethanesulfonyl fluoride (PMSF). Lysates were centrifuged at 30,000*g* for 15 min. The supernatant was used to purify recombinant annexin V and its mutant by two different methods described below. All purification steps were followed by SDS–PAGE and Western blot, using a polyclonal antibody against chicken cartilage annexin V. Amino acid analysis of the recombinant proteins was performed on a Beckman System 6300 analyzer after hydrolysis with 6 M HCl for 18 h at 110°C. N-terminal sequencing was done as described [Deutzmann et al., 1988].

Purification based on the interaction with liposomes. Annexins interact with phosphatidylserine liposomes in the presence of $CaCl_2$ [Kaplan et al., 1988]. Vesicles containing a mixture of 2 parts phosphatidylserine (PS) and 1 part phosphatidylcholine (PC) were prepared by sonication in buffer A, sedimented by centrifugation at 30,000*g* for 10 min, washed twice with the same buffer, and resuspended at 1,200 μg/ml in buffer A containing 1 mM $CaCl_2$. Phospholipid concentration was calculated according to Barlett [Barlett, 1959]. Equal volumes of vesicles and bacterial supernatants, containing the recombinant proteins, were mixed and the final free Ca^{2+} concentration was adjusted to 1 mM by addition of $CaCl_2$. After 15 min at room temperature, the mixture was centrifuged and the pellets were washed twice with buffer A containing 1 mM $CaCl_2$. The recombinant proteins were eluted from the vesicle pellets by resuspension in buffer A containing 5 mM EGTA

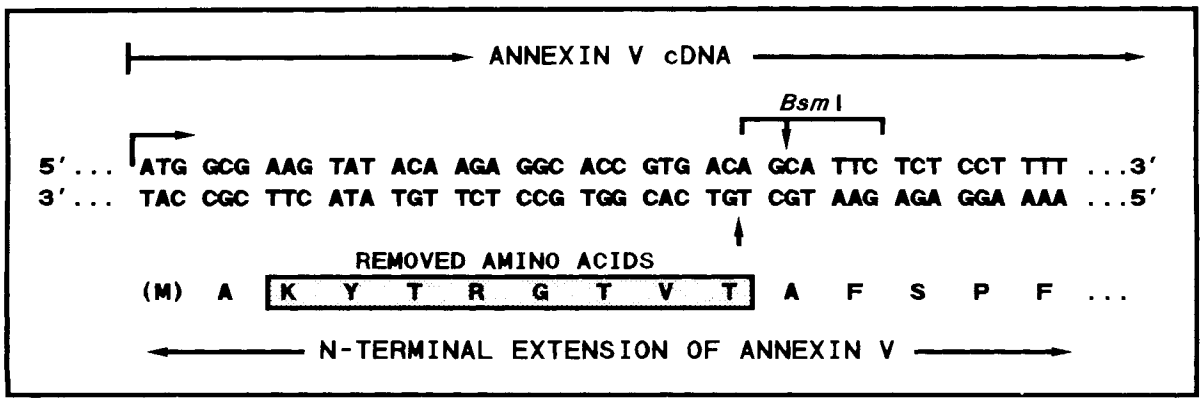

Fig. 1. N-terminal tail of chicken annexin V. The amino acid sequence and the cDNA sequence corresponding to the N-terminal extension of annexin V are shown. Amino acids removed in the dnt–mutant are boxed. The *Bsm*I site used for the construction of the mutated cDNA is indicated. The initiator Met (in brackets) is not present in the full-length recombinant nor in the dnt–mutant annexin V molecules.

(15 min, room temperature) and further centrifugation.

Chromatographic purification. The supernatants of the bacterial lysates, dialyzed against buffer A without NaCl, were chromatographically separated on a 1.5 × 10 cm DEAE-cellulose column (DE-52, Whatman, England). Retained proteins were eluted with a linear NaCl gradient (0–0.5 M). Fractions containing the recombinant proteins were pooled and dialyzed against 20 mM sodium acetate, pH 5.6. After dialysis, the recombinant proteins were loaded into a fast pressure liquid chromatography (FPLC) Mono-S cation-exchange column equilibrated in the same buffer and were eluted with a linear gradient of 0–430 mM NaCl in the same buffer. Pooled fractions of recombinant proteins were dialyzed against 50 mM Tris, pH 7.4, 50 mM NaCl, filtered through 0.22 μm, and stored at 4°C until used.

Spectroscopic Characterization

Only recombinant annexin V purified by FPLC on the Mono-S column was used for this purpose. Protein preparations were centrifuged at 100,000*g* for 10 min and filtered through 0.22-μm filters prior to spectroscopic analysis. Absorbance spectra were recorded on a Cary 118 spectrophotometer. Steady-state fluorescence emission spectra were recorded on an SLM spectrofluorimeter model 8000C, with the excitation wavelength set at 275 or 295 nm, with excitation and emission bandwidths at 4 nm. Spectra were recorded in the presence of 1 mM $CaCl_2$ or 1 mM EGTA; these solutions were obtained by the addition of the same volume of 0.5 M stocks of $CaCl_2$ or EGTA to a 0.1 mg/ml annexin V preparation in 50 mM Tris, pH 7.4, 50 mM NaCl (no changes in the pH of the solutions were detected). The baselines for spectra buffers alone were subtracted from each spectrum.

Circular dichroism (CD) spectra were recorded at 20°C on a Jobin Ivon Mark III dicrograph using the same protein preparations as for the fluorescence emission spectra. The average molecular weight per amino acid residue (109.6 Da) was calculated from the sequence data. Concentration was determined by amino acid analysis.

Calcium-Dependent Binding to Phospholipid Vesicles

Liposomes were prepared by sonication of 1.5 mg/ml PS (Sigma, St. Louis, MO) in 50 mM Tris, pH 7.4, 50 mM NaCl. Pelleted vesicles (10 min at 30,000*g*) were washed twice with buffer and resuspended at 1.5 mg/ml. Binding experiments were performed in the same buffer containing 6 μg of protein and 30 μg of PS liposomes in a total volume of 50 μl at Ca^{2+} concentrations ranging from 1 to 250 μM. After incubation at room temperature for 15 min, samples were centrifuged at 30,000*g* for 10 min. Bound protein was extracted from the vesicle pellet at room temperature for 15 min with 50 μl of buffer containing 5 mM EGTA instead of Ca^{2+}. The fraction of bound and unbound proteins was determined quantitatively by SDS–PAGE and subsequent densitometry of the Coomassie Blue-stained annexin V or dnt–mutant bands.

Collagen Purification

Type I and II collagens were isolated from chicken skin and chicken sterna, respectively. Collagens referred to as "native" were purified by neutral salt extraction and precipitation with 0.8 M NaCl in 0.5 M acetic acid, followed by DEAE-cellulose chromatography [Miller and Rhodes, 1982]. Collagens described as "pepsin-treated" were isolated after pepsin digestion followed by acidic salt precipitation as described [Chung and Miller, 1974]. Type X collagen from fetal bovine cartilage was a generous gift of Dr. T. Kirsch.

SDS–PAGE, Western Blot Analysis, and Protein Determination

Protein analysis was performed by 10% SDS–PAGE according to [Laemmli, 1970]. Proteins were stained with Coomassie brilliant blue R-250 or were electroblotted to nitrocellulose filters. After blocking with low-fat milk, recombinant annexin V, or the dnt–mutant were detected with a rabbit anti-chicken annexin V IgG obtained in our laboratory (1:1,000 dilution) followed by peroxidase-conjugated goat antirabbit IgG (BioRad, Richmond, VA) in a 1:1,000 dilution as second antibody and 3.3′-diaminobenzidine tetrahydrochloride dihydrate (DAB) and hydrogen peroxide as color substrates. The optical density of the color reaction was determined using a Cybertech CS1 densitometer. Silver staining was performed according to Oakley et al. [1980].

Protein concentration was calculated according to the method of Bradford [1976], using bovine serum albumin (BSA) as standard, by

ultraviolet (UV) spectroscopy or amino acid analysis.

Collagen Binding Assay

Binding of recombinant annexin V preparations to collagen was followed using the method of Kirsch and Pfäffle [1992] with minor modifications. Different amounts of collagen were applied to nitrocellulose filters (Schleicher and Schüll Co., Dassel, Germany) using a slot-blot apparatus. Filters were washed in 50 mM Tris, pH 7.4, 50 mM NaCl for 2 h and blocked with low-fat milk. After washing, the filters were incubated with purified recombinant annexin V or the dnt–mutant (7.5 μg/ml) in washing buffer containing either 5 mM $CaCl_2$ or 5 mM EGTA for 18 h at 4°C. Bound recombinant annexin V (or mutant) was immunostained as described for Western blot analysis of the recombinant proteins. Different amounts of annexin V also blotted onto the nitrocellulose membrane were used to normalize data from different filters.

RESULTS

Protein Production and Purification

The entire protein-coding regions of chicken annexin V or the dnt-annexin V variant were cloned into the *E. coli* expression vector pTrc99A, resulting in pACII.E (full-length recombinant chicken annexin V) and pdntACII.E (dnt–mutant). The ATG translation initiation codon is located in the *Nco*I site and immediately downstream of the strong *trc* promoter, which is inducible by IPTG and contains the *lac*Z ribosome binding site and the I^q repressor. The *Hind*III site in both constructs is located 417 bp downstream of the translational stop codon and immediately before the transcription terminators.

Bacteria transfected with either pACII.E or pdntACII.E yielded proteins of apparent molecular masses of 34 kDa (Fig. 2A, lane a) and 32 kDa (lane b), respectively. Only these proteins react with the antiserum against chicken cartilage annexin V (Fig. 2B, lanes a,b) and are absent in control cells (Fig. 2A,B, lane c). The production of the recombinant proteins is highly efficient, accounting from 2–5% of the total bacterial protein.

Recombinant proteins were purified following two different protocols. The first one exploits the ability of annexin V to interact with phospholipid vesicles in the presence of Ca^{2+} in a reversible manner [Schlaepfer et al., 1987; Kaplan et al., 1988]. This treatment eliminates the majority of the bacterial proteins, which remain in the supernatant (Fig. 2A, lanes d–f). Less than 5% of the full-length annexin V or dnt–mutant is lost (Fig. 2B, lanes d,e). Both proteins were almost quantitatively recovered from the washed vesicle pellet by extraction with 5 mM EGTA. Single protein bands in the EGTA supernatant (Fig. 2A, lanes g,h) show the appropriate molecular mass and give positive reaction with the specific antibody (Fig. 2B, lanes g,h). Additional bands were detectable only with silver staining but accounted for less than 2% of the total protein in the preparations (data not shown).

The recombinant proteins were also purified to homogeneity from the initial EGTA extracts by means of standard biochemical techniques using two chromatographic steps. Annexin V and dnt–annexin V elute from a DEAE-cellulose column at approximately 90 mM and 150 mM, respectively. Both proteins are already 70–80% pure at this stage, and degradation products are absent. On a subsequent purification by FPLC on a Mono-S cation-exchange column, the recombinant proteins emerge as almost symmetric peaks at 280 mM and 250 mM NaCl for full-length and dnt–mutant annexin V, respectively (Fig. 3). Fractions corresponding to these peaks (discarding those with possible contaminations by other minor proteins) were pooled and analyzed again by SDS–PAGE under nonreducing conditions. After silver staining single protein bands with apparent molecular masses of 34 and 32 kDa, which stain intensively with anti-annexin V antiserum (not shown), are present in the lanes corresponding to the material from bacteria expressing pACII.E (full-length) and pdntACII.E (dnt–mutant) constructs, respectively. No detectable degradation and/or aggregation is observed.

Both protocols yielded about 12 mg of recombinant annexin V and 10 mg of dnt–mutant from 500 ml bacterial culture with a similar purification degree (around 98% or higher). However, recombinant proteins isolated by the calcium-dependent phospholipid sedimentation always present more light scattering than the proteins purified by the two sequential chromatographic steps. This dispersion is probably due to a small contamination by very small lipidic micelles and can be eliminated by an additional chromatographic step on a Mono-S column (data not shown).

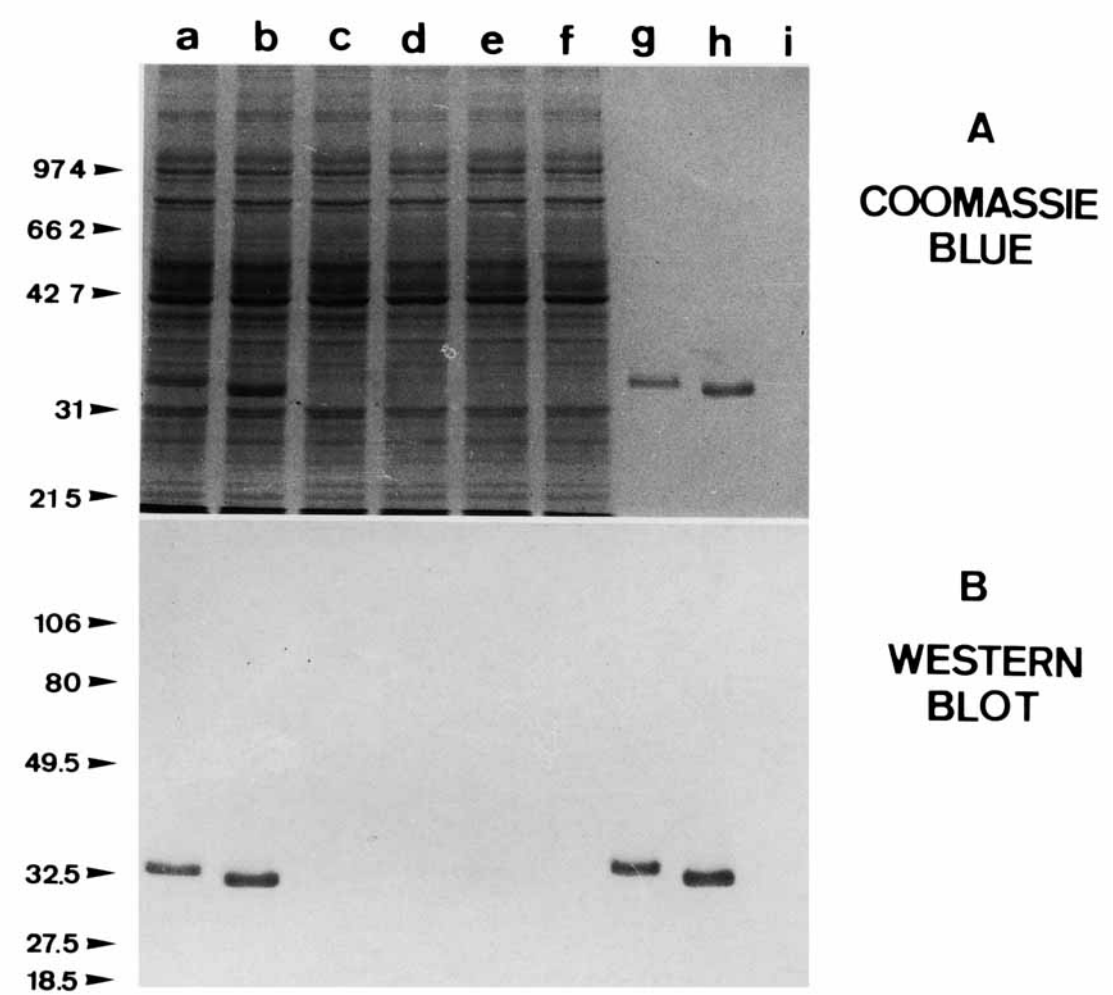

Fig. 2. Isolation of recombinant chicken annexin V. Recombinant full-length and dnt–mutant annexin V were purified from *E. coli* containing the recombinant plasmids pACII.E (**lanes a,d,g**) or pdntACII.E (**lanes b,e,h**). Lanes c,f,i, protein preparations of bacteria transformed with the expression vector alone. Aliquots of the crude bacterial lysates (**lanes a–c**), supernatants of the calcium/phospholipid sedimentation (**lanes d–f**), and proteins in the final EGTA-extraction supernatant (**lanes g–i**) were analyzed by 10% SDS–PAGE, followed by either (**A**) Coomassie Blue staining or (**B**) transfer to nitrocellulose and Western blot analysis. Molecular mass markers are indicated.

Amino acid analyses and N-terminal sequencing of recombinant annexin V and the dnt–mutant obtained by the two different protocols confirm their predicted sequence. The N-terminal methionine is not present in the recombinant proteins, thus suggesting that the proteins undergo proteolytic processing. Only Mono-S purified material was used for spectroscopical characterization to avoid possible interference of lipid microvesicles.

Spectroscopic Characterization

Several spectroscopic approaches have been used in order to determine whether recombinant chicken annexin V undergoes conformational changes as a consequence of Ca^{2+} binding. The UV absorbance difference spectrum of annexin V in the presence of 1 mM EGTA and 1 mM $CaCl_2$ showed no significant differences. From these spectra, a molar extinction coefficient of 22213 $M^{-1}cm^{-1}$ was calculated (Fig. 4A); protein concentration was determined by amino acid analysis. The CD spectra of annexin V recorded in the near UV (250–320 nm) revealed a characteristic maximum at 292 nm and three minimums at 262, 269, and 277 nm (Fig. 4B). The CD spectrum recorded at 200–250 nm in the presence of 1 mM $CaCl_2$ is typical for a protein with a very high content in α-helix and almost no β-pleated sheet structures (Fig. 4C). In fact, analysis of this spectrum using the CCA algorithm described by Perczel et al. [1991] pre-

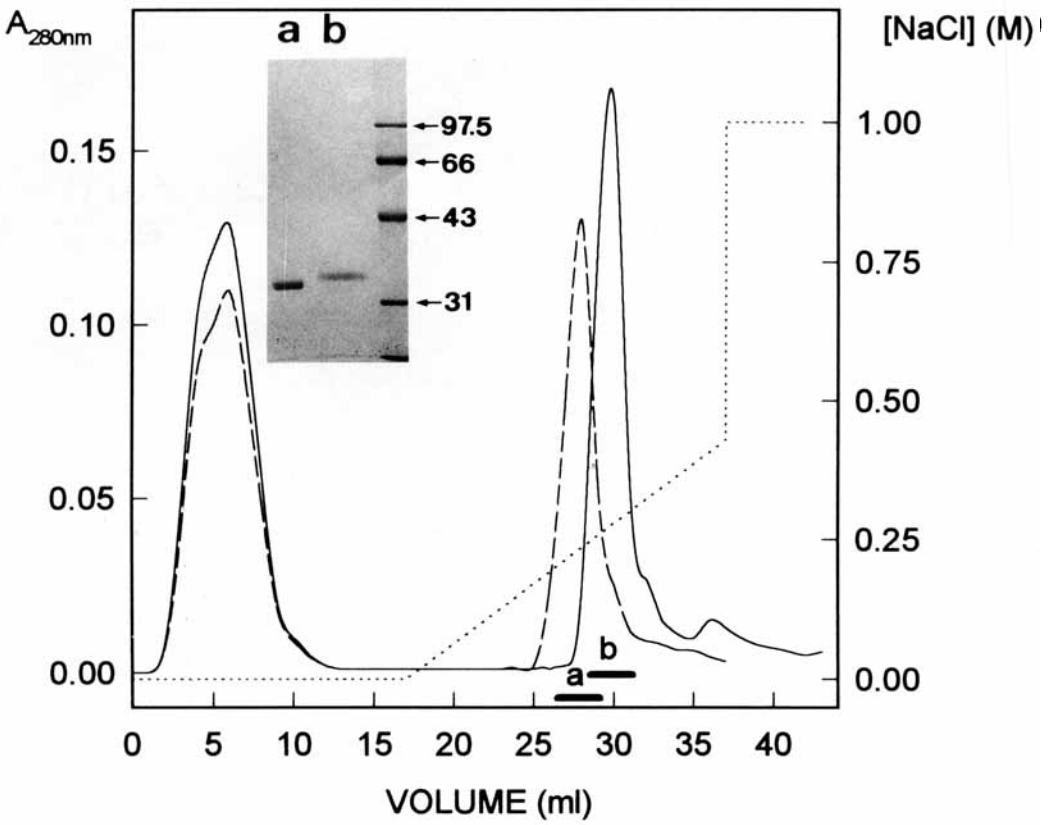

Fig. 3. Mono-S FPLC elution pattern of full-length and dnt–mutant chicken annexin V. Approximately 2–3 mg protein was injected on a Mono-S column with a flow rate of 1 ml/min and equilibrated in 20 mM sodium acetate, pH 5.6. Elution was performed with a linear 0–430 mM NaCl gradient. Fractions were monitored for absorbance at 280 nm. Full-length annexin V (solid line) elutes at 250 mM NaCl, whereas dnt–annexin V (dashed line) elutes at 200 mM. Fractions containing purified full-length (**b**) or dnt–mutant protein (**a**) were pooled, dialyzed against 50 mM Tris, pH 7.4, 50 mM NaCl, and stored at 4°C. The insert shows the results of gel electrophoresis (10% SDS–PAGE) of pool a and pool b after silver staining.

dicts that about 70% of the polypeptide chain is α-helical and 0–5% of it is folded in a β-sheet structure. These results are in good agreement with the crystallographic data obtained in the presence of Ca^{2+} [Bewley et al., 1993]. CD spectra repeated in the presence of 1 mM EGTA show almost no difference to those recorded in the presence of Ca^{2+}.

Possible conformational changes in the presence of Ca^{2+} were also analyzed by recording fluorescence emission spectra in the presence of 1 mM EGTA or 1 mM $CaCl_2$ (excitation wavelengths of 275 and 295 nm). Excitation at 275 nm results in an emission spectra that is due to the combined fluorescence of tryptophan 187 (W^{187}) and the nine tyrosine residues present in chicken annexin V. A maximum at 312 nm appears both in the presence of 1 mM EGTA or 1 mM $CaCl_2$; Ca^{2+} induces only a slight decrease in the fluorescence emission intensity (approx. 5%; Fig. 4D). More significant differences are induced by Ca^{2+} at an excitation wavelength of 295 nm (due only to W^{187}). A red shift from 325 to 328 nm is detected in the presence of Ca^{2+} parallel to an increase in the quantum yield of W^{187} (approx. 15.5%; Fig. 4D). These changes in the emission spectra must be interpreted in terms of a conformational change after binding of Ca^{2+}, which brings W^{187} to a slightly more polar environment. The increase in the quantum yield is probably due to an increase in the energy transfer from tyrosine residues located close to the tryptophan residue.

Binding to Phospholipids

Both the full-length recombinant and dnt–mutant annexin V cosediment with 2PS:PC (w/w) vesicles in the presence of Ca^{2+}, as we were able to purify both proteins exploiting this characteristic. To determine whether recombinant annexin V and the dnt-mutant had the same functional properties as cartilage annexin

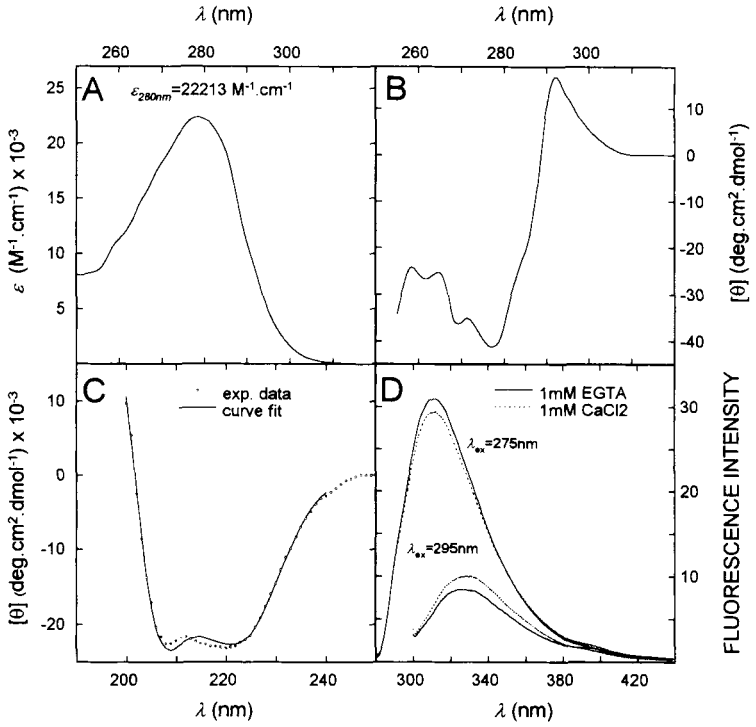

Fig. 4. Spectroscopical characterization of recombinant chicken annexin V. **A:** Absorbance spectrum of annexin V expressed as molar extinction coefficient. **B:** CD spectrum on the near UV region (250–320 nm) of annexin V dissolved in 50 mM Tris, pH 7.4, 50 mM NaCl. **C:** CD spectrum on the far UV region (200–250 nm) of annexin V. Open circles show the experimental spectrum; the solid line represents the curve fit according to the CCA algorithm described by Perczel et al. [1991]. No significant differences were detected when the spectrum was recorded in the presence of 1 mM Ca^{2+} or 1 mM EGTA. **D:** Fluorescence emission spectra of annexin V at 275 and 295 nm excitation wavelength, in the presence of 1 mM EGTA (solid line) or 1 mM Ca^{2+} (dashed line). Fluorescence intensity is expressed in arbitrary units.

V, Ca^{2+}-dependent binding to phospholipids was investigated in more detail.

Calcium affinities were analyzed by a Ca^{2+}-dependent liposome pelleting assay (Fig. 5). Half-maximal binding to PS liposomes is observed at a lower Ca^{2+} concentration for the dnt–mutant (55 μM) than for the full-length recombinant protein (77 μM). In both cases, binding to phospholipid vesicles occur in a highly cooperative manner with respect to Ca^{2+} concentration.

Binding of Annexin V to Collagen

Binding of recombinant annexin V to collagen was studied using an overlay assay [Kirsch and Pfäffle, 1992]. Collagen types I and II were isolated from chicken skin and sternal cartilage, respectively, and blotted onto nitrocellulose. After incubation of the nitrocellulose filters with annexin V in the presence of 5 mM EGTA (Fig. 6) or in the presence of 5 mM $CaCl_2$, almost no differences were found. Annexin V shows a high-affinity binding to native chicken type II collagen (Fig. 6B) and to bovine collagen type X (data not shown). It also binds to native collagen type I, but less efficiently (Fig. 6A). No binding to BSA was found. Thermal denaturation of collagen (30 min at 90°C) strongly decreases the interaction with both collagen types; the interaction is completely abolished when the collagen molecules are pepsin-digested at 4°C (Fig. 6A,B). Figure 6C,D shows the collagen binding activity of the dnt–annexin V mutant. No significant differences are observed when compared to the binding activity of the full-length protein. Binding is also Ca^{2+} independent and strongly dependent on the integrity of telopeptide extensions of collagen.

DISCUSSION

Up to now, only two annexins have been found to interact with collagen, annexin V [Mollenhauer and von der Mark, 1983; Mollenhauer et al., 1984; Pfäffle et al., 1988; Wu et al., 1991; Kirsch and Pfäffle, 1992], and annexin VI [Wu et al., 1991]. However, some reports suggest that also other annexins may interact with colla-

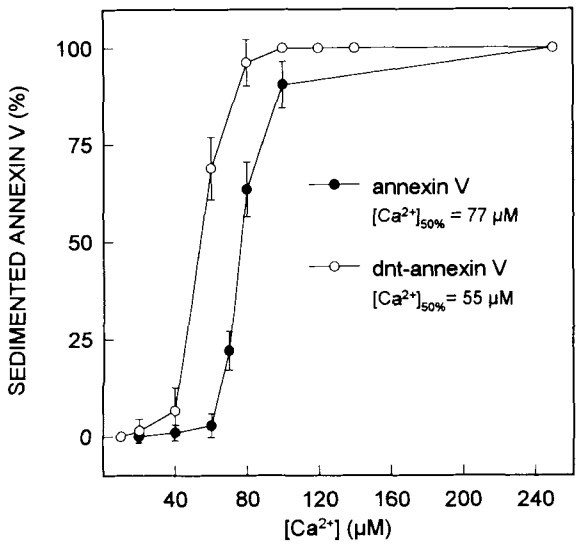

Fig. 5. Ca^{2+}-dependent phospholipid binding of full-length and dnt–mutant chicken annexin V. Proteins were assayed for binding to PS liposomes as described in Materials and Methods. Reactions were performed in buffer containing increasing amounts of Ca^{2+} and the fraction of bound (sedimented) and unbound (supernatant) protein was determined by 12% SDS–PAGE, followed by Coomassie Blue staining and densitometric analysis of the gels. Data are the calculated average of three independent experiments for each protein. Ca^{2+} concentrations at half-maximal PS binding is shown.

gen, as they are retained on a collagen–Sepharose column [Wirl and Pfäffle, 1988; Josic et al., 1990; Schwartz-Albiez et al., 1993]. All annexin protein cores are largely homologous, but the N-terminal tail is unique for each annexin; several investigators have suggested that the specific functions of annexins are located to the N-terminal domain. Annexin V contains the shortest N-terminus and the major differences appear in the first 10 residues. In order to study the collagen-binding activity of annexin V, we have cloned the full-length molecule, as well as a mutant lacking eight amino acid residues in the N-terminal tail that are unique for annexin V.

N-terminal sequencing and amino acid analysis confirmed the identity of the recombinant protein with chicken annexin V isolated from cartilage and the removal of the 8 amino acid residues in the truncated mutant. In both recombinant proteins, full-length and mutant, the processing of the initial Met residue gives rise to proteins with a free N-terminal amino group. By contrast, the N-terminus of chicken cartilage derived annexin V is blocked [Mollenhauer and von der Mark, 1983; Pfäffle et al., 1990]. The initiator methionine is probably cleaved, and the N-terminal alanine blocked by an N-acetyl group as reported for human and bovine brain annexin V [Funakoshi et al., 1987; Iwasaki et al., 1987; Learmoth et al., 1992]. Nevertheless, the specific functions of annexin V, such as the calcium-dependent phospholipid binding and the interaction with collagen are independent of whether the N-terminus is blocked. The presence of a blocked N-terminus, however, might be responsible for other properties of annexin V, such as stability, compartmentalization, or even secretion.

The high yield obtained in the purification of recombinant annexin V allowed a spectroscopical characterization. This analysis was carried out in order to evaluate possible changes in the secondary and tertiary structure of the protein after calcium binding. All spectra were recorded with protein purified by FPLC on a Mono-S column, resulting in preparations with negligible light scattering. Binding of Ca^{2+} to chicken annexin V does not induce significant changes in the absorbance spectrum nor in the CD spectrum in the far UV. The latter indicates that binding of Ca^{2+} does not modify the secondary structure of annexin V. In fact, binding of Ca^{2+} has been described to trigger only small conformational changes that affect mainly the loops between α-helices A and B in each of the Ca^{2+}-binding domains [Meers 1990; Concha et al., 1993; Meers and Mealy, 1993]. The annexin V four-repeat core domain is such a highly structured molecule that such small changes cannot be detected by circular dichroism. On the other hand, fluorescence emission spectra reveal a conformational change which affects the exposure to the solvent of W^{187} located in the endonexin fold in domain III. It has been suggested that chicken annexin V, in contrast to annexins I and II, does not bind Ca^{2+} on domain III, based on crystallographic data from protein crystals formed in the presence of Ca^{2+} [Bewley et al., 1993]. However, all four domains of rat annexin V bind calcium [Concha et al., 1993], and recently the X-ray analysis of human annexin V crystallized in the presence of high calcium revealed that domain III also binds Ca^{2+} [Sopkova et al., 1993]. Calcium binding in the third domain triggers key conformational changes that expose the single tryptophan residue to the solvent. Differences to annexin V molecules from other origins may, thus, be only due to differences in obtaining the crystals in the presence of Ca^{2+} [Huber et al., 1990a,b, 1992; Concha et al.,

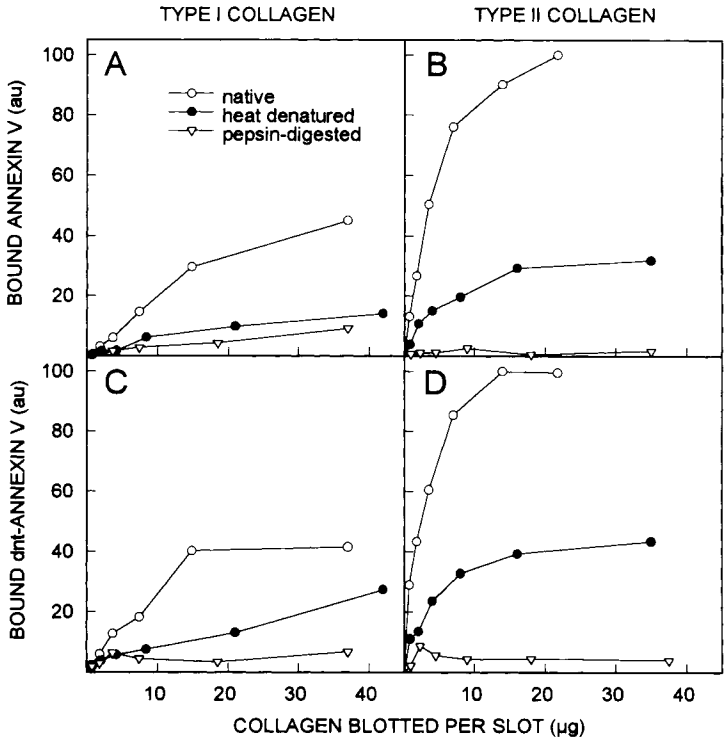

Fig. 6. Recombinant annexin V and dnt–annexin V binding to collagen. Increasing amounts of chicken collagen type I (**A,C**) and type II (**B,D**) were blotted onto nitrocellulose filters and incubated with purified recombinant full-length annexin V (**A,B**) or dnt–annexin V (**C,D**) at 7.5 μg/ml in 50 mM Tris, pH 7.4, 50 mM NaCl, containing 5 mM EGTA. No differences were detected when the incubation was performed in the presence of 5 mM $CaCl_2$ instead of EGTA. Collagen molecules were obtained by neutral salt extraction (native, ○) or by pepsin treatment (pepsin-digested, ▽). Neutral salt extracted collagens were heat-denatured (●) at 90°C for 30 min. Bound annexins were immunostained and the filters were analyzed by densitometry; data are expressed as integrated optical density for each band setting as 100 the value of maximum binding.

1993; Sopkova et al., 1993]. Thus, it is possible that in all annexin V molecules the tryptophan residue, which is buried in the protein core in the absence of Ca^{2+}, might be exposed to the solvent after Ca^{2+} binding to this domain as reported for rat annexin V. This could explain the red shift we have detected in the fluorescence emission spectrum of W^{187} in recombinant chicken annexin V, which is similar to that reported for human recombinant annexin V [Meers 1990; Meers and Mealy, 1993]. The increase in the fluorescence intensity of the tryptophan residue in the presence of 1 mM $CaCl_2$ may be explained by different mechanisms. It is possible that the conformational changes which expose W^{187} to the solvent also increase the energy transfer from tyrosines to the tryptophan residue. In fact, a parallel decrease in the tyrosine quantum yield is observed, which may account for the increase in the emission of the tryptophan residue. On the other hand, it may also be explained by a decrease in the internal quenching after calcium binding, as suggested [Meers, 1990].

One of the most important characteristics of annexins is their ability to interact with acidic phospholipids in the presence of Ca^{2+}. As a functional characterization, we have verified that recombinant annexin V and the dnt–mutant maintain the ability to interact with liposomes in a calcium-dependent manner. However, we analyzed in greater detail the calcium dependence of the interaction with PS liposomes to ascertain possible differences to the cartilage derived protein. Both recombinant proteins show a high cooperativity regarding the calcium binding, a necessary step required for binding to the liposomes. It has been proposed that the N-terminal tail plays a role in closing the structure by acting as a cinch holding together the first domain to the fourth domain [Huber et al., 1990a; Creutz, 1992]. Moreover, proteolytic cleavage of the annexin II N-terminal tail also reduces the calcium requirement for phospho-

lipid binding [Drust and Creutz, 1988; Ando et al., 1989]. Therefore, the differences detected between the two recombinant proteins, although not very large, may come from a more relaxed conformation in the annexin core of the dnt– mutant, which favors the interactions.

In previous studies, the affinity of annexin V isolated from cartilage to native collagen type II was shown by affinity chromatography after incorporation into liposomes [Mollenhauer and von der Mark, 1983] and by solid-phase overlay assay [Kirsch and Pfäffle, 1992]. These findings were confirmed by Wu et al. [1991]. Type X collagen also binds to annexin V. Using the recombinant full-length chick annexin V, we have confirmed these findings. However, the increased binding of annexin V to collagen type I in the absence of Ca^{2+} described in Kirsch and Pfäffle [1992] could not be confirmed. It is essential that the collagen molecules remain native and the telopeptides are retained. In our experience it is also very important that collagen solutions are freshly prepared before every binding experiment. Heat-denatured collagen type I or II binds poorly to annexin V, and pepsin-extracted collagens or native collagens treated with pepsin at 4°C (which totally or partially lack the telopeptide extensions) show almost no interaction with chick annexin V, indicating that the binding sites are localized in the telopeptide extensions of both types of collagen. This finding is consistent with rotary shadowing illustrations of collagen complexes with detergent-solubilized annexin V from chicken cartilage (anchorin CII), showing collagen molecules binding to annexin V only through their ends [Mollenhauer and von der Mark, 1983].

Our findings are not completely in accordance with the collagen binding data obtained with human annexin V (von der Mark et al., unpublished results). Human annexin V, which is 80% identical to chick annexin V, binds similarly to type I, II, and III collagen, in both the native or heat-denatured conformation of the collagen molecules. Also, pepsin-digested collagens bind to human annexin V. These data, however, were obtained using a different assay, based on an enzyme-linked immunosorbent assay (ELISA) test, and with different collagen concentrations and preparations.

Further studies with annexin V from various species, or with recombinant chimeric annexins, will have to be performed to consolidate the different collagen binding characteristics of chicken versus human annexin V. Among other annexins, only annexin VI was shown to bind to collagen type II and type X [Wu et al., 1991]. It is also interesting that the collagen binding activity of annexin V is Ca^{2+} and phospholipid independent, while almost all other activities of annexins require these cofactors.

For elucidation of the molecular mechanism of annexin V-collagen interactions it will be essential to identify the collagen binding sites in annexin V. Since there is a high degree of homology among tetrade repeats of different annexins, but almost no homology in length and sequence in the amino terminal ends, the possibility existed that the collagen binding site of annexin V was located in the N-terminal tail. Our studies with the mutant annexin V lacking 8 amino terminal residues, however, clearly demonstrate that the collagen binding sites must be located in the core repeats of annexin V. This is also supported by the fact that the amino ends of human and chick annexin V are identical, with the exception of three residues, while the binding characteristics of the two annexins to collagen seem to be rather different (see above). Additional studies using mutagenized and chimeric annexins will be required to identify their collagen binding sites.

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