The Roles of Annexins and Types II and X Collagen in Matrix Vesicle-mediated Mineralization of Growth Plate Cartilage*

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Annexins II, V, and VI are major components of matrix vesicles (MV), i.e. particles that have the critical role of initiating the mineralization process in skeletal tissues. Furthermore, types II and X collagen are associated with MV, and these interactions mediated by annexin V stimulate Ca\(^{2+}\) uptake and mineralization of MV. However, the exact roles of annexins II, V, and VI and the interaction between annexin V and types II and X collagen in MV function and initiation of mineralization are not well understood. In this study, we demonstrate that annexins II, V, or VI mediate Ca\(^{2+}\) influx into phosphatidylyserine (PS)-enriched liposomes, liposomes containing lipids extracted from authentic MV, and intact authentic MV. The annexin Ca\(^{2+}\) channel blocker, K-201, not only inhibited Ca\(^{2+}\) influx into fura-2-loaded PS-enriched liposomes mediated by annexins II, V, or VI, but also inhibited Ca\(^{2+}\) uptake by authentic MV. Types II and X collagen only bound to liposomes in the presence of annexin V but not in the presence of annexin II or VI. Binding of these collagens to annexin V stimulates its Ca\(^{2+}\) channel activities, leading to an increased Ca\(^{2+}\) influx into the liposomes. These findings indicate that the formation of annexin II, V, and VI Ca\(^{2+}\) channels in MV together with stimulation of annexin V channel activity by collagen (types II and X) binding can explain how MV are able to rapidly take up Ca\(^{2+}\) and initiate the formation of the first crystal phase.

Annexins are a family of proteins that has in common the ability to bind to acidic phospholipids in the presence of Ca\(^{2+}\) (1, 2). The family consists of at least 12 members, and three of them, annexins II, V, and VI, are highly expressed in calcifying cartilage and bone (3, 4). Annexin II and V each contain four to eight repeats form the conserved core region, which is responsible for the Ca\(^{2+}\)-dependent binding of the proteins to phospholipids. In contrast, the N-terminal regions of the annexins are highly variable and may contribute to the specific functions of the various annexins (1, 2).

Annexins II, V, and VI are major components of matrix vesicles (MV), which are particles that, after being released from the plasma membrane of hypertrophic chondrocytes or osteoblasts, have the critical role of initiating the mineralization process in cartilage and perhaps in bone (3, 5). Three independent lines of evidence indicate that annexins II, V, and VI exhibit distinct Ca\(^{2+}\) ion channel properties. First, when inserted into artificial phosphatidyserine bilayers they form voltage-dependent Ca\(^{2+}\) ion channels (6-8). Second, the crystal structures of these annexins are largely a-helical with parallel barrels of a-helical domains forming a hydrophilic, charged pore through the center of the protein (6, 8, 9). Third, annexins II, V, and VI are able to mediate Ca\(^{2+}\) influx into artificial liposomes (10;11). It was shown that annexin-mediated Ca\(^{2+}\) influx into liposomes is rapid during the first 20 min and then reaches a plateau after 20 min (10, 11).

The initial phase of MV-mediated mineralization is characterized by the uptake of mineral ions by these particles and the formation and growth of the first mineral phase inside the vesicles (5). Because MV are enclosed by a membrane, channel proteins are required to mediate the influx of mineral ions into these particles. Previous findings from our and other laboratories, showed that chymotrypsin treatment, which removes most of the annexins from MV, and zinc treatment, which inhibits annexin-mediated Ca\(^{2+}\) influx into phosphatidyserine (PS)-enriched liposomes, diminished MV Ca\(^{2+}\) uptake (12-15), suggesting that annexins II, V, and VI serve as ion channels in MV, enabling Ca\(^{2+}\) loading of the vesicles during the initial phase of mineralization.

Previous studies have revealed that collagen types II and X are associated with the outer surface of MV (16). We demonstrated that the selective removal of these collagens from the MV surface drastically reduced the ability of vesicles to take up Ca\(^{2+}\). The addition of purified type II or X collagen to these collagen-depleted MV restored their Ca\(^{2+}\) uptake ability to levels similar to MV containing these collagens (12). Types II and X collagen were shown to bind directly to annexin V (17, 18). Thus, it is possible that annexins II, V, and VI form Ca\(^{2+}\) channels in MV. In addition, binding of types II and X collagen to annexin V would anchor MV to the extracellular matrix, and it might further activate the annexin V channel properties. This would explain the rapid influx of Ca\(^{2+}\) into MV that is required for the formation and growth of the first crystal phase within the vesicles. To test this hypothesis, we isolated MV from growth plate cartilage and measured Ca\(^{2+}\) uptake by these particles in the absence or presence of K-201 (JTV519), a specific annexin channel inhibitor (19, 20). Furthermore, we

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The abbreviations used are: MV, matrix vesicles; GST, glutathione S-transferase; PS, phosphatidyserine; PE, phosphatidylethanolamine; SCL, synthetic cartilage lymph; TES, 2-(2-hydroxy-1,1-bis(hydroxy-methyl)ethyl)amino)ethanesulfonic acid.

1 The abbreviations used are: MV, matrix vesicles; GST, glutathione S-transferase; PS, phosphatidyserine; PE, phosphatidylethanolamine; SCL, synthetic cartilage lymph; TES, 2-(2-hydroxy-1,1-bis(hydroxy-methyl)ethyl)amino)ethanesulfonic acid.
tested whether annexin II, V, and VI are able to mediate Ca\(^{2+}\) influx into MV and whether binding of type II or X collagen to annexin V is able to stimulate its Ca\(^{2+}\) channel activity by measuring Ca\(^{2+}\) influx in fura-2 loaded liposomes comprised of extracted MV lipids, or PS and phosphatidylethanolamine (PE) in a molar ratio of 9:1, in the absence or presence of annexin II, V, or VI, or complexes of annexin V and type II or X collagen.

**EXPERIMENTAL PROCEDURES**

**Isolation of Matrix Vesicles**—MV were isolated from growth plate cartilage of 6- to 8-week-old broiler strain chickens and from cultures of non-mineralizing hypertrophic and mineralizing post-hypertrophic chondrocytes as described previously (12). Briefly, tissues were digested in 0.1% trypsin (type III, Sigma, St. Louis, MO) at 37 °C for 30 min followed by digestion with collagenase (200 units/g tissue, type IA, Sigma) at 37 °C for 3 h in synthetic cartilage lymph (SCL). Chondrocyte cultures were first incubated with 0.1% trypsin at 37 °C for 15 min followed by incubation with crude collagenase (500 units/ml) at 37 °C for 3 h in SCL. SCL contained 2 mM Ca\(^{2+}\) and 1.42 mM Pi in addition to 104.5 mM Na\(^{+}\), 133.5 mM Cl\(^{-}\), 63.5 mM sucrose, 16.5 mM 2-(2-hydroxy-1,1,1-trifluoroethyl)ethanesulfonic acid (TES), 12.7 mM K\(^{+}\), 5.55 mM D-glucose, 1.83 mM HCO\(_3\)^{-}, 0.57 mM Mg\(^{2+}\), and 0.57 mM SO\(_4\)^{2-}. To harvest MV, the partially digested tissue was vortexed and the suspension was subjected to differential centrifugation at 13,000 \(\times g\) for 20 min and 100,000 \(\times g\) for 1 h. Protein concentration of the vesicles was determined using the BCA assay (Pierce, Rockford, IL), and alkaline phosphatase activity was assayed as described previously (12).

**Ca\(^{2+}\) Uptake by MV Isolated from Tissue**—Ca\(^{2+}\) uptake was assayed by incubating MV aliquots (100 \(\mu\)g of protein) in 1 ml of Ca\(^{2+}\)-labeled SCL at 37 °C under mild shaking. After 24-h incubation, 100-\(\mu\)l aliquots of the SCL incubations were sampled by microfiltration through Millipore HA filters (0.45-\(\mu\)m pore size). After washing the filters twice, radioactivity associated with the filters was determined by scintillation counting. Ca\(^{2+}\) uptake was measured in the presence of various concentrations of K-201 (JTV519). This compound was generously provided by Drs. N. Kaneke and T. Tanaka.

**Ca\(^{2+}\) Uptake by MV Isolated from Chondrocyte Cultures**—After 24-h incubation of MV aliquots (100 \(\mu\)g of protein) in 1 ml of SCL in the absence or presence of 200 nm annexin II, V, or VI at 37 °C, MV were pelleted, washed twice in 150 mM NaCl, 10 mM TES (pH 7.4), and 200 mM EDTA (buffer 1), and resuspended in 1 ml of buffer 1 containing 1 \(\mu\)M fura-2. MV suspensions were then incubated with 1% Triton X-100 to burst matrix vesicles and to release intraluminal Ca\(^{2+}\) (blast method). Changes in the fluorescence ratio, 340:380 nm, were measured.

**Isolation of Lipids from Authentic MV**—To isolate lipids from authentic MV, lyophilized MV pellets isolated from growth plate cartilage were rehydrated with a chloroform/methanol mixture at a molar ratio of 9:1 or lipids extracted from MV (see above). After the lipids were dried down from chloroform, the lipid film was rehydrated and dried down from chloroform again, a dehydration/rehydration method and were loaded with fura-2 as described previously (11). Briefly, liposomes were suspended in 150 mM NaCl, 10 mM TES, pH 7.4, and 200 \(\mu\)M EDTA, and Ca\(^{2+}\) was added to a final concentration of 400 \(\mu\)M. The suspensions were warmed to 37 °C. Ca\(^{2+}\) influx was initiated by adding annexin II, V, or VI (200 nm) or complexes containing annexin V and collagen types II, X, or IX. In addition, Ca\(^{2+}\) influx was measured in the presence of annexin II, V, or VI by various concentrations of the fluorescence ratio at the two excitation wavelengths of 340 and 380 nm. The emission wavelength was kept constant at 510 nm as a function of time. The excitation wavelength of the Ca\(^{2+}\)-bound form of fura-2 was 340 nm, whereas the excitation wavelength of the Ca\(^{2+}\)-free form of fura-2 was 380 nm.

**Annexin V/Collagen/Liposome Binding Studies**—To test the binding of type II or X collagen to liposomes in the absence or presence of annexin II, V, or VI, a 200 nM concentration of these annexins was first incubated with liposomes in the presence of 400 \(\mu\)M Ca\(^{2+}\). Liposomes and liposomes without annexin were then incubated with type II or X collagen (10 \(\mu\)g) for 1 h at room temperature. Liposomes were quantitatively pelleted by centrifugation at 200,000 \(\times g\) for 15 min. The pellets were then washed twice and resuspended in 20 \(\mu\)l of TES buffer. Aliquots of these suspensions (5 \(\mu\)l) were dotted onto nitrocellulose membranes. After blocking with low-fat milk protein, the membranes were immunostained with antibodies specific for annexin II, annexin V, annexin VI, collagen type II, or collagen type X. The optical density of the color reaction was determined using a densitometer. To determine the amount of annexin II, V, or VI bound to liposomes, various defined concentrations of purified recombinant annexin II, V, or VI were incubated with MV. Protein binding was then assayed by putting the tissue samples on ice.

**Preparation of Liposomes**—Phospholipids were obtained from Avanti Polar Lipids (Birmingham, AL). Fura-2 was obtained from Molecular Probes (Eugene, OR). Large thin-walled liposomes were prepared using a dehydration/rehydration method and were loaded with fura-2 as described previously (11, 21). The liposomes contained either PS and PE in a molar ratio of 9:1 or lipids extracted from MV (see above). After the lipids were dried down from chloroform, the lipid film was rehydrated for 30 min in a stream of nitrogen saturated with water. The rehydrated phospholipids were overlaid with a solution containing 150 mM NaCl, 10 mM TES, pH 7.4, and 200 \(\mu\)M EDTA, and 100 \(\mu\)M fura-2. After incubation at 37 °C for 2 h, the suspension was washed four times and used for Ca\(^{2+}\) influx measurements within the next 24 h. In addition, aliquots of the liposome suspensions were extruded through membranes with various pore sizes (20, 2, and 0.2 \(\mu\)m) using the Extruder (Lipex Biomembranes).

**Measurement of Ca\(^{2+}\) Influx into Fura-2-Loaded Liposomes**—Ca\(^{2+}\) influx into fura-2-loaded liposomes was measured in the absence or presence of annexin II, annexin V, or annexin VI, or complexes of annexin V/type II collagen, annexin V/type X collagen, annexin V/type IX collagen in a fluorescence cuvette with a 1-cm path length using a method previously described (11). Briefly, liposomes were resuspended in 150 mM NaCl, 10 mM TES, pH 7.4, and 200 \(\mu\)M EDTA, and Ca\(^{2+}\) was added to a final concentration of 400 \(\mu\)M. The suspensions were warmed to 37 °C. Ca\(^{2+}\) influx was initiated by adding annexin II, V, or

**RESULTS**

Ultrastuctural analysis revealed that MV freshly isolated from growth plate cartilage of 6- to 8-week-old chickens were, in general, round to oval with diameters ranging from 100 to 35578 nm (Fig. 1A). Immunoblot analysis demonstrated that these vesicle preparations contained annexin II, V, and VI and that types II and X collagen were associated with the vesicles (Fig. 1B). These findings are in agreement with previous studies (12, 16, 30). Furthermore, these vesicle fractions contained a high content of alkaline phosphatase activity (data not shown) and showed significant Ca\(^{2+}\) uptake when incubated in SCL in vitro (Fig. 3).
To address the question of whether MV annexins mediate Ca\(^{2+}\) influx into these particles, we first determined whether annexin II, V, or VI mediates Ca\(^{2+}\) influx into fura-2-loaded PS-enriched liposomes. AnnexinII, V, or VI was able to mediate Ca\(^{2+}\) influx into liposomes containing PS and PE in a molar ratio of 9:1 (Table I and Fig. 2). These annexins not only mediated Ca\(^{2+}\) influx into large liposomes but also into liposomes, which were extruded through various pore size membranes, including liposomes extruded through a 0.2-μm pore size membrane (Table I). These unilamellar liposomes were similar in size to authentic matrix vesicles isolated from growth plate cartilage. There was a ~2-fold difference in the rate of annexin-mediated Ca\(^{2+}\) influx between large-sized versus small-sized liposomes. It is likely that this difference reflects the amount of annexin molecules bound per unit amount of phospholipid surface area or curvature. Based on these findings, that the annexins not only mediate Ca\(^{2+}\) influx into mixed-sized, multi-, and unilamellar liposomes but also into unilamellar liposomes with similar size as MV, and evidence presented below, that annexin II, V, or VI mediates Ca\(^{2+}\) influx into intact MV, it is reasonable to assume that the mixed-sized, multi-, and unilamellar liposomes provide an accurate and simple model to study annexin Ca\(^{2+}\) channel formation and activities in MV. Thus, mixed-sized, multi- and unilamellar liposomes were used in the subsequent experiments.

The 1,4-benzothiazepine derivative K-201, a specific annexin Ca\(^{2+}\) channel blocker (19, 20, 31), inhibited Ca\(^{2+}\) influx into liposomes mediated by annexin II, V, or VI in a dose-dependent manner (Fig. 2). Buffer containing only K-201 had no effect on Ca\(^{2+}\) flux across the membrane or leakage of fura-2 from the liposomes (Fig. 2). Interestingly, K-201 also inhibited Ca\(^{2+}\) uptake by authentic MV isolated from growth plate cartilage in a dose-dependent manner (Fig. 3), revealing that annexin II, V, and VI mediate Ca\(^{2+}\) influx into authentic MV.

To further test whether the lipid composition of the MV membrane allows annexins to form channels and mediate Ca\(^{2+}\) influx, we extracted lipids from MV using chloroform/methanol in a ratio of 2:1. The extracted lipids were then reconstituted into liposomes and loaded with fura-2. Table II shows that annexin II, V, or VI mediated Ca\(^{2+}\) influx into liposomes containing lipids extracted from authentic MV. The rate of Ca\(^{2+}\) influx mediated by these three annexins was similar (Table II). The rate of Ca\(^{2+}\) influx mediated by annexin II, V, or VI into liposomes containing lipids extracted from authentic MV (340:380 nm ratio of ~0.69, see Table II) was slightly higher than the rate of influx mediated by these annexins into liposomes containing PS and PE in a molar ratio of 9:1 (340:380 nm ratio of ~0.55, see Table I), suggesting that the MV membrane contains, besides PS, other lipids that might influence annexin binding and/or Ca\(^{2+}\) channel formation.

We next determined whether exogenous annexin II, V, and VI are able to mediate Ca\(^{2+}\) influx into intact vesicles isolated from non-mineralizing hypertrophic chondrocytes. These vesicles did not contain annexins II, V, and VI (4; see also Fig. 4A) and were not able to take up Ca\(^{2+}\) and to mineralize (4) (Fig. 4B, bar b). In contrast, MV isolated from mineralizing post-hypertrophic chondrocytes contained annexins II, V, and VI (Fig. 4A), and they took up significant amounts of Ca\(^{2+}\) when incubated in SCL for 24 h (Fig. 4B, bar a). The addition of annexin II, V, or VI to MV isolated from non-mineralizing hypertrophic chondrocytes led to influx of Ca\(^{2+}\) into these particles comparable to influx of Ca\(^{2+}\) into annexin-containing MV isolated from mineralizing post-hypertrophic chondrocytes (Fig. 4B, bars c–e). Thus, annexin II, V, and VI were not only able to mediate Ca\(^{2+}\) influx into liposomes containing PS and PE or lipids from authentic MV but also in intact authentic MV.

Previously, we and others have demonstrated that types II and X collagen bind to annexin V (16–18). In addition, removal of types II and X collagen from MV led to a drastic decrease in Ca\(^{2+}\) uptake by these particles (12). Thus, it is possible that binding of type II or X collagen to annexin V might stimulate its channel activities. To test this hypothesis, we first investigated whether type II or X collagen can bind to liposomes in the presence of annexin II, V, or VI. Liposomes containing PS and PE in a molar ratio of 9:1 were first incubated with 5 μg of annexin II, V, or VI in the presence of Ca\(^{2+}\), to allow the annexins to bind to the liposomes. Then, these annexin-containing liposomes were incubated with type II or X collagen. After washing the liposomes, aliquots of the liposome fractions were dotted onto nitrocellulose membranes, which were immunostained with antibodies specific for the annexins, type II and X collagen. All three annexins (II, V, and VI) bound to the liposomes in presence of Ca\(^{2+}\) in equal amounts. Approximately 0.7 μg of annexin II, 0.7 μg of annexin V, and 0.5 μg of annexin VI bound to liposomes containing 300 μg of total lipids. Type II or X collagen bound to liposomes that contained annexin V (Fig. 5, bars b), but not to liposomes that contained annexin II (Fig. 5, bars a) or annexin VI (Fig. 5, bars c).

Table I

| Annexin | ΔFluorescence ratio (340:380 nm) |
|---------|---------------------------------|
| II      | a 0.557 ± 0.023 b 0.538 ± 0.041 c 0.291 ± 0.014 d 0.210 ± 0.032 |
| V       | a 0.550 ± 0.036 b 0.601 ± 0.039 c 0.224 ± 0.032 d 0.207 ± 0.018 |
| VI      | a 0.541 ± 0.019 b 0.517 ± 0.028 c 0.295 ± 0.017 d 0.253 ± 0.027 |

FIG. 1. Ultrastructural and immunoblot analysis of MV. MV were isolated from growth plate cartilage of 6-day- to 8-week-old chickens as described under “Experimental Procedures.” A, electron micrograph of MV. Bar, 300 nm. B, aliquots of the MV fractions were analyzed by SDS-polyacrylamide gel electrophoresis and transferred electrophoretically to nitrocellulose membranes. Membranes were immunostained with antibodies specific for annexin II (AnII), V (AnV), and VI (AnVI) and types II (α1(II)) and X collagen (α1(X)).
or X collagen were not able to bind to liposomes in the absence of annexin V (Fig. 5, bars d).

Next, we determined whether binding of type II or X collagen to annexin V modulates its Ca\textsuperscript{2+} channel activities. Ca\textsuperscript{2+} influx into fura-2-loaded liposomes was measured in the presence of annexin V or complexes of annexin V and type II or X collagen. A significantly increased Ca\textsuperscript{2+} influx into liposomes was measured in the presence of complexes containing annexin V and type II (Fig. 6, bar b) or X collagen (bar e) compared with annexin V alone (bar a). Pepsin-treated type II (bar d) or X collagen (bar f) was less effective in stimulating channel activities than the non-pepsin-treated collagens containing telopeptide and globular regions. Denatured type II (bar d) or X collagen (bar g) did not stimulate annexin V-mediated Ca\textsuperscript{2+} channel activities.

Type IX collagen, which did not bind to annexin V (data not shown), was not able to stimulate its Ca\textsuperscript{2+} channel activities (bar h). Type II or X collagen alone did not mediate significant Ca\textsuperscript{2+} influx into liposomes (bars i and j).

**DISCUSSION**

Annexins II, V, and VI Mediate Ca\textsuperscript{2+} Influx into MV—Previous studies, including findings from our laboratories, have clearly demonstrated that annexins II, V, and VI form Ca\textsuperscript{2+} channels in planar lipid bilayers and artificial liposomes leading to Ca\textsuperscript{2+} influx into these particles (6–8, 10, 11, 32). In addition, a previous report showed the similarity in Ca\textsuperscript{2+} channel activity of annexin V and MV in planar lipid bilayers (14). These findings suggest that annexins II, V, and VI form channels in MV and mediate rapid Ca\textsuperscript{2+} influx into these particles (6–8, 14). In this study we provide several lines of direct evidence for this possibility. First, annexin II, V, and VI medi-
annexin V/type IX collagen (values are means ± S.E.) and mineralization of authentic MV isolated from growth plate cartilage, clearly establishing the role of these three annexins in mediating Ca\(^{2+}\) loading of MV.

MV-mediated mineralization of cartilage is restricted to a few cells layers close to the chondro-osseous border. Thus, cartilage mineralization is, and must be, under cellular control. Only post-hypertrophic chondrocytes release mineralization-competent MV, which contain annexin II, V, and VI and are able to initiate matrix mineralization. On the other hand, hypertrophic chondrocytes release vesicles that do not contain these annexins and cannot mineralize (4). However, the addition of exogenous annexin II, V, or VI to these non-mineralizing vesicles leads to Ca\(^{2+}\) influx into these particles, demonstrating that channel activities of annexins II, V, and VI enable MV to take up Ca\(^{2+}\). Thus, because of the absence of annexins, vesicles from non-mineralizing chondrocytes are not able to take up Ca\(^{2+}\) and to form the first intraluminal crystal phase. Most of the Ca\(^{2+}\) inside mineralization-competent vesicles is in a bound form (initial crystal phase) (33), thus permitting perpetual Ca\(^{2+}\) influx into the vesicles mediated by annexin II, V, and VI.

The annexins are cytosolic proteins that, in the presence of Ca\(^{2+}\), bind to acidic phospholipids. However, as we have recently demonstrated, binding of annexins to a membrane is not sufficient for channel formation, but a specialized lipid composition is required for annexin II, V, and VI to form Ca\(^{2+}\) channels (11). As shown in this study, the lipids extracted from MV provide an environment in which these annexins are able to form channels. By definition, a channel must span the membrane to allow flux of ions through a membrane. Annexin II and V monomers, however, are too small to span the membrane lipid bilayer. A recent crystal structure analysis of an annexin XII hexamer has shown not only that the spatial dimension of the hexamer is sufficient to span the membrane but that it also has a central pore lined with charged residues (34). On the basis of this structure, the authors proposed that an annexin V hexamer would also have a central pore lined with negatively charged residues (34). We have previously demonstrated that annexin II and V form hexameric structures in PS-rich liposomes and MV (11, 35). Thus, it is plausible that multimeric annexin units are needed to form functional ion channels. In addition, although each annexin by itself is capable of mediating Ca\(^{2+}\) influx into liposomes containing annexin V, whereas no significant binding was observed to liposomes containing annexin II or VI or to annexin-free liposomes.

**Fig. 5. Binding of type II or X collagen to liposomes in the presence of annexin II, V, or VI.** Liposomes containing PS and PE in a molar ratio of 9:1 were first incubated with annexin II, V, or VI in the presence of Ca\(^{2+}\) followed by incubation with type II or X collagen. In addition, liposomes were incubated with type II or X collagen only. Aliquots of the liposome fractions were dotted onto nitrocellulose membranes and immunostained with antibodies specific for type II or X collagen. The intensity of the bands was analyzed by densitometry. The optical density obtained for staining of annexin V-containing liposomes incubated with type II or X collagen followed by antibodies for type II collagen or type X collagen was set as 1. Data were obtained from five different experiments; values are means ± S.E. a, liposomes containing annexin II incubated with type II or X collagen; b, liposomes containing annexin V incubated with type II or X collagen; c, liposomes containing annexin VI incubated with type II or X collagen; d, liposomes incubated with type II or X collagen. Note that type II or X collagen bound to liposomes containing annexin V, whereas no significant binding was observed to liposomes containing annexin II or VI or to annexin-free liposomes.

**Fig. 6. Ca\(^{2+}\) influx into fura-2-loaded liposomes in the presence of annexin V or complexes of annexin V and type II or X collagen.** Ca\(^{2+}\) influx into fura-2-loaded liposomes containing PS and PE in a molar ratio of 9:1 was measured in the presence of annexin V (a), or complexes of annexin V/native type II collagen (b), annexin V/pepsin-treated type II collagen (c), annexin V/denatured type II collagen (d), annexin V/native type X collagen (e), annexin V/pepsin-treated type X collagen (f), annexin V/denatured type X collagen (g), annexin V/native type IX collagen (h), native type II collagen (i), or native type X collagen (j). Data were obtained from five different experiments; values are means ± S.E. Note the increased influx of Ca\(^{2+}\) into liposomes in the presence of annexin V/native type II or X collagen complexes compared with annexin V alone.
selective removal of surface-attached types II and X collagen from MV leads to a reduction in Ca\(^{2+}\) uptake by these particles, rendering the possibility that binding of type II and X collagen activates the Ca\(^{2+}\) channel activities of annexin V (12). In this study, we show direct evidence that binding of type II or X collagen to annexin V stimulates its Ca\(^{2+}\) channel activities, leading to an increased Ca\(^{2+}\) influx into liposomes. Pepsin-treated type II or X collagen is less effective in stimulating annexin V channel activities, which is in agreement with our previous findings that pepsin-treated type II or X collagen is less effective in binding to annexin V and stimulating Ca\(^{2+}\) uptake by MV (12, 18). Thus, the telopeptide regions of type II collagen and the globular domains of type X collagen seem to be required for optimal binding and stimulation of annexin V channel activities.

As shown in this study, type II or X collagen stimulates annexin V-mediated Ca\(^{2+}\) influx into liposomes by 2- to 3-fold. We have previously shown that the selective removal of types II and X collagen from MV reduced Ca\(^{2+}\) uptake by these particles from 30–35% to 5–7% of total \(45^{\text{Ca}}\) in the incubation solution (12). These findings indicate that the interactions between types II and X collagen and annexin V play a crucial role for Ca\(^{2+}\) loading and the formation of the first crystal phase inside MV and that the disruption of these interactions has severe consequences on mineralization of MV. In addition, these interactions may function to anchor MV to the extracellular matrix and might provide a bridge to facilitate the transfer of mineral from MV to extracellular matrix macromolecules.

Based on its restricted localization in hypertrophic and mineralizing growth plate cartilage, type X collagen is thought to play a role in the mineralization process. However, its exact functions in this process remain controversial. Two studies showed no association between type X collagen and focal calcification sites (36, 37). Furthermore, previous findings revealed that the non-helical domain of type X collagen must be removed to facilitate cell-mediated mineralization of the egg shell membrane, suggesting that type X collagen functions as an inhibitor of mineralization (38). In contrast, based on the findings that type X collagen expression precedes mineral formation (39), and its capacity to bind Ca\(^{2+}\) and MV (16, 40), it was proposed that type X collagen might prepare the matrix for subsequent mineralization. In addition, calcified cartilage of transgenic mice showed no association between type X collagen and focal calcification (36, 37).

In conclusion, our study provides new and important insights into the regulatory mechanisms involved in the initiation of mineralization of tissues during normal development and under pathological conditions and suggests new strategies for the prevention of pathological mineralization.

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MV are not only released from mineralizing post-hypertrophic chondrocytes, but also from osteoblasts and odontoblasts. We have isolated MV from fetal calvarial bone. These vesicle fractions also contained annexin II, V, and VI, and interestingly, types II and X collagen, which is in agreement with our previous study showing the transient expression of types II and X collagen in fetal calvarial bone (42). In addition, we and others have shown the presence of mineralized areas, MV, and the expression of annexin II and V and types II and X collagen in the upper zone of osteoarthritic cartilage (43–46). In that case, mineralization of articular cartilage is associated with this tissue’s progressive destruction. Matrix metalloproteinases, such as collagenases, are activated during cartilage destruction in osteoarthritis (47). Thus, it would be interesting in future studies to determine how these metalloproteinases affect annexin V- and types II and X collagen-stimulated Ca\(^{2+}\) influx into MV. MV were not only found in skeletal tissues, but also in mineralizing arteriosclerotic plaques of arteries (48, 49). As shown in this study, the MV annexins alone are sufficient to mediate Ca\(^{2+}\) influx into MV. In tissues, where types II and X collagen are present, Ca\(^{2+}\) uptake is greatly enhanced, probably leading to a more rapid initiation and progression of mineralization than in the absence of these collagenases.

In conclusion, our study provides new and important insights into the regulatory mechanisms involved in the initiation of mineralization of tissues during normal development and under pathological conditions and suggests new strategies for the prevention of pathological mineralization.

2 T. Kirsch, G. Harrison, E. E. Golub, and H.-D. Nah, unpublished observations.
