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Review

The roles of annexins and alkaline phosphatase in mineralization process✪

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In this review the roles of specific proteins during the first step of mineralization and nucleation are discussed. Mineralization is initiated inside the extracellular organelles–matrix vesicles (MVs). MVs, containing relatively high concentrations of Ca^{2+} and inorganic phosphate (P_i), create an optimal environment to induce the formation of hydroxyapatite (HA). Special attention is given to two families of proteins present in MVs, annexins (AnxAs) and tissue-nonspecific alkaline phosphatases (TNAPs). Both families participate in the formation of HA crystals. AnxAs are Ca^{2+}- and lipid-binding proteins, which are involved in Ca^{2+} homeostasis in bone cells and in extracellular MVs. AnxAs form calcium ion channels within the membrane of MVs. Although the mechanisms of ion channel formation by AnxAs are not well under-

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Abbreviations: 1,25-$(OH)_2$-D_3, 1α,25-dihydroxyvitamin D_3; AnxA, vertebrate annexin; ATPase, ATP hydrolase; ATRA, all-*trans* retinoic acid; BMP-2, bone morphogenetic protein 2; GPI, glycosylphosphatidylinositol; HA, hydroxyapatite; MV, matrix vesicle; NTP, nucleoside triphosphate; PE, phosphatidylethanolamine; PC-1, plasma cell membrane glycoprotein-1 (NTP pyrophosphatase phosphodiesterase isoenzyme); PS, phosphatidylserine; RAR/RXR, receptors of retinoic acid and its derivatives; T3, 3,5,3′-triiodo-L-thyronine; TGFβ, transforming growth factor β; TNAP, tissue-nonspecific alkaline phosphatase.

stood, evidence is provided that acidic pH or GTP contribute to this process. Furthermore, low molecular mass ligands, as vitamin A derivatives, can modulate the activity of MVs by interacting with AnxAs and affecting their expression. AnxAs and other anionic proteins are also involved in the crystal nucleation. The second family of proteins, TNAPs, is associated with P_i homeostasis, and can hydrolyse a variety of phosphate compounds. ATP is released in the extracellular matrix, where it can be hydrolyzed by TNAPs, ATP hydrolases and nucleoside triphosphate (NTP) pyrophosphohydrolases. However, TNAP is probably not responsible for ATP-dependent Ca^{2+}/phosphate complex formation. It can hydrolyse pyrophosphate (PP_i), a known inhibitor of HA formation and a byproduct of NTP pyrophosphohydrolases. In this respect, antagonistic activities of TNAPs and NTP pyrophosphohydrolases can regulate the mineralization process.

SKELETAL TISSUES

The two major skeletal tissues, cartilage and bones, are structurally and functionally different (Heinegard & Oldberg, 1989). Cartilage is highly hydrated and, except at the growth plates of long bones, rarely mineralizes, resulting in a permeable matrix of gel-like consistency. On the other hand, bone matrix routinely mineralizes to form a rigid impermeable matrix (Marks & Popoff, 1988). Proteoglycan and type II collagen are major matrix components in cartilage, while type I collagen is the major part of bone matrix (Marks & Popoff, 1988). In both cartilage and bone, cellular activities include matrix formation, mineralization and resorption.

In each tissue, different cell types (Fig. 1) perform distinct tasks, which sometimes overlap each other. Bone matrix is formed and mineralized by osteoblasts and resorbed by osteoclasts (Fig. 1A). Osteocytes participate in extracellular exchanges between different components of osseous tissue. Osteocytes are also involved in the mechanotransduction (Marks & Popoff, 1988). In cartilage, matrix formation results from the activity of chondrocytes (Marks & Popoff, 1988). Chondrocytes express hypertrophic and non-hypertrophic phenotypes (Fig. 1B). Hypertrophic chondrocytes are characteristic for developing bones and for so-called growth plate. Non-hypertrophic chondrocytes are also found in the growth plate and may participate in the formation of articular cartilage (Fig. 1C) (Poole, 2001). Growth plate chondrocytes undergo several series of differentiation events, including proliferation and hypertrophy. All these events are required for bone formation during endochondral ossification. Chondrocyte hypertrophy occurs at the expense of adjacent matrix and it requires matrix resorption.

Bone formation takes place in the organism not only during embryonic development (growth plate cartilage in the process of endochondral bone formation) and growth but throughout the life in the process of physiological bone remodeling (Lian & Stein, 1996).

CELL BIOLOGY

Chondrocytes and osteoblasts are of mesenchymal origin. Mesenchymal stem cells are able to generate progenitors with restricted developmental potential. From progenitor cells, various cell types can be differentiated into fibroblasts, adipocytes, chondrocytes and osteoblasts (Fig. 2). The two latter cell types under the influence of growth factors give rise to cells able to form calcified tissues. Hypertrophic chondrocytes and osteoblasts initiate the calcification process by releasing matrix vesicles (MVs) (Anderson, 2003). MVs of growth plate cartilage differ in lipid and protein composition from MVs produced by osteoblasts (Boyan *et al.*, 1988). It has been suggested that MV biogenesis, from growth plate hypertrophic chondrocytes, could be the result of programmed cell death. This would

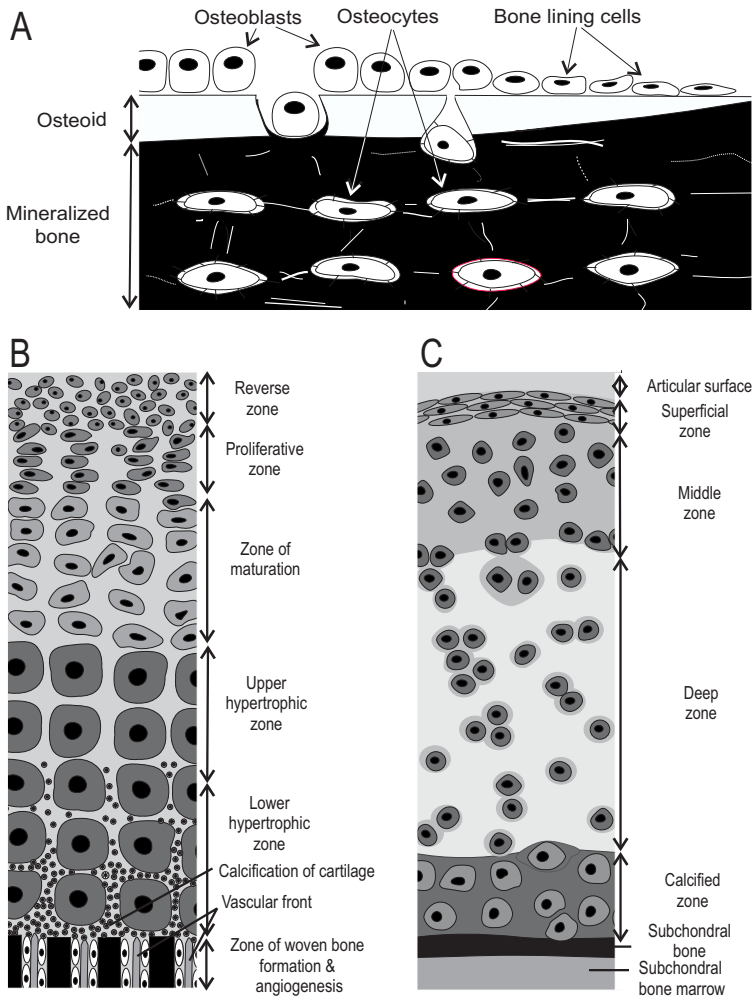

Figure 1. Regional organization and relationships among bone, growth plate and articular cartilage.

Panel A. Topographic relationship among bone cells. Osteoblasts are located on the lining layer of bone surface, actively producing matrix, which is not yet calcified (osteoid tissue). Osteocytes are the most mature or terminally differentiated cells of the osteoblast lineage. Osteocytes are embedded in the bone matrix. Panel B. primary mammalian growth plate showing progressive development of chondroblasts from the proliferative zone to the lower hypertrophic zone, where matrix synthesis stops and the extracellular matrix is calcified. Panel C. Regional organization of articular cartilage. The superficial zone contains thin collagen fibrils arranged parallel with the articular surface. The partly calcified cartilage of the calcified zone is indicated. Adapted from Marks & Popoff (1988) and Poole (2001).

appear not to be the case for MVs released from viable osteoblasts (Anderson, 2003). MVs initiate mineral formation starting from embryonic ossification to bone formation in adults (Hoshi & Ozawa, 2000).

MATRIX VESICLES

Several stages of mineralization were identified. The mineralization of bone and cartilage requires the presence of extracellular MVs (Anderson, 1995; 2003), since the first step of mineralization is initiated inside these organelles. MVs (in size between one hundred to several hundred nanometers in diameter) serve as a site for Ca^{2+} and P_i accumulation. MVs create a specific environment where deposition of initial amorphous mineral complexes (nucleation) occurs and where hydroxyapatite (HA) e.g. $Ca_{10}(PO_4)(OH)_2$, is produced and forms needle-like crystals on

the inner surface of the MV membrane. The extracellular matrix contains sufficiently high levels of Ca^{2+} and P_i concentrations to sustain the nucleation process and to propagate the mineralization (Anderson, 2003). Ion channels and transporters present in MV membrane are responsible for Ca^{2+} and P_i uptake into these organelles. After reaching a certain length, the needle-like HA crystals are released from MVs into the extracellular matrix. The mechanisms by which the HA crystals can break the membrane of MVs are not very well understood. One possible explanation is that the activity of phospholipases could be triggered, once HA crystals are formed, and may affect the MV membrane fluidity (Swain *et al.*, 1992; Schwartz & Boyan, 1988).

The second step of mineralization starts with a release of HA crystals. These crystals serve as a template for the formation of crystalline arrays, leading to a tissue calcification (Anderson, 2003). Although details of the mechanism are still unknown, assembly of mineral complexes depends probably on electrostatic, structural and stereochemical properties at the inorganic–organic interface. Subtle interactions between negatively charged domains of proteins, anionic phospholipids and mineral complexes are crucial in the propagation of arrays of crystals. All processes taking place in MVs require a dynamic but tightly regulated system to maintain Ca^{2+} homeostasis and P_i delivery. Many actors have been identified to date, among them vertebrate annexin (AnxAs), Ca^{2+}- and membrane-binding proteins, as well as alkaline phosphatase.

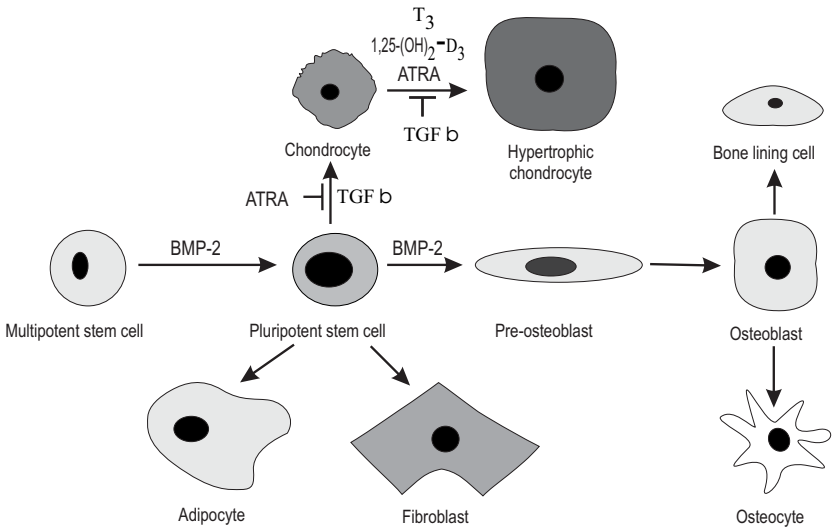

Figure 2. Lineage of osteochondroprogenitor cells.

Pluripotent stem cells develop from multipotential mesenchymal stem cells. The pluripotent stem cells are progenitors of all indigenous cells of connective tissues: fibroblasts, adipocytes, osteoblasts and chondrocytes. The influences of several physiological factors like transforming growth factor β (TGFβ), bone morphogenetic protein 2 (BMP-2), all-*trans* retinoic acid (ATRA), 1α,25-dihydroxyvitamin D_3 (1,25-$(OH)_2$-D_3), and 3,5,3'-triiodo-L-thyronine (T3) on the lineage of osteoblasts and chondrocytes are indicated in the figure. Osteoblasts exist in two forms as osteoblasts monolayer on the surface of growing bone tissue which synthesize and secrete organic components of matrix and produce mineralization competent matrix vesicles and osteocytes enclosed within bone matrix. Chondrocytes are characterized by two phenotypes: non-hypertrophic characteristic for articular cartilage, and hypertrophic in growth plate. Adapted from Lian & Stein (1996).

ANNEXINS IN MINERALIZING TISSUES

From twelve members of the annexin family of proteins present in mammalian organisms,

three were identified in MVs: annexin A2 (AnxA2), AnxA5 and AnxA6 (Cao *et al.*, 1993; Kirsch *et al.*, 1997a; 1997b; Kirsch & Claassen, 2000). Due to the high Ca^{2+} concentration both inside and outside MVs, and the high content of anionic phospholipids, mainly phosphatidylserine (PS), and cholesterol in MV membrane (Harder *et al.*, 1997; Wuthier, 1975; Ayala-Sanmartin, 2001; Ayala-Sanmartin *et al.*, 2001; De Diego *et al.*, 2002;), AnxAs can be associated with both outer and inner leaflets of MV membrane. AnxAs affect membrane stability in a Ca^{2+}-dependent manner (Goossens *et al.*, 1995). In addition, AnxAs could be involved in the Ca^{2+} transport, as ion channels inserted within the MV membrane.

During the first phase of MV-mediated calcification, mineral complexes appear on the inner surface of MV membrane. The high affinity for Ca^{2+} of PS is quite strong in the inner leaflet of the MV membrane enriched with anionic lipids (Majeska *et al.*, 1979; Taylor *et al.*, 1998). Accordingly, AnxA5 exhibiting Ca^{2+}-dependent PS-binding property was isolated with $PS–Ca^{2+}–P_i$ complexes from nucleation core of chicken growth plate MVs (Wu *et al.*, 1993; 1996; 1997a). Smaller amounts of AnxA2 and AnxA6, as well as other proteins, were co-purified with AnxA5 (Wu *et al.*, 1997a; 2002a).

AnxAs associated with the outer surface of MV and bone-derived cell membranes may interact with extracellular matrix molecules. AnxA2 and AnxA6 bind chondroitin sulfate in a Ca^{2+}-dependent manner (Ishitsuka *et al.*, 1998; Ishitsuka, 2000; Takagi *et al.*, 2002). AnxA5 binds types II and X collagens and C-propeptide of type II collagen (Kirsch & Pfäffle, 1992; von der Mark & Mollenhauer, 1997; Kirsch *et al.*, 2000a). The above described interactions may influence MV shape, thereby affecting crystal growth. Indeed, chondrosarcoma cells, expressing low quantities of AnxA5, are not able to bind type II collagen. This suggests that AnxA5 is a key molecule to promote extracellular matrix binding, which is essential for cartilage function (King *et al.*, 1997). In chicken growth plate, types II and X collagens enhance Ca^{2+} influx into MVs, promoting activity of ion channels formed by AnxAs (Kirsch & Wuthier, 1994; Kirsch *et al.*, 1994; 2000a). However, the presence of collagen is not essential for mineralization, as shown with knockout animals (Jacenko *et al.*, 1993), with reconstituted systems (Kirsch *et al.*, 1997a) and with purified MVs (Hsu & Anderson, 1978; Kirsch *et al.*, 2000a; Wang & Kirsch, 2002). Nevertheless, collagens could influence initialization and progression of mineral formation in MVs. In addition, *ANXA5–/–* mice were normal in respect of development of their skeletons (Brachvogel *et al.*, 2003), probably because other AnxAs could replace AnxA5 function in knockout animals.

AnxAs are specific markers of chondrocyte hypertrophy. Articular cartilage cells, in contrast to growth plate chondrocytes, maintain a stable phenotype. The upper zone of the articular cartilage (Fig. 1C) contains thin collagen fibrils and proteoglycan called aggrecan. In this zone, tensile forces connected with daily life are maximally concentrated. In lower zones, as in the middle and deep zones, the cell density decreases, collagen fibers are thicker and aggrecan content is higher. Calcified zones, where chondrocytes develop an hypertrophic phenotype, provide a link between subchondral bone and joint cartilage (Poole, 2001). Articular cartilage, unlike growth plate, usually does not undergo matrix calcification. However, mineralization frequently occurs during osteoarthritis and aging. In osteoarthritis, progressive damage and loss of articular cartilage matrix (especially in superficial zone) are observed. These events are accompanied by cell death and pathological matrix mineralization, leading to bone remodelling and to subchondral bone mass increase. In addition, an inflammatory process occurs, giving rise to pain and movement disabilities. The amount of AnxAs is scarce in normal articular cartilage, while it significantly increases during progression of osteoarthritis (Mollenhauer *et al.*, 1999;

Kirsch *et al.*, 2000b; Pfander *et al.*, 2001). Therefore, AnxAs could be specific markers of differentiation during osteoarthritis. For example, AnxA8, a protein not previously described in the growth plate, is expressed during inappropriate cell differentiation in osteoarthritis (White *et al.*, 2002). Relatively high annexin expression in articular cartilage chondrocytes is characteristic for hypertrophic cells and cells undergoing apoptosis (Kirsch *et al.*, 2000b; Kouri *et al.*, 2000) with the appearance of MVs or apoptotic bodies, respectively (Derfus *et al.*, 1998; Hashimoto *et al.*, 1998; Mollenhauer *et al.*, 1999; Kirsch *et al.*, 2000b). These events lead to mineralization of joint matrix (Gelse *et al.*, 2003) and expression of hypertrophy protein markers, as type X collagen and alkaline phosphatase (Hoyland *et al.*, 1991; Pullig *et al.*, 2000; Kirsch *et al.*, 2000b). MVs are present in articular cartilage from healthy subjects (Einhorn *et al.*, 1985; Derfus *et al.*, 1996). In osteoarthritis, MVs coexist in extracellular matrix with apoptotic bodies which are the products of chondrocytes at the terminal differentiation stage. There are no phagocytic cells in joint cartilage, therefore, apoptotic bodies remain in the cartilage unless the extracellular matrix becomes degraded.

FACTORS AFFECTING ANNEXIN ION CHANNEL FORMATION

The existence of a Ca^{2+} transport system in MVs is not well established. Possible candidates are AnxAs, since ion channels formed by these proteins *in vitro* have been described in literature (Berendes *et al.*, 1993; Arispe *et al.*, 1996; Kourie & Wood, 2000; Kirilenko *et al.*, 2002). To understand how AnxAs can mediate Ca^{2+} influx into MVs, factors affecting annexin activity in the mineralization process should be identified, as for example lipid composition of MV membrane.

MV membrane is distinct from plasma membrane (Wuthier, 1975). It is enriched in PS, diphosphatidylglycerol and lysophospholipids due to the difference in the rate of phospholipid degradation (Wuthier *et al.*, 1977; 1978). The anionic phospholipid content in calcified cartilage and bone is significantly higher than in non-calcifying cartilage zones (Wuthier, 1968; Wu *et al.*, 2002a). This may indicate that anionic phospholipids are involved in mineral formation. It is in agreement with the results of many experiments indicating that Ca^{2+}-dependent binding of AnxAs to model membranes is enhanced by the content of anionic phospholipids. Maximal Ca^{2+} influx mediated by AnxAs into liposomes occurs when they are prepared from PS and phosphatidylethanolamine (PE) mixture at 9:1 mole/mole (Kirsch *et al.*, 1997a). In addition, PS clustering may be induced by the high cholesterol content in MV membrane (Wuthier, 1975). AnxA5 interacts in a Ca^{2+}-dependent manner with cardiolipin in isolated mitochondria (Megli *et al.*, 1995; 2000). Since cardiolipin is present also in MV membrane (Wuthier, 1975), these interactions may occur in MVs.

AnxA2, AnxA5 and AnxA6 are abundant in acidified organic extracts of MVs (25–40% of extraction of AnxAs from crude preparations, as reported by Genge *et al.*, 1991), suggesting their presence in the hydrophobic core of lipid bilayer. This was also evidenced by using selective labeling of AnxA5 with photoactivable hydrophobic reagent, revealing that this protein inserts into the membrane hydrophobic core at mildly acidic pH (Isas *et al.*, 2000). At low pH, aspartate and glutamate residues of AnxAs are protonated. The protein surface becomes more hydrophobic, facilitating its insertion into lipid bilayer (Kohler *et al.*, 1997; Beermann ofm cap *et al.*, 1998; Isas *et al.*, 2000; 2003; Golczak *et al.*, 2001a; b).

Whether, the low pH-induced annexin ion channels in MVs may form during mineralization, remains to be elucidated. In fact, it is not clear which population of AnxAs may participate in ion channel formation: AnxAs associated with the external or the

internal leaflet of the MV membrane. It is possible that annexin channels are formed in plasma membrane before MV budding. The pH measurements made in tissue sections indicate that intracellular pH in chicken growth plate is dependent on the zone from which chondrocytes are derived. The lowest pH was observed in the periphery of late hypertrophic and calcifying cells (Wu *et al.*, 1997b). Moreover, protons are byproducts of HA formation in MVs. Low pH can prevent HA formation by increasing solubility of formed mineral for which the optimal pH for crystal formation is in the range of 7.4–7.8 (Valhmu *et al.*, 1990). However, extensive acidification during crystal formation is prevented by type II carbonic anhydrase (Stechschulte *et al.*, 1992; Sauer *et al.*, 1994).

Chondrocytes in the growth plate release NTPs that may regulate cell maturation and matrix mineralization (Hatori *et al.*, 1995; Hung *et al.*, 1997;). NTPs are also released by non-stimulated (Hatori *et al.*, 1995) and by stimulated osteoblasts in response to mechanical activation (Romanello *et al.*, 2001). AnxAs can bind nucleotides under *in vitro* conditions (Kirilenko *et al.*, 2001; 2002; Bandorowicz-Pikula *et al.*, 2001; 2003) but probably, with the exception of AnxA7 (Caohuy *et al.*, 1996), do not hydrolyze nucleotides. GTP in a millimolar concentration range induced AnxA6 channel formation in planar lipid bilayers (Kirilenko *et al.*, 2002). It was also shown that the AnxA5 ion channel activity in MV could be regulated by NTPs (Arispe *et al.*, 1996). However, the mechanism by which these channels are formed in MVs is not yet elucidated.

INTERACTIONS OF ANNEXINS WITH OTHER PROTEINS DURING MINERALIZATION

Changes in extracellular fluid composition, reductions in extracellular pH, increase in matrix synthesis, as well as morphological changes associated with local compaction of matrix around the cells, may affect chondrocyte proliferation and maturation (Buschmann *et al.*, 1995; Quinn *et al.*, 1998; Wu & Chen, 2000). For example, hyperosmotic stimuli was reported to affect protein synthesis in cartilage, as well as Ca^{2+} and H^{+} homeostasis (Dascalu *et al.*, 1996; Erickson *et al.*, 2001).

Additional factors that may affect annexin ion channel activity during mineralization are associated with their interaction with other proteins. Mobasheri *et al.* (2002) attributed perception of mechanical signals in cartilage to cell surface membrane mechanoreceptors. These receptors are composed of integrins and stretch activated ion channels. Multiple mechanosensitive ion channels were characterized in osteoblasts and chondrocytes. None of these channels revealed similarities with AnxAs (Davidson *et al.*, 1990; 1996; Duncan & Hruska, 1994; Guilak *et al.*, 1999; Koprowski & Kubalski, 2001; Biggin & Sansom, 2003; Shakibaei & Mobasheri, 2003). In osteoblasts, increase in $[Ca^{2+}]_{in}$ by oscillating fluid flow, was attenuated by the addition of anti-AnxA5 antibodies. This suggests that AnxA5 may be involved in mechanotransduction in bone (Yellowley *et al.*, 2002). Recently, it was observed that AnxA5 binds to the cytoplasmic part of $\beta 5$ subunit of bovine integrin $\alpha v \beta 5$ (Andersen *et al.*, 2002).

Homodimeric S100A and S100B proteins interact with AnxA5 and AnxA6 at 1 mole S100 dimer per 2 mole annexin stoichiometry (Donato, 2003). It was previously demonstrated by co-immunoprecipitation (Arcuri *et al.*, 2002) and inhibition of annexin- mediated Ca^{2+} fluxes (Garbuglia *et al.*, 1998; 2000). However, annexin–S100 interactions have not been investigated in cell systems able to perform mineralization. It was reported that calbindin D9k, an unusual monomeric member of S100 proteins, is present in MVs (Balmain, 1991; 1992; Balmain *et al.*, 1989; 1991; 1995). Calbindin

D9k is a vitamin D_3-dependent protein and its expression affects dietary Ca^{2+} accumulation in bones (Li *et al.*, 2001). The presence of this protein is important for interstitial Ca^{2+} absorption. In rat epiphyseal chondrocytes, calbindin D9k is highly expressed only in mature and hypertrophic chondrocytes (Balmain *et al.*, 1995). It is postulated that calbindin D9k takes part in mineral nucleation (Balmain, 1991). Besides, calbindin D9k reveals 47% and 37% identity and 64% and 55% homology in primary structure with S100A and S100B proteins, respectively. Such high similarity between proteins supports the hypothesis that calbindin D9k can interact with AnsAs during mineralization.

EFFECT OF RETINOIC ACID ON THE MATURATION OF CHONDROCYTES AND ON THE MINERALIZATION PROCESS

Recent findings reveal that growth plate chondrocytes proliferate and mature faster upon treatment with all-*trans* retinoic acid (ATRA) (De Luca *et al.,* 2000). It is accompanied by terminal differentiation of chondrocytes and production of mineralization competent MVs, rich in AnxAs and alkaline phosphatase (Wang & Kirsch, 2002; Wang *et al.*, 2003). ATRA, an agonist of receptors of retinoic acid and other retinoids (RAR/RXR), stimulates events leading to mineralization and matrix remodeling. In addition, it stimulates cell differentiation and apoptosis, as well expression of metalloproteinases (Nie *et al.*, 1998), type I collagen (expression of proteoglycans and type II and X collagens is inhibited), alkaline phosphatase and AnxAs (Wu *et al.*, 1997c; Wang *et al.*, 2003). Moreover, events characteristic for apoptosis, such as down-regulation of Bcl-2, activation of capsase-3 and DNA fragmentation occur after treatment with ATRA. These events are reversed by simultaneous treatment of cells with ATRA and BAPTA-AM (intracellular Ca^{2+} chelator) or K-201, a 1,4-benzothiazepine derivative that can inhibit ion channel activity of AnxAs (Kaneko *et al.*, 1997a; 1997b; Hofmann *et al.*, 1998; Wang *et al.*, 2003). This may indicate that annexin-mediated Ca^{2+} fluxes are responsible for events related to cell maturation, cell apoptosis and tissue mineralization. Recently, we observed that precursor of ATRA, all-*trans* retinol (vitamin A), binds to AnxA6 *in vitro* (Fig. 3), especially at acidic pH, providing a possible regulatory link with an annexin-mediated mineralization process. Addition of retinoids could promote the mineralization process not only by enhancing annexin expression but by direct interaction with AnxAs or by changing the membrane fluidity (Wang *et al.*, 2003). It has been also shown that 1α,25-dihydroxyvitamin D_3 binds to AnxA2 of rat osteoblast-like ROS 24/1 cells, inducing increases in intracellular Ca^{2+} concentration (Baran *et al.*, 2000).

ROLES OF ANNEXINS AND OTHER ANIONIC PROTEINS IN THE NUCLEATION PROCESS

Most non-collagenous proteins involved in initiation and regulation of biological mineral formation are anionic (Boskey, 1996). Among proteins synthesized by osteoblasts are osteonectin, osteopontin, osteocalcin and bone sialoprotein. Cartilage extracellular proteins are similar to bone, while both tissues differ in types of collagens. All these proteins share a high content of aspartic and glutamic acid residues (30–40%) and multiple phosphoryl and sialyl groups. They differ in their abilities to affect the formation of HA *in vitro* (Hunter *et al.*, 1996). Additionally, the phosphoproteins of bone are processed by limited proteolysis, then they are converted into more phosphorylated species that could facilitate mineralization (Suzuki *et al.*, 1996). AnxAs have several putative phosphorylation sites and

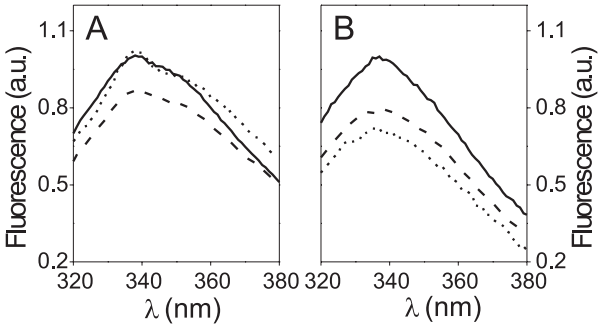

Figure 4. Binding of vitamin A (all-*trans* retinol) to AnxA6.

To determine binding of vitamin A to AnxA6, quenching of intrinsic fluorescence of the annexin was measured, using the same method as for the retinol carrier protein (Raghu *et al.*, 2003). Panel A. Human recombinant AnxA6 (1 μM) was incubated in 150 mM NaCl, 10 mM Tris/HCl, pH 7.4, without (bold line) or with (dashed line) 3 μM vitamin A added from concentrated stock solution in ethanol (final concentration of ethanol did not exceed 0.5%). Asolectin liposomes were also added in the presence of AnxA6 (protein/lipid ratio of 1:1000, by mole) and vitamin A (dotted line). Panel B. AnxA6 (1 μM) was incubated in 150 mM NaCl, 10 mM citric buffer, pH 6.0, without (bold line) or with (dashed line) 3 μM vitamin A. Asolectin liposomes were also added in the presence of AnxA6 (protein/lipid ratio of 1:1000, by mole) and vitamin A (dotted line). Samples were excited at 295 nm and fluorescence emission spectra were recorded at the wavelength range from 320 to 380 nm. All measurements were performed on a Fluorolog 3 spectrophotometer (Jobin Yvon Spex Edison, NJ) with 2-nm slits for both excitation and emission, at 25°C. Quenching of the AnxA6 intrinsic fluorescence by vitamin A is higher at pH 6.0 than at pH 7.4. After liposome addition, the protein fluorescence returns to the basic level only at pH 7.4, probably due to higher affinity of vitamin A for lipids than for AnxA6 (dissociation of protein–vitamin A complex). At pH 6.0, upon addition of liposomes, AnxA6 inserts within the hydrophobic core of the membrane lipid bilayer where it is still able to interact with hydrophobic vitamin A. The result of this experiment suggests that AnxA6 binds vitamin A *in vitro*.

some of them are phosphorylated *in vitro* (Grima *et al.*, 1994). In the case of AnxA6, phosphorylation mimicking mutation resulted in higher Ca^{2+}-binding affinity and conformational changes leading to increased protein flexibility in comparison with wild AnxA6 (Freye-Minks *et al.*, 2003).

It is not known how AnxAs can influence the nucleation sites at the membrane interface and which charged domains are responsible for electrostatic interactions taking place during nucleation. Crystal structures of AnxAs suggest the importance of flexibility for AnxA6 (Avila-Sakar *et al.*, 2000) and AnxA5 (Oling *et al.*, 2000; 2001) in the annexin–phospholipid interactions. Given these findings, it is tempting to suggest that AnxAs may influence molecular organization during nucleation formation, through changes in molecular flexibility or through protein–protein interactions. Such interactions with other MV proteins may favor accumulation of inorganic material.

ALKALINE PHOSPHATASE AND RELATED PROTEINS IN THE MATRIX VESICLES

Alkaline phosphatase is one of the most frequently used biochemical markers of osteoblast activity (Risteli & Risteli, 1993; Garnero & Delmas, 1996; Nawawi *et al.*, 1996; Magnusson *et al.*, 1999). Four genes encoding human alkaline phosphatase have been cloned (Kam *et al.*, 1985; Millán, 1986; Henthorn *et al.*, 1987; Millán & Manes, 1988) corresponding to three specific alkaline phosphatase genes located in chromosome 2 (germ-cell, placenta and intestinal) and one TNAP gene located in chromosome 1 (Moss, 1992). Alkaline phosphatases from all sources are homodimeric metalloenzymes which catalyze the hydrolysis of almost any phosphomonoester with release of P_i and alcohol (Fernley, 1971).

TNAP exists in three forms derived from bone, liver and kidney and differing in carbohydrate groups. Osseous TNAP localized in plasma membrane and in MVs, is a glycosylphosphatidylinositol (GPI)-anchored protein (Noda *et al.*, 1987; Pizauro *et al.*, 1994). Given

the different solubilization of TNAP from osteoblast plasma membrane, obtained from human primary bone cell culture, it was suggested that changes in TNAP activity result from age-related modifications. These changes could be associated with the post-translational modification of TNAP or with the membrane constituents (Radisson *et al.*, 1996; Bourrat *et al.,* 2000). The role of TNAP in mineral formation was evidenced in the case of hypophosphatasia, an inheritable disorder leading to a defective bone formation and characterized by a deficiency in TNAP (Whyte, 1994). Mice deficient in the gene encoding TNAP mimic a severe form of hypophosphatasia, indicating the importance of TNAP in hydrolyzing phosphate substrates, including PP_i, during mineral formation (Narisawa *et al.*, 1997). In addition, several mutations in TNAP occur around a calcium- binding site of the enzyme, not directly associated with the metal-binding site function for hydrolysis. It is suggested that these mutations result in TNAP misfolding (Mornet *et al.*, 2001).

TNAP appears to be a multifunctional enzyme and several of its properties may be important for the mineralization process (Bellows *et al.*, 1991; Hsu, 1992a; 1992b; Rattner *et al.*, 2000). Although TNAP is a well-known biochemical marker of mineralization, the nature of the substrate hydrolyzed by TNAP is not clearly established. It was proposed a long time ago that TNAP may supply P_i by hydrolyzing phosphate substrates (Robison, 1924). This proposal was further substantiated by the observation that supplementation of culture media with β-glycerophosphate, an exogenous TNAP substrate, induced osteogenesis and HA deposition (Tenenbaum, 1981; Ecaot-Chevrier *et al.*, 1983). Addition of levamisole, a specific inhibitor of TNAP activity, prevented β-glycerophosphate-induced mineralization *in vitro* (Tenenbaum, 1987).

TNAP purified from femur of chicken embryos induces the formation of HA in mineralization medium without P_i but containing Ca^{2+} and phosphate substrates (AMP, creatine phosphate, glucose phosphate and β-glycerophosphate). Under these conditions, addition of ATP does not promote the formation of HA (Hamade *et al.,* 2003). This finding is consistent with the fact that a specific ATPase, rather than TNAP, is responsible for ATP-dependent mineral formation within MVs isolated from bone and/or cartilage (Hsu & Anderson, 1995; 1996; Hsu *et al.*, 1999). The nature of ATPase involved in the ATP-dependent mineral formation is not known and it was proposed that a Ca^{2+}-ATPase could fulfil this role (Hsu & Anderson, 1996). Therefore, not only TNAP but also other enzymes are involved in the P_i homeostasis (Fig. 4). The local concentration of

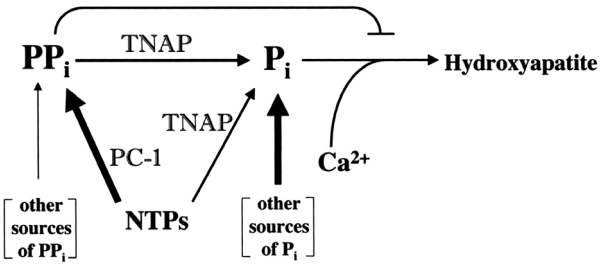

Figure 4. Production of pyrophosphate and inorganic phosphate and their antagonistic effects on the mineralization process.

PP_i, inhibitors of HA formation, are produced at least partly by plasma cell membrane glycoprotein-1 (NTP pyrophosphatase phosphodiesterase isoenzyme, PC-1) from the hydrolysis of NTPs. The activity of TNAP may boost the formation of HA, by hydrolyzing PP_i and eliminating its inhibitory effect on HA formation. P_i arises from distinct sources, including the hydrolytic activity of TNAP. Accumulation of P_i and Ca^{2+} can induce the formation of HA. Adapted from Hessle *et al.* (2002).

P_i can be increased by the activities of adenine monophosphodiesterases, ATP hydrolases (ATPases) and NTP pyrophosphohydrolases (Bartling & Chong 1999; Anderson, 2003).

Chondrocytes in the growth plate release ATP (Hung *et al.*, 1997). ATP is also released by non-stimulated bone cells (Hatori *et al.*, 1995) or in response to mechanical stimula-

tion (Romanello *et al.*, 2001). PP_i, which could result from the activity of several types of NTP pyrophosphohydrolases (Ho *et al.*, 2000; Huang *et al.*, 1994; Terkeltaub *et al.*, 1994; Johnson *et al.*, 1999a; 1999b) (Fig. 5), and biphosphonates are known inhibitors of HA formation (Tenenbaum, 1987; Skrtic & Eanes, 1994). Thus, it was suggested that TNAP may hydrolyse pyrophosphate groups (Rezende *et al.*, 1994; Camolezi *et al.*, 2002). Heritable deficiencies of the gene encoding NTP pyrophosphohydrolase could play an important role in the etiology of human ossification of the posterior longitudinal ligament of the spine and pathologic soft-tissue ossification, by decreasing the production of PP_i (Okawa *et al.*, 1998; Johnson *et al.*, 1999a; Nakamura *et al.*, 1999). The antagonistic regulation of PP_i concentration by the activities of TNAP and NTP pyrophosphohydrolase was confirmed by the experiments performed on knockout mice null for both TNAP and plasma cell membrane glycoprotein-1 (PC-1, e.g. NTP pyrophosphatase phosphodiesterase isoenzyme) genes (Hessle *et al.*, 2002). The double knockout mice were essentially normal (Hessle, 2002), while TNAP knockout mice mimicked the metabolic disease – hypophosphatasia (Whyte, 2001). These findings suggest that TNAP, together with other hydrolytic enzymes, participate in the P_i homeostasis. Deficiency in PC-1 may result in cartilage calcification, while lack of TNAP expression may result in hypophosphatasia. Both enzymes could be putative therapeutic targets for the treatment of bone mineralization diseases. It was proposed that inhibitors of PC-1 activity could be used for the treatment of hypophosphatasia (Hessle *et al.*, 2002).

P_i arising from extracellular matrix and from the hydrolytic activities of enzymes located either in MVs or in the plasma membrane of chondrocytes or osteoblast cells, is transported into the MVs to initiate the first stage of the mineralization process. Indeed, sodium-dependent P_i transporter responsible for the P_i uptake inside MVs has been identified (Montesuit *et al.*, 1991; Anderson, 2003). Recent findings indicate that other P_i transporters, not strictly sodium-dependent, are involved in the P_i uptake inside the MV from chondrocytes (Wu *et al.*, 2002b). The regulatory factors on the function of these transporters have not yet been identified.

CONCLUDING REMARKS

The prerequisite for the initial crystalline HA generation and its deposition, requires the continuous supply of Ca^{2+} and P_i inside the MVs. This is accomplished by the activities of several proteins that are involved in Ca^{2+} and P_i homeostasis, among them AnxAs and TNAPs. Although the functions of AnxAs are not well established, an emerging picture suggests that these proteins form calcium ion channels in MV membrane. At our present stage of knowledge, further investigations are needed to substantiate the mechanism of Ca^{2+} fluxes through annexin channels. In addition, AnxAs bind NTPs, probably regulating NTP supply outside of MVs, and providing a possible link to NTP hydrolysing enzymes including alkaline phosphatases. A further link between calcium homeostasis maintained by AnxAs and P_i supply is provided by the calcium-binding property of alkaline phosphatase, recently reported by Mornet and co-workers (Mornet *et al.*, 2001). One may suggest that AnxAs by affecting calcium homeostasis within MVs, directly or indirectly, finely tune up the structure and function of alkaline phosphatase. This is in accordance with the general idea that most MV proteins are miltifunctional by nature (Boskey, 1996). Complex interplays between these proteins are necessary to fulfill the highly ordered and tightly controlled mineralization process. An additional regulation can occur at the protein expression level or at the post-translational modification stage in response to stress or aging.

Summarizing, it becomes more obvious now that the interactions between AnxAs, TNAP and their ligands, as well as their respective localization within MVs, are important factors that may influence the calcification of osseous tissues.

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