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# Biomineralization of bone: a fresh view of the roles of non-collagenous proteins

**Jeffrey Paul Gorski**[1]

[1]Center of Excellence in the Study of Musculoskeletal and Dental Tissues and Dept. of Oral Biology, Sch. Of Dentistry, Univ. of Missouri-Kansas City, Kansas City, MO 64108

## Abstract

The impact of genetics has dramatically affected our understanding of the functions of non-collagenous proteins. Specifically, mutations and knockouts have defined their cellular spectrum of actions. However, the biochemical mechanisms mediated by non-collagenous proteins in biomineralization remain elusive. It is likely that this understanding will require more focused functional testing at the protein, cell, and tissue level. Although initially viewed as rather redundant and static acidic calcium binding proteins, it is now clear that non-collagenous proteins in mineralizing tissues represent diverse entities capable of forming multiple protein-protein nteractions which act in positive and negative ways to regulate the process of bone mineralization. Several new examples from the author's laboratory are provided which illustrate this theme including an apparent activating effect of hydroxyapatite crystals on metalloproteinases. This review emphasizes the view that secreted non-collagenous proteins in mineralizing bone actively participate in the mineralization process and ultimately control where and how much mineral crystal is deposited, as well as determining the quality and biomechanical properties of the mineralized matrix produced.

### Keywords

Biomineralization; PHEX; Phospho 1; Bone sialoprotein; DMP1; Type XI collagen; Fetuin

## 2. INTRODUCTION

Mineralization of bone represents an extracellular process in which osteoblastic and osteocytic cells remain viable embedded within a matrix of their making. While mature bone matrix is predominantly composed of type I collagen, the ubiquitous expression of collagen indicates that additional factors are needed to initiate and control the formation of hydroxyapatite crystals which are deposited predominantly between the collagen fibrils. In general, investigation of the process of bone formation and mineralization has been difficult because of the restricted location of the forming sites, the temporal movement of the mineralization front as mineralization proceeds, and the mineralized nature of the bone tissue. This has necessitated a reliance upon cell culture models to generate concepts and

Send correspondence to: Jeff Gorski, Department of Oral Biology, School of Dentistry, University of Missouri-Kansas City, 650 East 25th Street, Kansas City, MO 64108, Tel: 816-235-2537, Fax: 816-235-5524, gorskij@umkc.edu.

paradigms which are then tested in explant or whole animal models. This review represents an overview of both *in vitro* and *in vivo* model systems and attempts to integrate data from both sources to reach functional concepts regarding the roles of each of the major non-collagenous proteins in biomineralization of bone.

## 3. PROPOSED MECHANISMS OF MINERAL NUCLEATION IN BONE

New bone formation in the vertebrate embryo is produced by two distinct condensation processes: intramembranous, which forms and persists in the calvaria and jaws, and endochondral, which forms the long bones. In intramembranous bone formation, woven or primary bone is rapidly formed without a cartilaginous precursor, whereas endochondral bone uses a previously formed vascularized cartilage template (1). Primary bone is formed *in situ*ations where a rapid deposition of bone is required—embryogenesis, fracture healing, and adaptive bone gain after mechanical loading (~3000 microstrain) (2). In adult skeletons, the two distinct types of bone formation may have common but also unique molecular and cellular control mechanisms. Primary bone is defined as bone that forms where no bone existed previously. In contrast, lamellar or mature bone can be viewed as “replacement bone” formed when bone (either woven or lamellar bone) is resorbed by osteoclasts and then replaced by new lamellar bone. “Modeling” and “remodeling” are applied to the formation of primary bone and lamellar bone, respectively. In both primary and lamellar bone, initial hydroxyapatite crystal nucleation is mediated by organic molecules. There are two essential events in mineralization: the first is nucleation of mineral to produce crystals with a C-axis length of between 15 and 20 nm (3) and the second is mineral crystal propagation, also known as the crystal growth phase (4). Several cell-derived structures have been proposed to be responsible for the initiation and control of the mineral nucleation event. These include: matrix vesicles (5–8), biomineralization foci (9–11), ‘crystal ghosts’ (12), calcospherulites (13), phosphoproteins-collagen fibrils (14, 15) and fetuin (16, 17). The contribution of each of these to the initial nucleation event continues to be debated but it is likely that their relative contributions will vary in different tissues or developmental stages.

### 3.1. Biomineralization Foci

We have shown that 10–25 micron in diameter BMF are the sites of initial mineral nucleation in three osteoblast cell lines, in developing periosteum, and in new primary bone produced by marrow ablation model (9, 10). Interestingly, pre-BMF can be visualized prior to addition of extra phosphate to the cultures and, following addition, nucleation of hydroxyapatite crystals occurs exclusively within BMF starting as soon as 12 h later. Proteomic analyses on laser capture micro dissected mineralized BMF indicate they are enriched in acidic phosphoproteins BSP and BAG-75, along with a range of different sized vesicles (50 nm to 2 μm) including matrix vesicles (11). Banded collagen fibrils do not appear to be present within these pre-BMF. Vesicles within BMF are the sites of initial mineral nucleation (9–11. 18, 19). Although BSP is associated with the first needle-like crystals produced in developing bone or culture models, it has not been reported to be a component of matrix vesicles. In this way, BSP appears to define a biochemically distinct population of particles which are larger than matrix vesicles and which can also become mineralized (10, 18). Importantly, based on a shared enrichment in BSP and their structure

and tissue distribution, Bonucci recently concluded that crystal ghosts are a mineralized tissue equivalent of BMF in osteoblastic cultures (20).

Recently we showed that mineral nucleation within UMR106-01 and fetal mouse calvarial osteoblastic cells could be blocked by 100 μM AEBSF, a covalent serine protease inhibitor, without impacting cell viability (11). In contrast, fifteen general and specific inhibitors against acidic, metallo-, and sulfhydryl proteases were without effect. Partial fragmentation of BMF biomarkers BSP and BAG-75 was also inhibited by AEBSF, along with that for procollagen C-terminal propeptidase enhancer1 (PCOLCE1) and 1,25-vit. D3-MARRS protein (Membrane Associated, Rapid Response Steroid binding receptor). The 50 kDa fragment of BAG-75 was detected using antibodies against the N-terminal peptide (VARYQNTEEEE) (21); in separate studies we have shown that these antibodies detect a 50 kDa band in serum which is induced 3-fold in rats stimulated to undergo new bone formation (unpublished result, Gorski). Subsequent work has sought to establish the mechanism for AEBSF action.

A detailed literature search identified a candidate protease sensitive to AEBSF inhibition, proprotein convertase SKI-1 (S1P) (22). Analysis of BSP, 1,25-D3-MARRS (Membrane Associated, Rapid Response Steroid binding receptor), and procollagen C-proteinase enhancer protein 1 (PCOLCE1), three proteins whose cleavage is blocked by AEBSF (14), reveals each contains several SKI-1 candidate cleavage sequences which appear capable of generating the observed fragments (unpublished result, Huffman, Seidah and Gorski). We have also recently shown that a catalytically active 105 kDa form of SKI-1 (23, 24) is highly expressed within laser capture microscope purified BMF (25).

### 3.2. Calcospherulites

Midura *et al.* (29, 30) recently isolated calcospherulites (from the mineralization front of tibial periosteum) and used them to induce the mineralization of type I collagen *in vitro*. Calcospherulites were identified as 0.5 um diameter particles which had taken up calcein within the 24 h following *in vivo* injection. The particles exhibited a Ca/P ratio of 1.3 which rose to 1.6 after treatment with ice cold ethanol and were enriched in bone matrix phosphoproteins BSP and BAG-75. When incubated in collagen hydrogels, calcospherulites seeded a mineralization reaction on type I fibrils. When compared with BMF from osteoblastic cells, we conclude that calcospherulites represent mineralized vesicles comparable to those which are the sites of initial mineral deposition within BMF. We believe their membrane bilayer acts as a barrier to proteolysis and permits their isolation based upon their mineral and calcein content, whereas we presume other BMF components are degraded by the dispase used (28,29). In this way, calcospherulites likely represent a protease resistant, mineral containing fraction of BMF (9–11, 18).

### 3.3. Matrix vesicles

First identified and characterized by Bonucci (31) and by Anderson (32), matrix vesicles have a long and controversial history in bone. However, recent work with BMF and calcospherulites indicates that matrix vesicles, and other vesicles, are present in bone and in mineralizing osteoblastic cultures (9–11, 18, 28, 29). However, while recent work shows

that mineral crystals in developing bone are deposited within vesicles (8, 33), it does not show that matrix vesicles are the exclusive sites of mineral deposition in bone. Rather, careful analysis suggests that different sizes and distinct compositional populations of vesicles exist in mineralizing bone, including matrix vesicles, and participate in the initial mineral deposition.

Xiao Z *et al.* (34) recently characterized the proteome of extracellular matrix vesicles isolated from mineralizing MC3T3-E1 osteoblasts using proteolysis. This confirmed a number of well known matrix vesicle components, including annexin V, alkaline phosphatase, aminopeptidase A, and Emilin-1. In addition, over 125 other proteins were identified in common between the two matrix vesicle preparation methods compared. The results indicate that the protein composition of matrix vesicles is the result of a molecular sorting process which yields a different more specialized composition than expressed on the plasma membranes of differentiated osteoblastic cells (34).

## 4. THE ROLE OF INDIVIDUAL NON-COLLAGENOUS PROTEINS

As the most prevalent protein in the body, type I collagen is distributed widely among mineralized and non-mineralized tissues. On this basis, it is apparent that type I collagen alone cannot be solely responsible for the unique capacity of bone to mineralize. With the abovementioned initiation systems in mind, we now turn to a consideration of the functional roles of individual non-collagenous proteins. The reader is referred to previous reviews for a more comprehensive treatment of the older literature (3, 35–37).

### 4.1. Bone Sialoprotein (BSP)

Originally cloned by Fisher and colleagues (38), BSP is thought to be related to the sialoprotein isolated by Herring in 1964 (39). Based on the work of Goldberg and colleagues (40–44), BSP is generally believed to function in bone as a crystal nucleator, although a number of studies demonstrate it is able to signal through its RGD integrin binding site and a second, cryptic cell adhesion site (45, 46). Importantly, Aubin and colleagues have produced a BSP null mouse and characterized its bone phenotype (47–51) with and without skeletal challenge. BSP null mice breed normally but their weight and size is lower than wild type mice. Hypomineralized bone is evident in embryonic and young adult bone, but not in mice over 1 year old (51). At 4 months of age, cortical bone from BSP (−/−) mice is thinner than wild type, but with greater trabecular volume with a very low bone formation rate. BSP null bone also displays fewer osteoclasts. In addition, BSP deficient mice exhibited a delay in healing response at 2 and 3 weeks after formation of cortical defects. In contrast, OPN null mice responded no differently than controls when analyzed by MicroCT or histology. Interestingly, BSP null mice lose bone normally in unloading studies, while OPN null mice do not lose bone (48). In summary, the absence of BSP results in impaired new bone formation and osteoclast activity (47–51). However, the lack of a more dramatic phenotype has been suggested to be due to a functional redundancy with other acidic phosphoproteins in bone.

Gordon *et al.* (52, 53) have shown that the RGD motif is required to for BSP to induce osteoblastic differentiation. When BSP was over-expressed in primary calvarial osteoblastic

cells using a adenoviral vector, Runx2 and Osx expression, alkaline phosphatase, and mineralization were all increased via a pathway involving focal adhesion kinase and MAP kinase associated ERK ½ proteins. These findings seem at odds however with the phenotype of transgenic mice over-expressing BSP using the CMV promoter. The mice display mild dwarfism, lower BMD, and lower trabecular bone volume, while expressing higher levels of bone resorption markers indicating increased osteoclastic bone resorption. An explanation for these unexpected *in vivo* results is not yet evident.

BSP also has effects on bone resorption of osteoclasts. Curtain *et al.* (54) showed that dephosphorylated bone BSP preferentially inhibited the differentiation of osteoclasts in a live bone organ culture model via an intracellular tyrosine phosphorylation signaling pathway. Native bone BSP had little effect on osteoclast formation and bone resorption, whereas dephosphorylated BSP eliminated PTH-induced bone resorption. These effects were only partially blocked by integrin receptor antagonist GRGDS. Mechanistically, the effects of dBSP appeared to be mediated by down regulation of osteoblastic RANKL production in this bone organ culture system. Physiologically, Curtain *et al.* (54) believe that dBSP released/produced by active osteoclastic resorption of bone matrix could feedback to cells in the local environment to block or limit the resorption phase. It should be noted that Mintz *et al.* (45) first identified a non-RGD, cryptic cell adhesion site on BSP which was exposed upon cleavage. In addition, Sato *et al.* (55) also showed that BAG-75 inhibited osteoclast differentiation as well as resorption by preformed osteoclasts.

Recent work with recombinant BSP demonstrates that phosphorylation of serine-136 is critical for BSP-mediated nucleation of hydroxyapatite crystals in a steady state assay (44). BSP binding to native type I collagen increased calcium binding by 10-fold compared with free BSP. Serine-136 is a site adjacent to one of two Glu-rich regions previously implicated in hydroxyapatite nucleation, e.g., residues 42–100 and 133–205 (56). Boskey and colleagues have also used diffusion studies within a semi-solid collagenous gel to establish the ability of BSP to nucleate hydroxyapatite crystals (57).

While these data indicate that BSP is the most potent protein nucleator of hydroxyapatite, a role for BSP in the mineralization of collagen is limited by a lack of a mechanism to bind BSP specifically to the hole or gap regions of fibrillar collagen as first proposed by Glimcher (16) and by Veis and Schleuter (15). Since initial mineral crystals were found to be associated with the hole regions in the turkey tendon model of mineralization and since type I collagen is a ubiquitous component of mineralizing as well as non-mineralizing extracellular matrices, these authors argued that the associated phosphoprotein(s), bound to the hole zone of collagen fibrils, acted as a mineralization nucleator. In this way, mineralization of bone extracellular matrix was proposed to be initiated by the formation of seed crystals in the interfibrillary space which would serve to propagate crystal growth within the broader bone matrix. While an attractive hypothesis, the proposal has yet to be validated largely due the absence of a mechanism to localize phosphoproteins to the hole region. However, recent work from our laboratory has provided an interesting potential mechanism for binding of BSP near the hole region of type I collagen fibrils.

As shown in Figure 1, osteoblastic cells in culture initiate mineralization within spherical extracellular complexes we have termed biomineralization foci (BMF). These BMF are compositionally complex and contain matrix vesicles and larger sized vesicles in which the first mineral crystals are formed. We have used laser micro dissection microscopy to isolate and characterize the proteome of BMF. Interestingly, type XI collagen was found to be enriched within micro dissected BMF, specifically, a 60 kDa fragment containing the 6b alternative splice variant sequence (Figure 2). In contrast, another splice variant, 8, while expressed by UMR106-01 osteoblastic cells as a full length 105 kDa alpha 1 chain, is not enriched within BMF (Figure 2). Both the 6b and 8 sequences are part of the N-terminal domain of the type XI alpha 1 chain (58) (Figure 3). The charge character of the NTD can be changed dramatically by alternative splicing since the 6b sequence has an isoelectric point of 11.3 and unusual arginine triplet repeat sequences. In contrast, the alternating 6a sequence is acidic in character. The NTD is a large globular extension. The NTD variable region containing the 6b and 8 sequences survives BMP-1 processing and exists as part of the extracellular, mature type XI collagen trimer (Figure 3) (59).

6b sequence:
**KKK**SNYT**KKK**RTLATNS**KKK**SKMSTTPKSEKFAS**KKKK**RNQATAKAKLGVQ

6a sequence: YAPEDIIEYDYEYGETDYKEAESVTEMPTFTEETVAQT

Alternatively, as shown by our culture data, the 6b region can be cleaved off as a 60 kDa fragment (Figures 2 and 3). As illustrated in Figure 4, the 60 kDa 6b containing type XI NTD fragment associates specifically with bone sialoprotein. Mineralized osteoblastic cell cultures were extracted with EDTA, which removes predominantly material associated with BMF (11), and then immunoprecipitated with anti-BSP antibodies. Interestingly, BSP and the 60 kDa type XI NTD region were only recovered from cultures which mineralized, not those inhibited with AEBSF (Figure 4). Other studies show that the 60 kDa band reacts with anti-6b type XI collagen antibodies and anti-Npp antibodies establishing it as the complete NTD domain (data not shown) (Figure 3).

Taken together we believe these results provide a new novel mechanism by which to localize mineral nucleator protein BSP to the surface of collagen fibrils. Specifically, type XI collagen has been shown to play a critical role in the initiation of type I and II collagen fibrillogenesis, and, in the regulation of fibril diameter (60). As indicated by the model in Figure 5, the 6b sequence of the variable region of the COL11 A1 chain is believed to be expressed on the surface of type I collagen fibrils (61). We propose that BSP can bind to the cationic 6b sequence either as a 60 kDa fragment within mineralizing BMF (Figures 2 and 4) or as present in the BMP-1 processed COL A1 chain exposed on the surfaces of type I collagen fibrils (Figure 5). In this way, BSP/type XI collagen complexes could act as local nucleators to initate mineralization of collagen fibrils. The fact that BSP and the 6b containing 60 kDa type XI collagen fragment are also enriched within BMF suggests that BMF and collagen mineralization may share in part a common mechanism for mineral crystal nucleation.

### 4.2. Noggin

Noggin, along with Chordin, is a bone morphogenetic protein (BMP) antagonist. During mouse embryogenesis, Noggin is expressed at multiple sites, including developing bones. Interestingly, Nog (−/−) mice die at birth from multiple defects that include bony fusion of the appendicular skeleton (62, 63). Groppe J *et al.* (64) reported the crystal structure of the antagonist noggin bound to BMP-7, which showed that noggin blocks the molecular interfaces of the binding epitopes for both type I and type II receptors. Importantly, the affinity of site-specific variants of noggin for BMP-7 correlated with changes in bone formation and apoptosis in chick limb development, showing that these complexes are biologically inactive.

### 4.3. Chordin

Chordin dorsalizes early vertebrate embryonic tissues by binding to bone morphogenetic proteins and, like noggin, sequestering them in latent complexes. Scott *et al.* (65) showed that over-expression of BMP1 and TLL1 in Xenopus embryos counteracted the developmental effects of chordin. Mechanistically, BMP1 and mTLL-1 cleave chordin at sites similar to procollagen C-propeptide cleavage sites. Other BMP-1 family members mTLD and mTLL-2 do not, however, cleave chordin. Interestingly, Wardle *et al.* (66) showed that noggin was not affected by over-expression of BMP-1 (and presumably not cleaved by it). Based on expression and its enzymatic activity, BMP-1 is the major chordin antagonist in early mammalian embryogenesis and in pre- and postnatal skeletogenesis.

### 4.4. Osteopontin (OPN)

OPN is an RGD-containing, variably phosphorylated member of the SIBLING family of non-collagenous matrix proteins. It is expressed in bone and bone marrow by osteoblastic and osteoclastic precursor cells, multi-nucleated osteoclasts, osteocytes, and osteoblasts (67–69). Franzen *et al.* (70) showed that OPN null mice did not experience age related bone loss. Specifically, measures of osteoclast function were several fold lower, despite an increase in the volume and number of osteoclastic cells in the lower tibial metaphysis which was hypothesized as a compensatory change to overcome the observed inactivity of osteoclasts. These findings indicate that OPN is required for osteoclastic resorption of bone (70). Asou *et al.* (71) also found that osteopontin deficient calvarial bone was inefficiently resorbed when implanted in osteopontin null mice compared with controls. The osteoclast deficiency observed here seemed to be due to reduced angiogenesis in the OPN null mice since it could be reversed by applying OPN protein to the deficient calvarial bone surface prior to its implantation in OPN deficient mice. These results demonstrate that OPN is required both for vascularization and subsequent osteoclastic resorption of bone (71).

A central role for OPN in the metabolic regulation of bone is also supported by the finding that PTH induced bone resorption does not occur in the absence of OPN (72). OPN deficiency also enhanced the formative response of long bones of null mice to intermittent PTH injections relative to that of control mice (73).

OPN has long been believed to function as an inhibitor of calcification reactions (74) by binding specifically to the growing faces of mineral crystals. However, the extent of

phosphorylation is a critical modulator of OPN-mineral interactions (74, 75). For example, recombinant OPN and dephosphorylated OPN had no effect on hydroxyapatite crystal formation or growth. In contrast, milk OPN, which contains >30 phosphates/mole, promotes hydroxyapatite formation in static assays (74). In this context, Hunter *et al.* (76) has recently reviewed the topic of non-collagenous protein mediated biomineralization, e.g., the interaction of nucleator proteins with calcium and phosphate ions. They conclude that the widely held concept of epitaxic crystal initiation may not fit current evidence. Their review of existing data and new energy minimization calculations indicates that most known crystal-bone acidic protein interactions are electrostatic in nature and depend primarily upon the inherent high electronegative charge density of these interactions rather than upon a particular stereochemical conformation. As a result, Hunter *et al.* (76) have put forth a thoughtful alternate hypothesis, e.g., the flexible polyelectrolyte hypothesis of protein-biomineral interaction.

### 4.5. Osteopontin, bone sialoprotein, and DMP1 form individual complexes with MMPs

Fedarko and colleagues have shown that three SIBLING proteins form specific high affinity complexes with several individual proMMPs (pro-metalloproteinases) and their active forms expressed in skeletal tissues (77). BSP complexes with proMMP-2 and active MMP-2, while osteopontin binds proMMP-3 and active MMP-3, and DMP1 binds proMMP-9 and active MMP-9. For MMP-2, BSP is also able to induce catalytic activity in latent pro-MMP-2 as well as to rescue active enzyme from TIMP2-MMP-2 inhibitory complexes (78, 79). All complexes were disrupted by competition with serum complement factor H suggesting that analogous physiological SIBLING complexes are likely to be limited to sequestered areas near cells. Interestingly, bound MMPs displayed a diminished susceptibility to their respective natural and synthetic inhibitors (77) which may be particularly relevant to the invasive behavior of cancer cells which jointly express high levels of MMPs and these SIBLING proteins (80).

### 4.6. Bone acidic glycoprotein-75

BAG-75 is defined by its N-terminal sequence (LPVARYQNTEEEE) and its immunoreactivity with an anti-protein antibody, an anti-peptide antibody against its N-terminal sequence, and a monoclonal antibody (21, 81, 82). A highly acidic, phosphoprotein isolated from and enriched in primary or intramembranous bone (3), BAG-75 binds more calcium ions (133 atoms/mole) than either BSP or OPN on a molar basis. Functionally, when added to differentiated osteoclasts or to differentiating osteoclast culture models, BAG-75 inhibits bone resorption (55). In contrast to BSP or OPN, BAG-75 displays a propensity to self-associate into macromolecular complexes which have the capacity to also trap or sequester large numbers of phosphate ions (83). Operationally, BAG-75 has served as a useful immunoreactive biomarker for areas in osteoblastic cultures and in healing bone which will subsequently become mineralized (11, 19).

### 4.7 Dentin matrix protein 1

Originally thought to be expressed only in cementoblasts, ameloblasts, osteoblasts, and odontoblasts (84), DMP1 is now known to be also enriched in osteocytes (85). DMP1 is

cleaved by BMP-1 into a 55 kDa C-terminal fragment and 35 kDa N-terminal fragment (86, 87). Using furin-deficient cells, von Marschall and Fisher (86) compared three different isoforms of BMP1 and each displayed different activities. In contrast to collagen cleavage by BMP-1, DMP1 cleavage showed no enhancement by procollagenase enhancer protein. DMP1 mediates cell adhesion via its RGD site with $alpha_v beta_3$ receptors on cells. RGD binding was the same for full length and C-terminal fragment, however, a proteoglycan form of DMP1 was much less active in cell adhesion assays. (88).

Tartaiz *et al.* (89) showed that non-phosphorylated recombinant DMP1 acted as a hydroxyapatite nucleator in a gelatin gel static diffusion system while DMP1 phosphorylated *in vitro* has no effect on crystal growth or formation. Phosphorylated DMP1 synthesized by marrow stromal cells (30 phosphates/mole) however acted as an inhibitor of hydroxyapatite crystal growth and formation. These results seem to indicate that the position of phosphorylation is important for DMP1 to function as a mineralization inhibitor. *In vivo*, DMP1 exists predominantly in tissues as two highly phosphorylated fragments of 57 kDa and 35 kDa. The 57 kDa fragment also acted as a hydroxyapatite nucleator. Gajjeraman *et al.* (90) has also shown that the N-terminal fragment of DMP1 acted as a mineralization inhibitor. However, in contrast to Tartaiz *et al.* (89), they also found that both full-length recombinant DMP1 and post-translationally modified native DMP1 were able to nucleate hydroxyapatite in the presence of type I collagen. This disparity may be due in part to the purity of the DMP1 fragment tested since it is now appreciated that two fragments are derived from the N-terminus, a chondroitin sulfate linked fragment and a 37 kDa polypeptide (91). The proteoglycan fragment acts as a mineralization inhibitor while the remaining 57 kDa and 37 kDa fragments both promoter hydroxyapatite crystal formation and growth *in vitro*.

Physiologically, the function of DMP1 in mineralized tissues is closely tied to systemic phosphate regulation. DMP1 null mice and patients with DMP1 mutations exhibit autosomic hypophosphatemic rickets (92, 93). Over-expression of either full-length DMP1 or the 57 kDa C-terminal fragment in the DMP1-null strain largely rescued the skeletal phenotype in mice suggesting that the latter fragment is able to recapitulate the function of full-length DMP1 (94). It can be assumed that the reduced mineralization detected in DMP1 null bone (95) may be largely due to the hypophosphatemia which is a characteristic of the skeletal phenotype (96). The hypophosphatemia of DMP1 null mice is associated with elevated levels of FGF23, a well known negative regulator of systemic phosphate concentration, although the exact mechanism remains unclear (96).

### 4.8. Osteocalcin

Thought to be the most bone restricted protein produced by osteoblastic cells, osteocalcin is unusual in that it is post-translationally modified at up to three glutamic acid residues (GLA)/mole by gamma-carboxylation in a vitamin K dependent enzymatic mechanism (97). Due to its acidic character and GLA residues osteocalcin was initially presumed to be an important regulator of bone mineralization. However, the phenotype of osteocalcin knockout mice is mild and displays no major bone abnormalities (98). These results raised serious questions regarding the function of osteocalcin in bone. Recently, Karsenty and colleagues

have suggested that osteocalcin functions as a hormone released from bone into the blood to regulate insulin secretion by the beta-cells of the pancreas and to control insulin sensitivity by adipocytes (99). Part of a new complex metabolic feedback loop controlling bone, pancreas, and fat tissue, the active form of osteocalcin is undercarboxylated. In this way, the content of active, undercarboxylated osteocalcin in blood is believed to be derived via osteoclastic bone resorption of bone matrix. The process of osteoclastic resorption is believed to expose bone matrix derived osteocalcin to low pH conditions (<4.5) which lead to its chemical decarboxylation and release to the blood (99). The full pathway remains to be completely elucidated since the receptor on pancreatic and fat cells which recognizes undercarboxylated osteocalcin has not yet been identified. Also, the mechanism requires validation to show that acid mediated decarboxylation of osteocalcin is compatible with the temporal sequence of osteoclastic resorption *in vivo*. While this pathway has been largely worked out through genetic ablation of various genes in mice, a growing body of supporting evidence indicates that osteocalcin serves as a metabolic hormone in humans as well (100). Thus, this novel osteocalcin paradigm raises the intriguing question whether other bone matrix proteins may also possess hormonal functions regulating other metabolic systems, e.g., systemic calcium and phosphorus ion concentrations, fat storage, etc.

### 4.9. Fetuin (alpha2HS-glycoprotein)

Fetuin is produced by the liver and by osteoblastic cells (101). Highly acidic, fetuin has the capacity to bind large numbers of calcium ions. As such, fetuin is believed to be a major systemic inhibitor of ectopic calcification in the blood plasma and in tissue fluids (102). As a consequence, fetuin null mice develop micro-calcifications in small blood vessels and increased soft tissue calcification deposits. Interestingly, fetuin also forms a colloid or suspension of fine particles with insoluble calcium phosphate crystals which have been termed calciprotein particles or CPPs (102–105). Jahnen-Dechent and McKee (106) have suggested that CPPs participate in a circulating pathway in plasma to solubilize crystalline calcium deposits and transport finely divided crystal particles among sites of calcium loss (the intestine, kidney, bone). In this sense, recent stopped flow experimental data from this group suggests that the ability of fetuin to inhibit calcification is due to its ability to shield and stabilize early stage protein-mineral complexes as small CPP-like particles rather than permit aggregation of nuclei with subsequent mineral crystal precipitation (107). While very intriguing, the physiological significance CPPs in the process of osteoblast mediated bone formation and mineralization remains to be established. Although likely to be involved, the capacity of fetuin to form and stabilize particulate microcrystals of calcium phosphate (hydroxyapatite) has not been formally tested in the “mineralization by inhibitor exclusion” model proposed by Price *et al.* (17) (see above). Furthermore, the “calcification activity” detected in serum from humans to fish and sharks and partially characterized by Price and colleagues (108) also seems likely to be due, at least in part, to the fetuin in serum and its associated finely suspended colloidal particles of calcium phosphate.

### 4.10. Periostin

Kashima *et al.* (109) showed that periostin is a biomarker for intramembranous bone and is preferentially expressed in the bone produced by individuals with fibrous dysplasia, a benign condition, while not expressed in endochondral bone. Periostin is a disulfide linked GLA-

containing 90 kDa protein secreted by osteoblasts and osteoblastic cell lines whose expression is down regulated by phosphate ion and is restricted to the periosteum and periodontal ligament (109, 110). Periostin shows some homology to Betaig-h3, a protein that promotes adhesion and spreading of fibroblasts (111). Betaig-h3 also contains GLA residues. Gamma-carboxylated periostin localizes to mineralized bone nodules formed by mesenchymal stem cells. Periostin knockout mice expressing LacZ were grossly normal at birth, however, 14% of the deficient mice died before weaning. Since all of remaining LacZ positive mice were growth retarded, periostin is required for normal postnatal bone growth (112). In this regard, Bonnet *et al.* (113) showed that periostin is required for the anabolic response of bone to mechanical loading and physical activity. In its absence, long bones in periostin null mice do not respond anabolically to axial compression loading, whereas periostin (+/+) mice respond normally by increasing bone formation activity especially in the periosteum. Importantly, the biomechanical response of periostin null mice was rescued by injection of sclerostin-blocking antibody. These results indicate that periostin acts to block the actions of sclerostin on bone formation. When combined with the skeletal retardation observed in periostin null mice (112), these results show that periostin is a regulator of bone mass and microstructure in response to loading.

Maruhashi *et al.* (114) also showed that periostin stimulated the proteolytic activation of lysyl oxidase. Lysyl oxidase mediates the formation of reactive aldehydes on collagen chains which participate in formation of chemical cross-links. However, lysyl oxidase requires proteolytic activation by BMP-1. Interestingly, the amount of active lysyl oxidase was reduced in calvarial osteoblasts from periostin null mice, while over-expression of periostin promoted release of its propeptide and enzymatic activation. Since direct binding studies showed BMP-1 binds to periostin, these results indicate that periostin acts in analogous way to PCOLCE-1, an enhancer protein which increases by up to 20-fold the rate of procollagen processing by BMP-1 (115).

### 4.11. Tissue Nonspecific Alkaline Phosphatase

While its expression is not restricted to bone, alkaline phosphatase has long been a prominent component of bone mineralization mechanisms due to its high expression in mineralizing tissues, its enrichment within matrix vesicles, and the ease of its functional analysis. TNAP is associated with the external face of surfaces of osteoblastic and chondrocytic cells via a phosphatidylinositol anchor (116, 117) which can be released by a specific phospholipase D (118). The functional role of alkaline phosphatase in mineralization has become somewhat refocused by very recent studies combining the TNAP knockout with that for Phospho 1.

### 4.12. Phospho 1 phosphatase

Phospho 1 is a phosphatase whose actions are restricted enzymatically to mineralizing cells where it displays a catalytic preference for substrates phosphatidylethanolamine and phosphatidylcholine (119). Phospho 1 is expressed specifically at sites of mineralization of cartilage and bone in osteoblasts and newly formed osteocytes (120). Phospho 1 null mice display growth plate abnormalities, spontaneous fractures, bowed long bones, osteomalacia, and scoliosis in early life. The fact that over-expression of TNAP does not prevent the

development of skeletal abnormalities in the Phospho1 null mice, despite correction of plasma PPi levels and of greatly elevated TNAP levels, suggests that Phospho 1 functions through a pathway that is distinct from that of TNAP (8).

As a component of matrix vesicles, it has been suggested that Phospho 1 could produce free phosphate for initiation of matrix mineralization using phosphatidylethanolamine and phosphatidylcholine lipids in matrix vesicles (121). Previous models of mineralization in bone and cartilage have emphasized a role for alkaline phosphatase. However, while deficiency of alkaline phosphatase does diminish the mineral density of bone and growth plate cartilage in young TNAP knockout mice, careful transmission electron microscopic analyses revealed that initiation of mineralization still occurred within vesicles of TNAP deficient bone (32). These findings suggested that hypo-mineralization in TNAP-deficient mice results primarily from an inability of mineral crystals to proliferate beyond the limits of the vesicular membrane. This failure of the second (proliferation) stage of mineralization may be caused by an excess of pyrophosphate inhibitor in the surrounding extracellular fluid. As proposed by Millan and Terkeltaub and colleagues, the concentration of mineralization inhibitor pyrophosphate is regulated by the balance between its breakdown by alkaline phosphatase and its production by PC-1 (ENPP1) (122).

Functionally, Phospho 1 is required to initiate *de novo* mineral crystals within matrix vesicles. For example, treatment of mesenchymal micromass cultures with lansoprazole, a small inhibitor of Phospho 1 resulted in reduced Alizarin red staining. *In situ* hybridization showed that expression of Phospho 1 preceded mineralization (and correlated with TNAP). Importantly, treatment of developing embryos for 5 days with lansoprazole completely inhibited mineralization of all leg and wing long bones as assessed by Alcian blue/Alizarin red staining (123). Yadav *et al.* (8) simultaneously knocked out Phospho 1 and TNAP and showed that the double knockout was completely devoid of skeletal mineralization and exhibited perinatal lethality, despite relatively normal systemic phosphate levels. They concluded that Phospho 1 has a non-redundant functional role during endochondral ossification and proposed an inclusive model of calcification that places Phospho 1 phosphatase action within vesicles in the initiation of mineralization and the functions of TNAP, nucleotide pyrophosphatase phosphodiesterase-1 and collagen in the extravesicular progression (propagation) of mineralization. The reader is

### 4.13. Ectonucleotide Pyrophosphatase/Phosphodiesterase (ENPP1)

A transmembrane glycoprotein with a short cytoplasmic tail, single transmembrane segment, and an extracellular C- terminal domain, ENPP1 helps to regulate bone and cartilage mineralization by producing inorganic pyrophosphate. The enzyme exhibits both 5′-nucleotide phosphodiesterase I and nucleotide pyrophosphohydrolase activities (124). Based on the results of a double knockout of alkaline phosphatase and ENPP1, the Millan and Terkeltaub labs suggested that these two enzymes each served to balance the activity of the other. Specifically, the double knockout corrects bone mineralization abnormalities which occur in single deletions of each individual enzyme (122). As noted above, recent work with Phospho 1 has clarified the potential roles of ENPP1 both as a 1) producer of pyrophosphate mineralization inhibitor and 2), under conditions of limiting alkaline phosphatase activity, as

a producer of extracellular inorganic phosphate via its phosphodiesterase activity to feed the growth and propagation of mineral crystals (8).

### 4.14. Biological Effects of Hydroxyapatite on Bone Matrix Proteins

Hydroxyapatite is the major crystalline form of calcium phosphate in bone and cartilage tissues. However, hydroxyapatite is thought to be biologically inert. Several studies raise the prospect that hydroxyapatite crystals can modify cellular MMP production and function in bone. First, exposure of fibroblasts or breast cancer cells to hydroxyapatite, calcium pyrophosphate, or basic calcium phosphate crystals induces mitogenesis as well as increases the transcription and expression of MMP-1 and MMP-3 (125–127). In all cases, endocytosis is required for crystals to induce synthesis of MMPs. The effect of calcium phosphate crystals could be abrogated by phosphocitrate, although the mechanism is unclear. Second, we have shown that hydroxyapatite crystals are able to alter dramatically the functional activity of MMP-1 and MMP-3 (83). Specifically, these MMPs, when bound, changed their substrate specificity such that each catalyzed their own autolytic degradation in an intermolecular, calcium-dependent process. Interestingly, other proteases including MMP-2, trypsin, chymotrypsin, papain, and zinc-dependent thermolysin were all unaffected by exposure to hydroxyapatite crystals (83).

Recent work with PHEX (phosphate regulating endoprotease homolog, x-linked) further supports an active role for hydroxyapatite crystals in bone function. PHEX is a single pass transmembrane endoprotease which regulates systemic phosphate metabolism since its mutational inactivation causes x-linked hypophosphatemia (128). Specifically, we have found that a 60 kDa, catalytically active fragment of PHEX is enriched within mineralized biomineralization foci purified by laser micro dissection from osteoblastic cultures (Figure 6). BMF are the initial sites of mineral nucleation within at least three different osteoblastic culture models (9–11, 18, 19). Initial proteomic characterization revealed that mineralized BMF were physically enriched in phosphoproteins BSP and BAG-75 (2–4). However, we have continued to investigate over 35 other matrix proteins and calcium or phosphate channels/transporters as candidate constituents of BMF. Thus far, only four proteins have been found to be physically enriched within BMF complexes, e.g., BSP (9–11, 18), BAG-75 (9–11, 18), Phex, and type XI collagen (see Figure 2 above). This point is illustrated in Figure 6, which shows the specificity of this enrichment through comparisons with 110 kDa full length MEPE and a 57 kDa fragment of DMP1, which are notably not enriched in mineralized BMF. Because BMF are detectable before mineralization and become the exclusive sites of initial mineral production/deposition in osteoblastic cultures, we hypothesize that these enriched proteins and their fragments work together to mediate this unique function of BMF complexes, e.g., mineral crystal nucleation.

PHEX is an endoprotease which is structurally related to the M13 family of proteases. We have found that PHEX also exhibits a limited homology with MMP-3 (matrix metalloproteinase-3 or stromelysin), another zinc-dependent metalloprotease. A two way BLAST comparison of these sequences

Phex: 538-**N**A**F**YSAST**N**Q I **R** - - F**P**A**G**-553 (identities noted with bold symbols)

MMP-3: 358-**N**Q**F**WAIRG**N**EV**R**AGY**P**R**G**-375

reveals a statistically significant homology of 33% with the #538–553 PHEX peptide region depicted; four other peptides were also found to share a 30–40% identity with MMP-3 (not shown). Homologies >25–30% are generally considered “real” and likely reflect at least a partial shared evolutionary history. The homology with the #538–553 peptide may be particularly important because this region is believed to represent part of the PHEX substrate binding domain (10), whereas in MMP-3 it comprises part of the C-terminal hemopexin domain.

This newly identified structural relationship suggests that PHEX may share functional properties with MMP-3. Because PHEX is localized preferentially to mineralized BMF (Figure 6) and because the substrate binding domain of PHEX exhibits a limited structural homology with MMP-3 (see above), we asked whether the 60 kDa fragment of PHEX observed in BMF was produced by autolytic cleavage induced by exposure to hydroxyapatite crystals. Figure 7 shows that 60 kDa PHEX is preferentially produced only when recombinant 98 kDa PHEX is incubated with hydroxyapatite crystals in the presence of ASARM peptide. Recombinant 98 kDa PHEX is missing its single transmembrane spanning domain near the N-terminus and a small intracellular domain at the N-terminus of the native protein (129, 130). We conclude that PHEX was induced to cleave itself, as seen for MMP-1 or MMP-3 (83), upon exposure to hydroxyapatite crystals. Since a similar sized 60 kDa fragment was detected by Western blotting of both recombinant PHEX (Figure 7) and of laser micro dissected BMF complexes (Figure 6), we hypothesize that a single hydroxyapatite induced autolytic cleavage site for PHEX is located in the N-terminal half of the sequence yielding a 60 kDa fragment sharing the same C-terminus for both. This is further supported by the fact that Western blots used anti-PHEX antibodies specific for a peptide very near the zinc-binding domain of PHEX (gift of Dr. P. S. Rowe, Univ. of Kansas). Second, we hypothesized that the 60 kDa fragment contained the catalytic region (zinc binding domain and substrate binding site) of PHEX (130), e.g., the 60 kDa fragment was catalytically active.

As shown in Figures 8A and 8B, both PHEX activity and the 60 kDa PHEX fragment content are both 2–3 fold higher in crude osteoblast plasma membrane preparations isolated from mineralized UMR106-01 osteoblastic cultures exposed to hydroxyapatite crystals as compared with that where mineralization was blocked by serine protease inhibitor AEBSF (11) or by omission of exogenous phosphate. Our results strongly suggest that an active 60 kDa PHEX fragment is produced by exposure to hydroxyapatite crystals during the process of mineralization occurring within biomineralization foci. We speculate that the 60 kDa PHEX could exhibit altered enzymatic properties relative to the 98 kDa recombinant isoform and/or the 105 kDa full-length membrane bound isoform. In this way, 60 kDa PHEX could provide a local or systemic feedback signal marking the initiation of mineralization, e.g., to mobilize additional phosphate ions to feed the growth of the new mineral phase.

### 4.15. Sclerostin

Encoded by its gene *Sost*, Sclerostin is a secreted glycoprotein highly expressed by osteocytes. Inactivating mutations in humans leads to a rare high bone mass condition called sclerosteosis. Transgenic knockout of Sclerostin in mice also leads to increased bone formation and bone strength (131). These findings demonstrate that Sclerostin functions as an anabolic inhibitor, a blocker of bone formation. Its mechanism of action on osteoblasts/osteocytes appears to involve strong protein-protein interactions/complexes with Periostin, Noggin, and cysteine-rich protein 61. Cysteine-rich protein 61 (Cyr 61) regulates mesenchymal stem cell proliferation and differentiation, osteoblast and osteoclast function, and angiogenesis (132). Bonnet *et al.* (113) used mechanical stimuli to show that periostin is required for the anabolic response to mechanical loading and physical activity. Administration of anti-Sclerostin blocking antibody to periostin (−/−) null mice rescued the anabolic bone formation response to biomechanical stimuli which had previously been blocked by this transgenic deletion (131). Availability of Sclerostin is also regulated by its interactions with Noggin, with whom it forms tight, mutually inhibitory complexes ($2.9 \times 10^{-9}$ M) (133). The formation of the Sclerostin-Noggin complex directly neutralizes BMP binding activity and attenuates the effects of these two BMP antagonists. As a result, the net biological effect of Sclerostin may depend upon its balance between various binding partners in the extracellular matrix.

### 4.16. Tenascin C

A large oligomeric extracellular matrix glycoprotein, Tenascin is also known as hexabrachion or cytotactin due to its hexameric quaternary structure with six arms linked to a central globular domain (134). Its expression is restricted to vertebrate development where it appears during neural crest cell migration and later at sites of cartilage, bone, and tendon formation. Tenascin can function to promote neurite outgrowth *in vitro* and can inhibit cell interactions with fibronectin, where each of these functional interactions is mediated by distinct cell binding sites. Tenascin-C has been proposed to be involved in the development of articular cartilage and in the mechanism by which articular chondrocytes avoid the endochondral ossification process (which occurs with bulk of metaphyseal and diaphyseal chondrocytes) (135). Condensation is a crucial stage in skeletal development. It occurs when a previously dispersed population of cells gathers together to differentiate into a single cell or tissue type (136). Extracellular matrix molecules, receptors and adhesion molecules such as fibronectin, tenascin-C, syndecan, and N-CAM initiate condensation and set condensation boundaries. Hox genes and other factors like BMPs regulate the proliferation of cells within condensations. Growth of a condensation ceases when Noggin inhibits BMP signaling—which sets up the next stage in development which is overt cell differentiation (135). However, tenascin-C deficient mice generated by two groups (137, 138) show no apparent skeletal phenotype, e.g., normal development and adulthood, life span, and fecundity, as well as normal healing of skin and nerves.

### 4.17. Phosphate-regulating neutral endopeptidase (PHEX)

Identification of the genetic targets of X-linked hypophosphatemia, autosomal dominant hypophosphatemic rickets (ADHR), X-linked hypophosphatemia (XLH), autosomal

recessive hypophosphatemia (ARHP), and hereditary hypophosphatemic rickets with hypercalciuria (HHRH) has identified at least eight genes whose function is required either indirectly or directly to control the systemic phosphate concentration in blood (139–141). One of these is zinc endoprotease PHEX (phosphate-regulating gene with homologies to endopeptidases on the X chromosome) (142). Loss of function mutations in the *Pex* gene cause X-linked hypophosphatemic rickets. A *Pex* mutation is also responsible for the 'hyp' mouse skeletal phenotype: hypophosphatasia and elevated FGF23 expression (143). The precise function of PHEX is unclear although two genetic rescue studies using bone restricted promoters have demonstrated it has a direct, local effect on bone mineralization as well suggest it has an indirect effect on systemic phosphate homeostasis (129, 144). This conclusion is also supported by results of transplantation of mutant Hyp or control periostea into wild type or phosphate supplemented Hyp mice and into control or phosphate-depleted normal mice (145). Also, over-expression of human PHEX under the beta-actin promoter seemed to rescue the bone mineralization defect, but did not affect phosphate homeostasis suggesting again independent physiological mechanisms control hyperphosphaturia and bone mineralization in XLH and Hyp (146). Since all three transgenic attempts to replace mutant PHEX in the Hyp mouse were able to at least partially rescue bone mineralization, it is widely believed that PHEX has a role in mineralization of bone.

Using a small peptide substrate, Liu *et al* (147) showed that MEPE binding to PHEX inhibits the peptidase activity of the latter enzyme. Subsequently, McKee and co-workers showed that PHEX can cleave and inactivate the mineralization inhibitory activity of some ASARM peptide isoforms derived from MEPE (148). However, the most highly phosphorylated forms, which are also the predicted to be the most effective minhibins (mineral crystal growth inhibitors), were not effectively cleaved by Phex. Minhibins is the term coined by Rowe and colleagues to refer to the ASARM peptides representing highly conserved, acidic, protease-resistant C-terminal regions of SIBLING proteins DMP1, BSP and MEPE (149). This latter group has shown that degradation of MEPE, DMP1, and release of SIBLING ASARM-peptides (minhibins) is dramatically increased in the HYP (Phex-deficient) mouse and that ASARM-peptide(s) are directly responsible for defective mineralization in the HYP skeleton (148, 149). It appears that cathepsin B, a neutral protease, may mediate the accelerated release of ASARM peptides in HYP mice since cathepsin inhibitors rescue the defect (150). While these findings explain a number of features of the bone phenotype associated with loss of PHEX in man and mice, they do not clarify the role of PHEX in normal mineralization of bone.

### 4.18. Matrix extracellular phosphoglycoprotein (MEPE, OF45)

MEPE, matrix extracellular phosphoglycoprotein, was first isolated from tumor tissue from a patient with oncogenic hypophosphatemia and is capable of inhibiting kidney tubular phosphate re-absorption (151) and absorption by the intestine (152)—features ascribed to phosphatonins, substances acting on multiple organs to regulate phosphate metabolism. It is also highly expressed in osteocytes as compared to osteoblasts. Deletion of this gene in mice results in increased bone formation and bone mass and less trabecular bone loss with age (153) whereas over-expression of MEPE caused a growth and mineralization defect with altered vascularization of bone and kidney (154). MEPE contains a highly phosphorylated

C-terminal region called the ASARM peptide. Based on work with recombinant proteins, PHEX binding to MEPE regulates the proteolytic release of ASARM peptides which act as potent inhibitors of mineralization in the HYP mouse (147) and in X-linked hypophosphatasia (155).

### 4.19. Functional importance of proteolysis in activation of transglutaminase and PCOLCE

Transglutaminase and PCOLCE [procollagen C-proteinase enhancer] are both required for normal bone formation and mineralization. PCOLCE is a 55 kDa precursor with an elongated structure that enhances the C-terminal processing of type I, II, and III collagen precursors catalyzed by BMP-1 (procollagen C-terminal propeptidase) (156). Following proteolytic activation, a 36 kDa active PCOLCE fragment mediates the effect on BMP-1 via its two CUB domains (157, 158). The C-terminal netrin-like domain of PCOLCE shares sequence homology with a similar domain in metalloproteinases. Although inactive with MMP-2, BMP-1, and ADAMTS, the netrin-like domain can accelerate procollagen processing in the presence of heparin or heparan sulfate glycosaminoglycan chains (159). Thus, PCOLCE alone can increase collagen processing about 10-fold (157), whereas a further increase in rate can be accomplished in association with cell surface heparan sulfate proteoglycans (159). Heparan sulfate proteoglycans are common components of most cell surfaces including osteoblasts.

Interestingly, PCOLCE (−/−) male mice possess larger long bones with altered geometries that increase resistance to loading compared with wild type bones. Female (−/−) long bones did not show this change. Under mechanical testing, null male long bones revealed diminished material properties which apparently were compensated for by adapative changes in bone geometry, e.g., making the bones bigger. For PCOLCE (−/−) vertebrae, both sexes exhibited diminished mechanical properties relative to controls. Similar to the male long bones, knockout vertebrae were thickened and had more numerous trabeculae with abnormal mineral crystal morphology. All of these findings appear consistent with the observed presence of abnormal collagen fibrils in both mineralized and non-mineralized tissues (160). Thus, PCOLCE is also a determinant of the mechanical and geometrical properties of bone in mammals mediating its effects through fibrillar collagens I, II, and III. It is particularly interesting that treatment of mineralizing osteoblastic cells with covalent serine protease inhibitor AEBSF blocks the activation of PCOLCE-1, while also blocking mineralization (11). Based on these results, activation of PCOLCE represents a protease regulated step in the mineralization pathway resulting in increased collagen fibrillogenesis and deposition.

Since transglutaminases, of which seven TGase genes have been cloned thus far, also require proteolytic activation, we questioned whether this step is also regulated similarly to that for PCOLCE. All TGase enzymes possess a conserved cys-his-asp catalytic triad and a calcium-binding domain although individual differences exist, e.g., TGM1 is a transmembrane enzyme and TGM2 contains a unique C-terminal GTPase/ATPase signaling region (161). The properties and enzymatic functions of TGase I, II, III, and Factor XIII are the most thoroughly studied. Individual TGases are the target genes in several human syndromes: Lamellar Ichthyosis of the skin (TGM1); Peeling Skin Syndrome (TGM5); and

unstable blood clots and increased bleeding tendency (Factor XIII). However, at present, a requirement for transglutaminase for mineralization of bone is yet to be firmly established since the TGase II (−/−) mouse exhibits no developmental or postnatal skeletal defects (162, 163). In view of the multiple copies of TGase and their wide distribution, it is likely that functional redundancy could be operative particularly since Factor XIII is expressed by osteoblast lineage cells (164) and is present in plasma.

Despite this, the work of Kartinen and colleagues suggests that transglutaminase II is functionally important for bone formation. They showed that mineralization of MC3T3-E1 (subclone 14) pre-osteoblastic cultures could be blocked completely by treatment with cystamine, a TGase inhibitor (165). Osteoblast differentiation was also blocked as expression of bone sialoprotein, osteocalcin and alkaline phosphatase was diminished. Within bone and in osteoblastic cultures, osteopontin, collagen, and osteocalcin have been consistently shown to be substrates for transglutaminase catalyzed crosslinking (166, 167). Intramolecular crosslinking of osteopontin increases its collagen binding properties five-fold (168). While these, and other (169), studies imply TGase activity may influence bone cell signaling directly or indirectly, supporting conclusive mechanistic evidence is not available. Furthermore, it is unclear whether other transglutaminase isoforms, e.g., TGase III, could also be expressed by osteoblastic cells *in vivo*. Finally, other than for Factor XIII and thrombin, the identity of the cleavage site and of the physiological protease mediating initial activation of bone transglutaminases remains unknown.

### 4.20. Neutral Proteases in Bone

While specific examples of protease actions in bone development and mineralization are given above, it is likely that other functions for proteases in bone will be identified, aside from those associated with osteoclastic bone resorption. For example, during *in vitro* differentiation of tibial derived rat osteoblasts, expression is increased for MMP-2 and MMP-9 and membrane gelatinase MMP-14. These proteases, along with TIMP-1 and TIMP-2, are developmentally regulated in accordance with the maturation stage of the osteoblastic cells. MMP-14 is expressed in differentiating osteoblastic cells and is localized in cells forming nodules. MMP-14 expression *in vivo* in rat tibias (day 7) also shows the highest levels among osteogenic cells, in lining osteoblasts on the newly formed trabeculae under the growth plate and on the endosteal surface of cortical bone (170). MMP-2 (gelatinase-A) and MMP-9, also known as 72 kDa and 92 kDa type IV collagenase, respectively, specifically cleave type IV collagen a major component of basement membranes. No bone phenotype has yet been reported in MMP-2 or MMP-9 null mice (171, 172). BMP-1, a $Zn^{+2}$ metalloproteinase, catalyzes biosynthetic processing of most collagens expressed by bone cells as well as other extracellular proproteins. Actually, BMP-1 comprises a family of four closely related enzymes which may display a redundancy in function (65). This latter point may explain the absence of a dramatic phenotype in BMP-1 null mice (173). Interestingly, transgenic deletion of caspase-3 leads to delayed ossification and decreased bone mineral density (174) due in part to impaired differentiation of bone marrow stromal stem cells. MT1-MMP is a transmembrane protease which acts on extracellular matrix components in the pericellular environment. Its deletion causes no detectable embryonic abnormality, but rather brings about progressive postnatal changes,

e.g., craniofacial dysmorphism, osteopenia, arthritis, and dwarfism as well as fibrosis of soft tissues (175, 176). Clarification of a potential mechanism for these changes was provided by Holmebeck K *et al* (177). They showed that collagen cleavage in osteocytes from mice deficient in MT1-MMP was disrupted and ultimately led to the loss of formation of osteocyte cell processes called dendrites important in inter-cellular communication. A potential implication from these static studies is that MTI-MMP mediates an active invasive collagen degradation process during osteocyte embedding.

SKI-1 is a member of the proprotein convertase family of serine proteases (178). PCs are required to process or mature the structure of growth factors, neuropeptides, toxins, glycoproteins, viral capsid proteins, and transcription factors. Transmembrane transcription factor precursors SREBP-1 and -2 are activated in a sequential process involving first SKI-1 cleavage and then S2P protease cleavage (179). After activation, the N-terminal fragments of SREBP-1 and SREBP-2 can be imported into the nucleus where they regulate gene expression by binding to transcriptional promoters containing consensus SRE sequences (180). CREB/ATF family transcription factors [ATF-6, LUMAN (CREB3), OASIS/BBF2H7 (CREB3L2), CREB-H, CREB-4, and AIbZIP/Tisp40 (CREB3L4)] also include transmembrane proteins which require SKI-1 catalyzed release of an N-terminal transcriptionally active fragment. SKI-1 is also an initiator of the unfolded protein response and acts generally to decrease cellular stress by increasing chaperone production, influencing ER-associated degradation of proteins, and regulating membrane remodeling (181).

Recent studies suggest a functional role for SKI-1 in normal bone formation (22). First, the skeletons of mice deficient in SKI-1 activatable transcription factor OASIS exhibit a severe osteopenia characterized by a type I collagen-deficient bone matrix and reduced osteoblastic activity (182, 183). Second, mice over-expressing ICER, a dominant negative effector of CREB/ATF transcription factors, displayed dramatically reduced trabecular bone and a markedly reduced femoral bone formation rate (184). Third, SKI-1 is required for normal cartilage morphogenesis in zebra fish (185). In a similar way, conditional inactivation of SKI-1 in mice using a type II collagen Cre recombinase transgene displayed abnormal calcification of the growth plate (186). Homozygous germ-line deletion of SKI-1 is embryonic lethal in mice (187). Finally, we have recently shown that inhibition of SKI-1 in osteoblastic cultures blocks the deposition of mineral crystals by blocking the expression of mineralization related proteins PHEX, type XI collagen, fibronectin, and DMP1 (188).

## 5. SUMMARY AND PERSPECTIVE

The role of extracellular non-collagenous proteins, cell surface and secreted, in biomineralization has evolved dramatically from initial considerations of calcium binding and cell adhesion. While detailed structure/function relationships are still under investigation, it is now clear that some proteins, such as osteocalcin, act as systemic hormones which serve to integrate the metabolic function of bone, fat and nervous tissues. Others, such as sclerostin, periostin, act as local regulators of the Wnt pathway regulating the osteocyte's response to mechanical loading. Fetuin is now well established as a product of bone cells as well as hepatocytes and plays a role in vascular calcium particle transport.

Interestingly, it is now possible to assign roles during biomineralization. Phosphatase Phospho 1, a vesicular component, is required to form the initial mineral crystals *in vitro* and *in vivo*, while alkaline phosphatase and ENPP1 appear to be needed to propagate or grow these initial crystals. Phex, DMP1, and MEPE all participate in the regulation of systemic phosphate, an ion critical for forming calcium hydroxyapatite. Newly identified protein-protein interactions like that proposed for bone sialoprotein and a specific, cationic, alternative splice variant of type XI collagen allow us to localize phosphoprotein nucleators at the gap regions of collagen fibrils within mineralizing osteoid. Also, bone sialoprotein is capable of rescuing catalytically active MMP-2 from previously inactive TIMP-MMP-2 complexes. Recent studies also suggest that hydroxyapatite crystals themselves may directly influence the catalytic function of metalloproteinases present within mineralized bone and cartilage. Finally, it now appears that expression of a number of mineralization related extracellular matrix proteins may be regulated in a common pathway. However, further work is needed to determine the extent to which the catalytic and inhibitor specificities of individual enzymes such as PHEX can be modified. Conceptually as well, the model of bone biomineralization proposed by Hunter *et al.* (76) has moved from a fixed epitaxic view of protein-mineral crystal interactions consistent with invertebrate shells to a less organized “flexible polyelectrolyte” model which reflects two decades of structural studies on acidic phosphoproteins such as osteopontin and bone sialoprotein.

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## Abbreviations

| | |
|---|---|
| **BMF** | biomineralization foci |
| **BSP** | bone sialoprotein |
| **AEBSF** | 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride |
| **SKI-1** | site-1, MBTPS1, or subtilisin/kexin isoenzyme-1 |
| **PCOLCE1** | procollagen C-terminal propeptidase enhancer1 |
| **1,25-vit.** | D3-MARRS protein, membrane associated, rapid response steroid binding receptor |
| **BMP** | bone morphogenetic protein |
| **TGase** | transglutaminase |
| **ADHR** | X-linked hypophosphatemia, autosomal dominant hypophosphatemic rickets |
| **XLH** | X-linked hypophosphatemia |
| **ARHP** | autosomal recessive hypophosphatemia |

| | |
|---|---|
| **HHRH** | hereditary hypophosphatemic rickets with hypercalciuria |
| **ENPP1** | PC-1, ectonucleotide pyrophosphatase/phosphodiesterase |
| **Fetuin** | alpha$_2$HS-glycoprotein |
| **TNAP** | tissue nonspecific alkaline phosphatase |
| **DMP1** | dentin matrix protein 1 |
| **OPN** | osteopontin |
| **NTD** | N-terminal domain |
| **GLA** | gamma-carboxyglutamic acid |
| **SIBLING** | small integrin-binding ligand, N-linked glycoprotein |
| **BGP** | beta-glycerolphosphate |
| **MMP** | matrix metalloproteinase |
| **BAG-75** | bone acidic glycoprotein-75 |
| **PC** | proprotein convertase |
| **Osx** | osterix |
| **BSA** | bovine serum albumin |
| **FBS** | fetal bovine serum |

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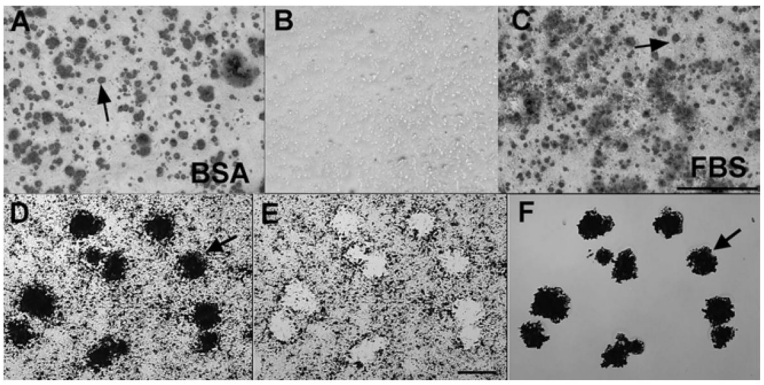


**Figure 1.**

Biomineralization foci can be isolated from total cell layers by laser capture microscopy. A and B, UMR-106-01 osteoblastic cells were cultured in serum-depleted conditions (BSA), or C, the presence of serum (FBS). Cultures were stained with Alizarin red S to detect hydroxyapatite crystals. B, both conditions failed to mineralize in the absence of BGP. Arrows point to mineralized BMF (A and C). Scale bar: 500 μm. D–F, laser capture microscopy of Alizarin red S stained BMF from UMR-106-01 culture. Arrows refer to the same BMF structures in all panels. D, microscopic view of field to be laser-captured. E, appearance of the residual cell layer left behind after laser dissection of mineralized BMF. F, purified BMF temporarily affixed to the “cap” used for laser capture. Scale bar: 25 μm. (Reproduced with permission of the J. of Biological Chemistry).

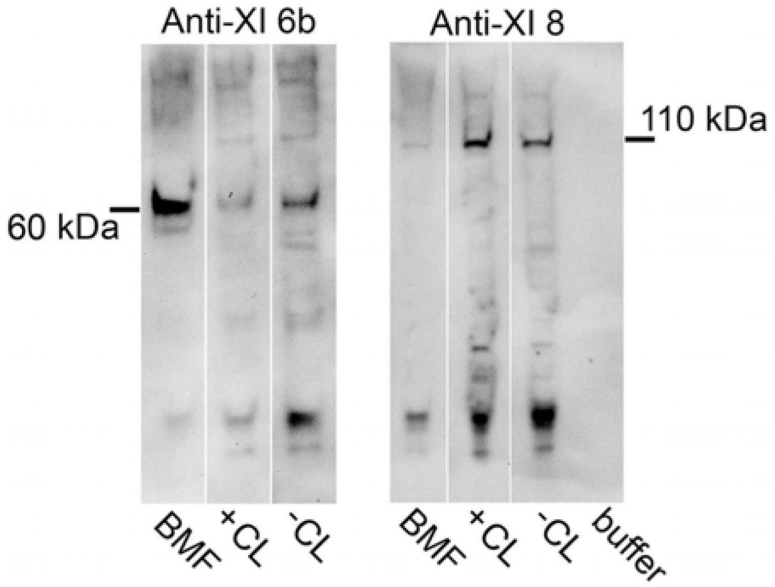


**Figure 2.**

Type XI collagen 6b splice variant is enriched in biomineralization foci. Fractions from osteoblastic cultures in Figure 1 were extracted and solubilized and subjected to Western blotting. A 60 kDa N-terminal 6b immunoreactive fragment of the COL11A1 chain was enriched in mineralized BMF (see F, Figure 1) compared to that from the total cell layer from mineralized (see D, Figure 1) or un-mineralized cultures (see B, Figure 1). In contrast, immunoreactive splice variant 8 was not enriched in BMF. KEY:BMF, extract of laser micro dissected biomineralization foci; +CL, extract from cell layer from mineralized culture; −CL, extract from cell layer from un-mineralized culture. Antibodies kindly provided by Dr. J. T. Oxford.

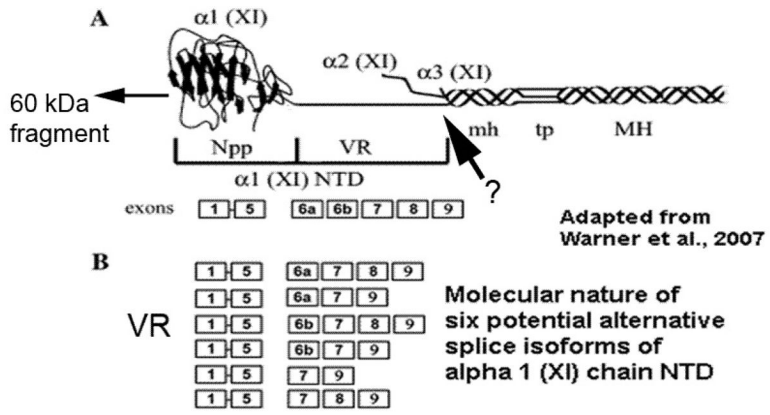


**Figure 3.**

Model of type XI collagen structure [adapted from Warner *et al.* (58)]. A, Proposed trimeric structure showing large non-helical N-terminal domain for alpha 1 chain. Arrow demarks approximate position of proteolytic cleavage by unknown protease which generates 60 kDa N-terminal fragment found enriched within biomineralization foci (see Figure 2). B, Description of the composition of six different possible splice variant sequences for the N-terminal domain. KEY: VR, variable region; NTD, N-terminal domain; Npp, N-terminal propeptide.

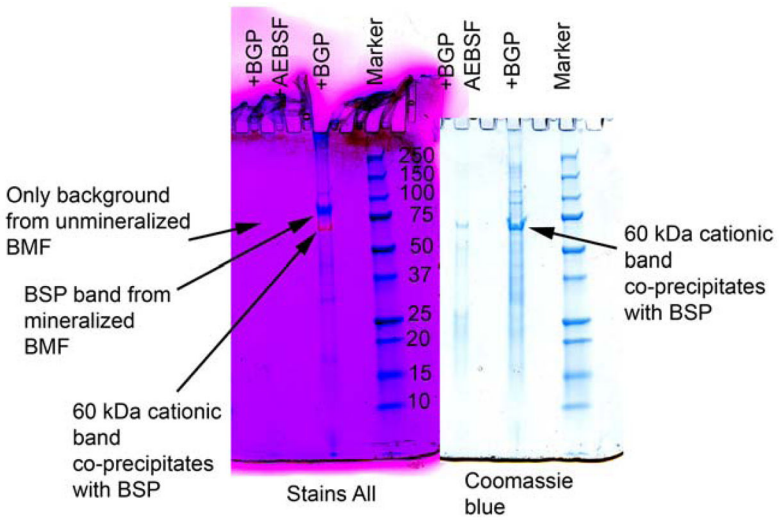


**Figure 4.**

Bone sialoprotein is immunoprecipitated together with 60 kDa cationic type XI N-terminal fragment only from mineralized osteoblastic cultures. Extracts of mineralized and un-mineralized osteoblastic cultures were immunoprecipitated with anti-bone sialoprotein antibodies. Immunoprecipitates were subjected to SDS-PAGE and the same gel was stained sequentially with Stains All (left) and then with Coomassie blue dye (right). KEY: +BGP +AEBSF, extract from culture where mineralization was inhibited by 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride; +BGP, extract from mineralized culture; marker, molecular weight standards.

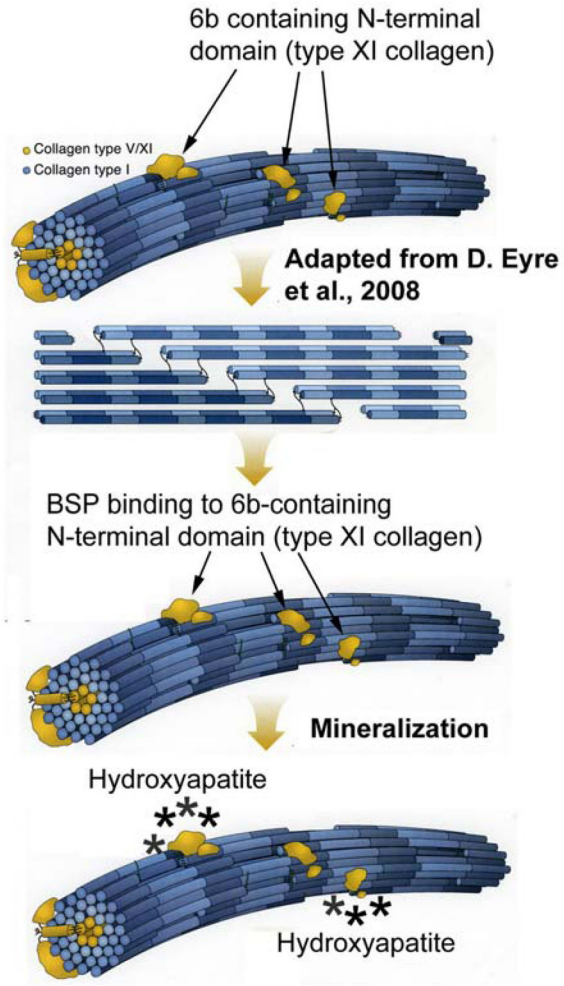


**Figure 5.**

Model for mineralization of collagen fibrils involving 6b splice variant containing type XI collagen and phosphoprotein BSP. Adapted from image by D. Eyre *et al.* (61), cationic 6b containing N-terminal domains are positioned on the surface of type I fibrils where they can bind mineralization nucleator BSP. We propose this mechanism may fulfill the 1964 phosphoprotein-collagen hypothesis of Glimcher, and, Veis and Schleuter.

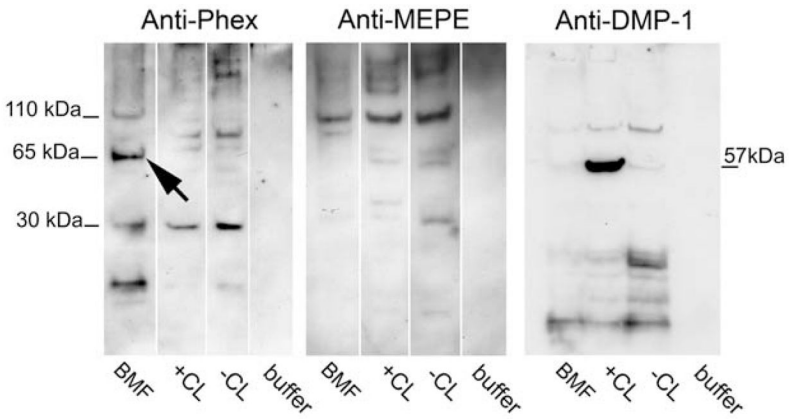


**Figure 6.**

Immunoblotting shows that a 60 kDa fragment of PHEX is specifically enriched in biomineralization foci whereas MEPE and DMP1 are not. BMF were isolated from Alizarin red stained cell layers using laser micro dissection (see Figure 1), and along with total cell layer fractions, were extracted as described by Huffman *et al.* (11). Six micrograms of total protein were applied to each lane and Western blotted with monospecific antibodies using chemiluminescent detection. Large arrow denotes band specifically enriched in purified BMF. KEY: BMF, extract of laser micro dissected biomineralization foci; +CL, extract from cell layer from mineralized culture; −CL, extract from cell; and, buffer alone, extraction buffer only. Antibodies were kindly provided by Drs. P.S. Rowe and L.D. Bonewald. referred to the publication by Yadav *et al.* (8) for a more complete description of their “unified model of mineralization”.

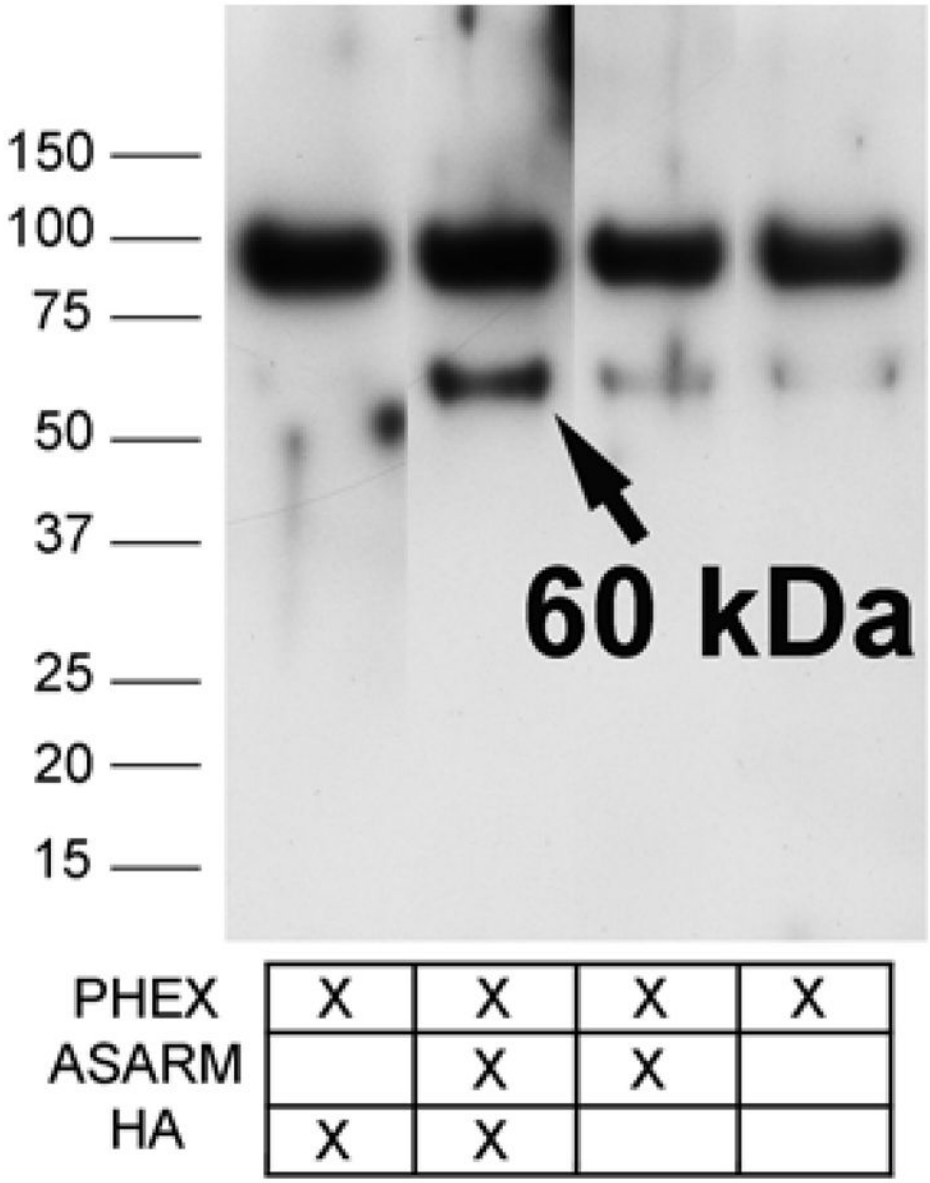


**Figure 7.**

Hydroxyapatite induces autolytic cleavage of PHEX in presence of ASARM peptide. KEY: PHEX, recombinant 98 kDa secPHEX; ASARM, ASARM peptide; and HA, hydroxyapatite crystals. Proteins were kindly provided by Dr. Peter S. Rowe.

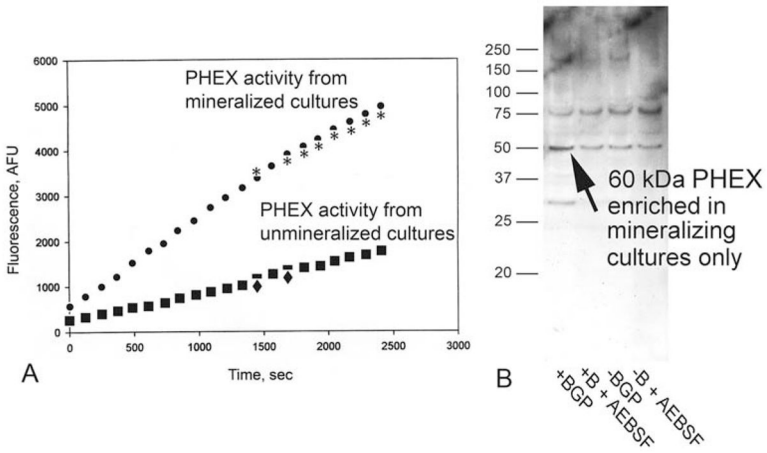


**Figure 8.**

PHEX endoprotease activity and 60 kDa PHEX fragment content are higher in membranes from mineralized osteoblastic cultures versus un-mineralized controls. A, Crude membranes were isolated and assayed for PHEX endopeptidase activity with a specific fluorescent substrate assay as previously described (147). Cultures assayed were either mineralized by addition of BGP (● and *) or treated with AEBSF to block mineralization (■ and ◆) (188). Individual symbols on the graph represent duplicate analyses. Activity was normalized based on the protein content of membrane preparations. (Data obtained in collaboration with Drs. S. Liu and D. Quarles). B, Crude membranes from mineralizing cultures are enriched in the 60 kDa PHEX band. Comparing the data in Figures 8A and 8B leads to the conclusion that the 60 kDa PHEX band is catalytically active. KEY: +BGP, mineralized; +B + AEBSF, mineralization blocked with AEBSF; −BGP, unmineralized (without BGP); −BGP + AEBSF (without BGP but with AEBSF). (Data obtained in collaboration with Drs. S. Liu and D. Quarles.)