Association of Specific Proteolytic Processing of Bone Sialoprotein and Bone Acidic Glycoprotein-75 with Mineralization within Biomineralization Foci*

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Mineral crystal nucleation in UMR 106-01 osteoblastic cultures occurs within 15–25-μm extracellular vesicle-containing biomineralization foci (BMF) structures. We show here that BAG-75 and BSP, biomarkers for these foci, are specifically enriched in laser capture microscope-isolated mineralized BMF as compared with the total cell layer. Unexpectedly, fragments of each protein (45–50 kDa in apparent size) were also enriched within captured BMF. When a series of inhibitors against different protease classes were screened, serine protease inhibitor 4-[(2-aminoethoxy)benzenesulfonyl]fluoride HCl (AEBSF) was the only one that completely blocked mineralization within BMF in UMR cultures. AEBSF appeared to act on an osteoblast-derived protease at a late differentiation stage in this culture model just prior to mineral deposition. Similarly, mineralization of bone nodules in primary mouse calvarial osteoblastic cultures was completely blocked by AEBSF. Cleavage of BAG-75 and BSP was also inhibited at the minimum dosage of AEBSF sufficient to completely block mineralization of BMF. Two-dimensional SDS-PAGE comparisons of AEBSF-treated and untreated UMR cultures showed that fragmentation/activation of a limited number of other mineralization-related proteins was also blocked. Taken together, our results indicate for the first time that cleavage of BAG-75 and BSP by an AEBSF-sensitive, osteoblast-derived serine protease is associated with mineral crystal nucleation in BF and suggest that such proteolytic events are a permissive step for mineralization to proceed.

Bone is a vascularized tissue that uniquely becomes mineralized as part of its developmental program (1). Mineralized bone serves essential vertebrate functions, including structural support, reversible storage for calcium and phosphorus, and as a reservoir for toxic metals and carbonate (2). Bone tissue is composed of osteoid; osteoblasts, which produce and mineralize new bone; osteoclasts, which resorb bone; and osteocytes, mature osteoblasts that maintain bone viability (1–3). Osteoid is a type I collagen-rich extracellular matrix enriched in acidic noncollagenous proteins (4). Using fetal rat calvaria cell cultures, Bellows et al. (5) showed that osteoid is unmineralized when initially deposited, and mineral crystals form within nodular structures over the following 48–72 h. Bone matrices can be classified as lamellar, based on a highly organized layered structure, or woven bone. Woven bone is formed during embryonic development, fracture healing, and at sites receiving mechanical stimulation in excess of 3,000 microstrain (6); lamellar bone replaces woven bone later in development.

The question of whether bone mineralization is under direct osteoblastic control or whether it is purely a passive chemical process is under active investigation. Schinke et al. (7) have proposed that calcification reactions in vivo are passive physiochemical processes occurring readily where local mineralization inhibitors are overwhelmed. In support of this hypothesis, Murshed et al. (8) produced a calcified dermal layer in transgenic mice expressing alkaline phosphatase in skin under the control of the type I collagen α chain promoter (2). Similarly, Luo et al. (9) and Murshed et al. (10) showed that matrix GLA protein is a passive local inhibitor of vascular calcification because deficient mice calcify their thoracic aorta. The latter approach emphasizes the formation of hydroxyapatite crystals as the primary experimental outcome.

A second view focuses on the active role of local extracellular nucleation complexes such as biomineralization foci (11, 12), crystal ghosts (13, 14), matrix vesicles (15), and the hole regions of collagen fibrils (16) with matrix vesicles (17, 18) or with extracellular matrix phosphoproteins (12, 19, 20). We have proposed that mineralization can be divided

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* This work was supported by National Institutes of Health Grants R21 DE14619 (to J. P. G.) and R01 AR052775 (to J. P. G.) and small grants from the Women’s Council of the University of Missouri, Kansas City (to N. T. H.). Parts of this research were presented in preliminary form at the annual meeting of the American Society for Biochemistry and Molecular Biology, June 12–16, 2004, Boston, MA; the American Society for Bone and Mineral Research, September 23–27, 2005, Nashville, TN; and the American Society for Bone and Mineral Research, September 15–19, 2006, Philadelphia, PA. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S3.

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into a cell-mediated nucleation phase within BMF, followed by passive growth and expansion of these initial crystals (11, 12). In this model, once the initial crystals reach sufficient size and number, the BMF barrier function is abrogated, facilitating the passive growth and expansion of the initial mineral phase into the larger, territorial collagenous matrix. The latter research focuses on the in vivo functionality of the mineralized bone product (10–19). In this context, hydroxyapatite crystal formation is envisioned to occur in a manner that facilitates subsequent vascular access to the crystals and placement of crystals within the organic matrix so as to facilitate mechanical support for organs, joints, muscles, and tendons.

Bone osteoid is enriched in phosphoproteins, acidic glycoproteins, and proteoglycans, some of which like BSP or its fragments are nucleators of hydroxyapatite crystals (20, 21). We have shown that phosphoglycoprotein BAG-75 expression delineates future extracellular sites of mineralization in vivo within woven bone and in vitro termed BMF (11, 12). BMF are 15–25-μm spherical extracellular structures containing several sizes of vesicles (15), which are sites of the first mineral crystals in the UMR osteoblastic model (12). Following plating, UMR cells proliferate and differentiate over the first 60–64 h and attain a competency to initiate mineralization in BMF, if supplemented with a phosphate source (22). BSP has also been localized to mineralizing nodules termed crystal ghost aggregates in rat bone, which are analogous to BMF (13, 14, 23). BSP is incorporated into BMF just prior the appearance of mineral crystals (12, 22). Based on these findings, we proposed that BMF structures function in an active mineralization process initiated and controlled by osteoblastic cells.

To better understand the role of BMF in bone mineral nucleation, we have begun to characterize the proteome of mineralized BMF isolated by laser capture microscopy. Our results show that isolated BMF are not only physically enriched in BAG-75 and BSP but also fragments of each. Screening inhibitors of the different classes of proteases revealed for the first time that serine protease inhibitor AEBSF completely blocked cleavage of BAG-75 and BSP, as well as mineral crystal nucleation within BMF. Two-dimensional SDS-PAGE comparisons of AEBSF-treated and control cultures suggested that activation of procollagen processing may also be inhibited. Taken together, our results demonstrate an association between serine protease cleavage of mineral nucleator BSP and mineral crystal nucleation within biomineralization foci and mineralization nodules.

**EXPERIMENTAL PROCEDURES**

**Materials**

Antibodies were from several sources as follows: nonimmune rabbit IgG (EMD Biosciences), anti-BAG-75 (number 503) (anti-peptide antibody) rabbit serum (24); anti-BAG-75 (number 504) (anti-protein antibody) rabbit serum (24); antiblem, bone sialoprotein LF-100 antiserum (Larry Fisher, NIDCR, National Institutes of Health); and monoclonal anti-BSP (WV1D1(9C5)) antibody (NIH Developmental Studies Hybridoma Bank, University of Iowa).

**Methods**

**Cell Culture**—UMR 106-01 BSP cells were passaged and cultured at 37 °C and 5% carbon dioxide as described previously (12, 22) and updated briefly here. Cells were seeded at a density of 1.0 × 10⁴ cells/cm² in Growth Medium (Eagle’s MEM supplemented with Earle’s salts, 1% nonessential amino acids (Sigma), 10 mM HEPES (pH 7.2), and 10% fetal bovine serum (Hyclone)). After 24 h, the medium was exchanged with Growth Medium containing 0.5% BSA (catalog number A-1933, Sigma) instead of FBS. Sixty four hours after plating, the culture medium was exchanged with Mineralization Media (Growth Medium containing either 0.1% BSA or 10% fetal bovine serum and 7 μM BGP). Cultures were then incubated for an additional 24 h, at the end of which (88 h) the cells were either subjected to MTT assay or fixed in 70% ethanol and then extracted for protein. In some experiments, protease inhibitors, including serine protease inhibitor AEBSF [(4-(2-aminoethyl)-benzenesulfonylfluoride HCl)] (EMD Biosciences), were added to cultures at 64 h after plating in Mineralization Media. Alternatively, AEBSF was added at 44 h after plating; inhibitor was then removed and exchanged for Mineralization Media at 64 h and the amount of mineralization analyzed at 88 h.

Primary mouse osteoblasts were isolated from calvaria of 5–7-day-old mice using a modification of the method described previously (25, 26). Briefly, the calvaria were aseptically harvested, and four sequential 20-min digests were performed in 0.05% trypsin, 0.2% collagenase in Hanks’ balanced salt solution. Fractions 2–4 were pooled, centrifuged, and resuspended in α-MEM containing 10% fetal bovine serum, 2 mM l-glutamine, 100 units/ml penicillin, and 30 μg/ml gentamicin (α-Growth Medium). 2 × 10⁴ cells were plated per T-75-cm² flask and allowed to reach confluency (3–4 days). Confluent flasks were then trypsinized and plated into 12- or 24-well culture dishes for experiments at a density of 20,000 cells per cm² growth area using media and supplements as described above. At confluency, the media were changed to α-MEM containing 5% FBS, 50 μg/ml ascorbic acid, 5 μM BGP, and other supplements as described above. BGP was omitted from some wells that served as an un-mineralized control. To test the effect of AEBSF, identical duplicate cultures were treated on days 3, 6, or 9 with 0.003 to 0.1 μM AEBSF. Phase contrast images were taken of living cultures on days 3–12. On day 12 after plating, one set of cultures was incubated with MTT as described below to determine cell viability. A second set of cultures was fixed on day 12 with 70% ethanol and processed for quantitative Alizarin red S staining as described below.
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**MTT Assay**—Culture wells were washed with Eagle’s MEM supplemented with Earle’s salts and then incubated with a solution of 0.5 mg/ml MTT in Eagle’s MEM for 1–2 h at 37 °C (27). Residual MTT solution was removed; the cells were disrupted by mixing briefly with dimethyl sulfoxide, and free reduced dye was read at 490 or 540 nm in a spectrophotometer.

**Quantitation of Mineralization**—After fixation in 70% ethanol, the cell layer was rinsed and stained with Alizarin red S dye as described previously (22). The same procedure was also used for serum-depleted cultures with the following modified washing protocol, e.g. the stained cell layer was rinsed once with 1 mm HEPES in nanopure water. A standard curve for Alizarin red S dye was constructed for each analysis, and amount of bound dye/culture well determined.

**Statistical Methods**—All statistical tests were performed using SigmaStat 3.1 software (Systat Software, Inc.). A one-way analysis of variance test was used to determine whether a statistical difference existed between the viability of UMR-106-01 cultures or the amount of mineral deposited. Subsequent pairwise multiple comparison tests were performed with the Student-Newman-Keuls or the Kruskal-Wallis method.

**Extraction of Cell Layer Fraction; One-step Method**—Cells were dislodged by scraping and then extracted with 75 mM potassium phosphate buffer (pH 7.2), containing 10 mM CHAPS, 75 mM sodium chloride, 50 mM tetrasodium EDTA, 10 mM benzamidine hydrochloride, 2 mM dithiothreitol, and 0.02% sodium azide for 1 h at 4 °C. Each extract was then homogenized briefly using a motorized pestle and clarified by ultracentrifugation at 30,000 rpm for 1 h at 4 °C. The resultant dialyzed extracts were used for comparative blotting studies where identical protein amounts were loaded per gel lane.

**Protein Determination**—Protein concentration of BMF extracts was determined using the NonInterfering Protein Assay by Geno-Technology Inc. (St. Louis, MO).

**Mass Spectrometric Analyses**—Protein bands and spots were detected by staining with Coomassie Blue G dye or with Sypro Ruby dye according to the manufacturer’s instructions (Bio-Rad). Excised gel bands/spots were reduced and alkylated followed by digestion with trypsin for 6–16 h (30). Peptides were extracted and subjected to reverse phase capillary liquid chromatography-mass spectrometry with a linear 2–70% acetonitrile gradient over 45 min in 50 mM acetic acid, in a 50 µm inner diameter × 7 mm Phenomenex C18 Jupiter Proteo capillary column. The column eluted directly into an LTQ linear ion trap mass spectrometer as described previously (30). The instrument was operated in the data-dependent mode in which one mass spectrum and eight collision-induced dissociation spectra were acquired per cycle. The data were analyzed using Mascot protein identification software (Matrix Science), with manual inspection of data base matches for validation. The Mascot identification program (Matrix Science) uses a statistical method to assess the validity of a match (31). Criteria used for protein identifications include matching the peptide based on the following: 1) the precursor (peptide) mass, and 2) MS/MS fragment masses present in the scan, coinciding with the predicted masses of peptides (and peptide fragment masses) from a data base entry. Protein searches are currently based on comparison to all or a subset of (rodent, for example) the sequences present in the MSDB data base, filename MSDB_20050227.fasta (February 27th, 2005 version). Protein
Mineralization of UMR Osteoblastic Cells Is Unchanged in Serum-depleted Conditions—To limit contamination by serum proteins in isolated BMF, we tested whether use of serum-free conditions would affect the amount or morphology of mineralization in UMR cultures. No differences were noted in the amount or morphology of mineralized BMF when conditions of serum depletion were compared with serum-replete conditions (compare Fig. 1, A versus C). As expected (22), few mineral crystals are evident when BGP is omitted (Fig. 1B). Quantitation of the amount of Alizarin red stain bound per well also revealed no significant differences (not shown). Manual counts of mineralized BMF formed under serum-containing and serum-depleted conditions showed no statistical difference (103 foci/cm² ± 6.56 S.D. versus 105 mineralized foci/cm² ± 6.08 S.D., p = 0.486 using one-way analysis of variance followed by Kruskal-Wallis method). These results confirm that the mineralization potential is unchanged in conditions of serum depletion.

**RESULTS**

Proteolytic Processing Is Essential for Mineralization of BMF—To specifically target proteins involved in the early stage of osteoblastic differentiation, 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. Gel images are representative of multiple analyses on two separate BMF preparations. Scale bar = 25 μm.

identifications made contain at least two peptides match in the MS/MS scans that meets or exceeds the threshold values for a 95% confidence level.

**Two-dimensional PAGE**—Gels were run according to the method of Witzmann et al. (32) and stained with either colloidal Coomassie Blue G, Pro-Q Emerald 300 glycoprotein stain (Invitrogen), or Pro-Q Diamond phosphoprotein stain (Invitrogen). PD-Quest (Bio-Rad) software was used to digitally analyze the colloidal Coomassie Blue G–stained gels comparing AEBSF–treated with nontreated cell layer and media fractions to identify proteins differentially expressed in one condition versus another.

**RESULTS**

Mineralization of UMR Osteoblastic Cells Is Unchanged in Serum-depleted Conditions—To limit contamination by serum proteins in isolated BMF, we tested whether use of serum-free conditions would affect the amount or morphology of mineralization in UMR cultures. No differences were noted in the amount or morphology of mineralized BMF when conditions of serum depletion were compared with serum-replete conditions (compare Fig. 1, A versus C). As expected (22), few mineral crystals are evident when BGP is omitted (Fig. 1B). Quantitation of the amount of Alizarin red stain bound per well also revealed no significant differences (not shown). Manual counts of mineralized BMF formed under serum-containing and serum-depleted conditions showed no statistical difference (103 foci/cm² ± 6.56 S.D. versus 105 mineralized foci/cm² ± 6.08 S.D., p = 0.486 using one-way analysis of variance followed by Kruskal-Wallis method). These results confirm that the mineralization potential is unchanged in conditions of serum depletion.

**BAG-75 and BSP and Fragments of Each Are Enriched within Purified Mineralized BMF**—Mineralized BMF, which appear as dark spots about 20–25 μm in diameter, were isolated from ethanol-fixed, Alizarin red-stained UMR cultures by laser capture microscopy (Fig. 1, D–F). Use of Alizarin red staining for identification provides a direct connection to previous work that defined BMF structures as sites of initial mineral crystal nucleation (12, 22). After laser capture of mineralized BMF, Fig. 1E depicts residual “holes” that are devoid of cells and show the underlying glass surface. Finally, Fig. 1F provides an image of the resultant captured BMF preparation with individual foci arranged in the same relative orientation as they appeared on the original stained slide (Fig. 1D). Visual inspection of captured populations revealed an absence of obvious cellular contamination. Furthermore, attempts at direct isolation of cells from the fixed cell layer using an LCM approach proved unsuccessful demonstrating that the fixed UMR cells adhere too tightly to the glass slide to permit their capture from this surface.

Following capture, ~6200 pooled BMF were extracted by mixing with 0.1 M Tris acetate buffer (pH 7.8), containing with 0.5% octyl glucoside, 0.05% SDS, 0.05 mM EDTA, and 0.02% sodium azide; BMF, represents proteins extracted from purified BMF (see Fig. 1F); + CL, cell layer extract of cultures after 24 h mineralization in β-glycerol phosphate (see Fig. 1D); − CL, cell layer extract of cultures not treated with β-glycerol phosphate (see Fig. 1B).

SDS-PAGE results show substantial enrichment of 75-kDa glycoproteins and phosphoproteins in the BMF extract when compared directly with + CL control (Fig. 2, see bands with asterisks). Sypro Ruby staining showed enrichment of bands in the 65-kDa range in BMF. In contrast, bands in the 10–15-kDa range appeared to be shared by both the BMF and total cell layer samples (Fig. 2). Although not quantitative, this comparative analysis is designed to identify those proteins substantially enriched within mineralized BMF. Our approach is based upon the hypothesis that BMF are structures assembled for the specific purpose of nucleating hydroxyapatite crystals in culture.

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and in primary bone (11, 12). Because mineral nucleation is a specialized function, our hypothesis predicts that BMF should exhibit a specialized proteome. Existence of clear differences (more than 5–10-fold) in 75-kDa glyco- and phosphoproteins between the BMF proteome and that of the +CL control supports this hypothesis. The absence of similar post-translationally modified proteins in the −CL control re-enforces this finding (Fig. 2).

Immunoblotting studies (Fig. 3, A–E) revealed that the 75-kDa glycoprotein phosphoproteins BAG-75 and BSP were both dramatically enriched in BMF only in the presence of BGP. Closer inspection reveals BMF fractions also contain a higher relative content of BAG-75 and BSP fragments (Fig. 3, B, D, and E, arrows). In the case of BAG-75, this was detected through use of an N-terminal 3-13 anti-peptide antibody (number 503), which is known to preferentially recognize a 50-kDa fragment (24). For BSP, a 45–50-kDa fragment was observable when the full-length BSP band was purposely overloaded (Fig. 3, C–E). Non-mineralizing cultures also contain a much smaller amount of the 45–50-kDa fragment (Fig. 3, D and E, −CL), although the gel band pattern is different from that for cultures treated with BGP (+CL). These findings validate the use of laser capture microscopy as a means to purify mineralized BMF from UMR 106 cell monolayers. Enrichment of full-length protein within BMF links BAG-75 and BSP with mineral nucleation, whereas localization of their cleavage fragments at the site of initial crystal nucleation raises a question as to whether proteolytic cleavage of BAG-75 and BSP is required for mineral nucleation within BMF.

Results with whole animals indicate that BAG-75 and BSP are two major glycoproteins in rat bone. Specifically, total 4 m guanidine HCl, 0.5 m EDTA extracts of the mineralized compartment of bone (33) contain a single 75-kDa glycoprotein band reactive with MAA lectin (Fig. 3F). This result parallels that obtained upon glycoprotein staining of UMR fractions (Fig. 2). Bone extracts, like UMR extracts, also contain a major phosphoprotein of this size revealed after Stains All staining (Fig. 3F). Finally, as shown in Fig. 3H, both purified BSP and BAG-75, but not a characteristic 50-kDa fragment of BAG-75 (24), strongly react with MAA lectin. As a result, we conclude that BAG-75 and BSP together compose the 75-kDa glycoprotein band whose cellular distribution specifically reflects the state of mineralization in the UMR culture model.

Serine Protease Inhibitor AEBSF Inhibits Mineral Crystal Nucleation in UMR 106 and in Primary Mouse Calvarial Cultures—To investigate the nature of the protease activity responsible for BAG-75/BSP cleavage and the relationship of cleavage with mineralization, we tested a variety of protease inhibitors (Table 1) in the UMR model. Individual inhibitors were added to confluent cultures at 64 h after plating, and the amount of mineral deposited within BMF was quantitated 24 h later. UMR cultures are not competent to mineralize until 60–64 h after plating, reflecting an osteogenic differentiation process that leads to the production of spherical pre-BMF structures.4

4 J. P. Gorski and R. J. Midura, manuscript in preparation.
Only one inhibitor, AEBSF, blocked mineralization in BMF (Table 1 and Fig. 4A). AEBSF is a covalent serine protease inhibitor (34) and was capable of completely blocking mineral nucleation at concentrations as low as 0.04 mM. None of the other protease inhibitors tested, which included inhibitors of thrombin, plasmin, plasminogen activator, furin, and matrix metalloproteinases, diminished mineralization in the UMR system when used at their optimal recommended dosage (Table 1). When added at 64 h after plating, AEBSF was similarly effective regardless of whether serum was included in the culture media or not (Fig. 4A), indicating that the source of the mineralization-related, AEBSF-sensitive protease is the UMR 106 cells themselves. However, the time at which AEBSF was added dramatically influenced the outcome. Assuming a control mineral level represented by 150–170 nmol of Alizarin red dye/well, the inhibitor was 10-fold less effective if present during the period in which the cells are actively proliferating and differentiating (44–64 h after plating) rather than during the mineralization period (64–88 h after plating) (22) (Fig. 4A).

To exclude the possibility that the effects of AEBSF were because of cell toxicity, AEBSF-treated and nontreated control cultures were analyzed using the MTT assay, a widely accepted method (44–64 h after plating) rather than during the mineralization period (64–88 h after plating) (22) (Fig. 4A).

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TABLE 1

| Inhibitor | Target protease(s) | Range of concentration tested | Specific inhibition of mineral deposition |
|-----------|--------------------|------------------------------|------------------------------------------|
| AEBSF     | Trypsin, chymotrypsin, plasmin, thrombin, kallikrein, proprotein convertases | 0.01–0.4 mM | Yes |
| Aprotinin | Trypsin, chymotrypsin, and plasmin | 0–3 μg/ml | No |
| Antipain  | Papain, trypsin, and plasmin | 100 μM | No |
| C1s inhibitor | Activated complement protein C1s | 0.1–100 μg/ml | No |
| Elastatin | Elastase and elastase-like proteases | 100 μM | No |
| GM 6001  | Matrix metalloproteinases 2, 3, and 9 | 10 μM | No |
| Hexa-α-arginine | Furin | 1–10 μM | No |
| Hirudin   | Thrombin | 0.5–10 ATU | No |
| Leupeptin | Trypsin-like proteases and some cysteine proteases | 100 μM | No |
| Pefabloc PL | Plasmin and plasma kallikrein | 1–100 μM | No |
| Pefabloc urokinase-type | Urokinase plasminogen activator | 1–100 μM | No |

* One antithrombin unit (ATU) will neutralize 1 NIH unit of thrombin at 37 °C, based on direct comparison with an NIH thrombin reference standard.

The 75-kDa glycoprotein band is likely composed of BAG-75 and BSP because they are the only two proteins of this molecular weight in total bone extracts shown to react with digoxigenin-labeled MAA lectin (see Fig. 3H). The 75-kDa phosphoprotein band is presumed to be predominantly composed of BAG-75 because BSP from bone exhibits a low phosphate content, whereas BAG-75 contains 44 phosphates/mol (35). Loss from the media fraction only occurs when mineralization is ongoing and not when it is blocked by inclusion of AEBSF or when BGP is omitted (Fig. 6A). Although similar analyses of the cell layer demonstrate that a...
75-kDa glycoprotein is taken up only when mineralization is progressing, a comparable increase in phosphoprotein (e.g. 75-kDa glycoprotein is taken up only when mineralization is progressing) occurred in serum-sufficient cultures. Although approximately one-half of the BSP is lost from the media fraction during mineralization (+BGP), a comparable amount of BSP becomes associated with the cell layer. Although BAG-75 protein was also lost from the media fraction, its recovery in the cell layer fraction was lower than expected. This is contrary to the known presence of BAG-75 antigen in BMF and nodular complexes prior to and during their mineralization in osteoblastic cell cultures (12). As a result, we

FIGURE 4. AEBSF inhibits mineral nucleation both in UMR 106 osteoblastic cultures and in primary mouse calvarial osteoblasts. A, with UMR cells, AEBSF blocks mineralization similarly in both serum-containing and serum-depleted conditions, whereas a 10-fold increase in effectiveness was observed when comparing 64–88 h versus 44–64 h cultures. For 64–88 h cultures: □, MTT absorbance in serum-depleted conditions; ■, MTT absorbance in serum-containing media; ○, amount of Alizarin red bound in serum-depleted conditions; ●, amount of Alizarin red bound in serum-containing media. For 44–64 h cultures: ▼, amount of Alizarin red bound in serum-containing media; △, amount of Alizarin red bound in serum-depleted conditions. Data are represented as the mean ± S.D. B, AEBSF completely inhibits mineralization within nodules of primary mouse calvarial cultures. MTT assay results and the amount of Alizarin red S bound to mineral deposits within cultures on day 12 are plotted versus the concentration of AEBSF added to cultures on day 9. Error bars represent the means ± S.D. 8, AEBSF completely inhibits mineralization within nodules of primary mouse calvarial cultures. MTT assay results and the amount of Alizarin red S bound to mineral deposits within cultures on day 12 are plotted versus the concentration of AEBSF added to cultures on day 9. Error bars represent the mean ± S.D.

FIGURE 5. Phase contrast microscopy of mineralized and AEBSF-treated primary calvarial osteoblastic cultures. Primary calvarial osteoblastic cells were harvested and cultured as described under “Methods.” On day 9 after plating, some of the culture wells were treated with 0.01 mM AEBSF, whereas control cultures were re-fed normal media. Unstained cultures were photographed on day 12. A, phase contrast image of control cultures. Mineralized nodules (arrows) appear as dark deposits under these conditions. B, phase contrast image of culture treated with 0.01 mM AEBSF. No mineralized nodules were visible. Results shown are representative of multiple wells and were consistent in three separate experiments. Scale bar ≈ 500 μm.

FIGURE 6. One-step extraction of UMR osteoblastic cell layer is unable to account for the quantitative loss of BAG-75 from the media fraction occurring during mineralization. A, extraction of cell layers with 50 mM EDTA/CHAPS extraction buffer (0.1 M Tris acetate buffer (pH 7.8), containing with 0.5% octyl glucoside, 0.05% SDS, 0.05M EDTA, and 0.02% sodium azide) reveals the loss of 75-kDa glycoposphoproteins from the media and the subsequent uptake of this band into the cell layer. Treatment of the cultures with AEBSF blocks the cell layer uptake of this same band. B, media and cell layer extracts from cell cultures treated with or without β-glyceraldehyde-phosphate treated cultures and unaccounted for in the cell layer extract from the same cultures. Recovery of full-length 75-kDa BSP from the cell layer of β-glyceraldehyde-phosphate treated cultures, along with that for conditioned media, is comparable with that without β-glyceraldehyde-phosphate; however, no BSP fragment (45–50-kDa) was detected in the cell layer fraction.
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FIGURE 7. Two-step extraction method yields increased recoveries of 75- and 50-kDa glycoprotein and phosphoprotein bands. UMR cell layers were extracted first with 0.05 M EDTA and then with urea-CHAPS as described under "Experimental Procedures." The extracts were then processed for SDS-PAGE and the gels stained with Pro-Q Emerald and Pro-Q Diamond fluorescent stains, or with Coomassie Blue. Compared with results with the one-step extraction method (Fig. 6A), increased recoveries of 75- and 50-kDa glycoprotein and phosphoprotein bands are denoted by arrows. For reference, the appearance of relevant conditioned media gel lanes is depicted in Fig. 6A; the conditioned media were unaffected by choice of cell layer extraction method.

reasoned that the one-step extraction method resulted in a lower than expected recovery of BAG-75.

As an alternative, a two-step sequential extraction protocol was used. To dissolve mineral crystals and release bound proteins, the cell layer was first extracted for 2 h at 4 °C with 0.05 M EDTA (pH 7.8). The residual cell layer was then treated vigorously with 8 M urea and 2% CHAPS (pH 7.8). Each extract was processed separately and subjected to SDS-PAGE, and the gels were stained with either Coomassie Blue dye or for glycoproteins or phosphoproteins. Urea/CHAPS extracts showed few differences among the four different experimental conditions (Fig. 7). In contrast, EDTA extracts of mineralizing cell layers grown only in the presence of BGP displayed dramatically increased glycoprotein- and phosphoprotein-stained bands at 50 and 75 kDa when compared directly with nonmineralizing cultures grown in the other three conditions (Fig. 7). Interestingly, total protein staining with Coomassie Blue showed a comparable pattern for all culture conditions suggesting the absence of large scale proteolysis accompanying mineral nucleation within BMF (Fig. 7). Taken together, these findings indicate that the two-step extraction method improves recoveries of unaccounted for 75- and 50-kDa glycoproteins from the cell layer of mineralized cultures. These results indicate that one or more 75-kDa glycoprophosphoproteins present in the serum-free media compartment of UMR 106-01 cultures are specifically taken up by the cell layer (+BGP) during the mineralization period (64–88 h) (Figs. 6 and 7). Because LCM-captured BMF are highly enriched in a similar glycoprophosphoprotein band of 75 kDa (Fig. 2), we propose that this band is taken up from the media into the cell layer where it is specifically localized within the BMF structures. When mineralization is blocked with AEBSF, the 75-kDa glycoprophosphoprotein band remains in the media fraction (Fig. 7). Likewise, in the absence of BGP, the 75-kDa band remains in the media compartment (−BGP and −BGP + AEBSF) (Fig. 7).

AEBSF Inhibits the Proteolytic Cleavage of BAG-75 and BSP That Accompanies Mineralization—In view of the identification of BSP and BAG-75 as 75-kDa glycoproteins involved in mineral nucleation and the enrichment of 45–50-kDa fragments within LCM-captured BMF (Fig. 3), it was of interest to establish whether their cleavage was also susceptible to AEBSF inhibition. UMR cultures were grown in the presence or absence of AEBSF and of BGP. Resultant cell layer fractions were extracted with the two-stage extraction protocol of 0.05 M EDTA followed by 8 M urea, 2% CHAPS (see "Methods"). For comparison, all media and cell layer fractions were electrophoresed in adjacent lanes and blotted with either MAA lectin, antibody 503 (recognizes N-terminal residues number 3-13 of BAG-75), antibody 504 (recognizes BAG-75 protein), or anti-BSP antibodies (Fig. 8, A–D).

Consideration of these blots revealed several interesting points. First, full-length BAG-75 and BSP are taken up by the cell layer only in the presence of BGP (Fig. 8, B and C). Second, 45–50-kDa fragments of BAG-75 (Fig. 8A) and BSP (Fig. 8C) were detected in the cell layer only when mineralization occurs. Importantly, cleavage is blocked by AEBSF coincident with inhibition of mineralization. Third, MAA lectin, which recognizes both BSP and BAG-75 (Fig. 3H), also recognizes 45–50- and 75-kDa forms in mineralized cell layer fractions (Fig. 8D). Finally, direct analyses of LCM-captured BMF have shown the 75- and 45–50-kDa fragment forms of BAG-75 and of BSP are both predominantly localized to BMF complexes (Fig. 3). In summary, AEBSF blocks uptake and cleavage of BAG-75 and BSP, as well as mineral nucleation within BMF.

In view of the known affinity of BSP and BAG-75 for hydroxyapatite crystals (35, 36), it is possible that some of the uptake by the +BGP cell layer is because of direct binding to mineral. However, a significant portion of these proteins taken up by the +BGP cell layer also occurs in the absence of mineral and of cleavage (+BGP + AEBSF) (Fig. 8, B and C). Control blots developed with MAA lectin confirm our earlier glycoprotein staining results showing redistribution of a 75-kDa glycoprotein coincident with mineral crystal nucleation (Fig. 8D). In this way, we suggest that the amount of direct protein binding to mineral crystals is represented by the difference between the respective 75-kDa bands in the +BGP versus +BGP + AEBSF lanes (Fig. 8, B and C). Although the percentage of cleaved fragment relative to full-length BAG-75 or BSP in the cell layer of mineralized cultures (+BGP) is less than 50%, the absolute amount of these stained fragments is similar to that for uncleaved precursor proteins (Fig. 8, A and C) from nonmineralized cultures (+BGP + AEBSF). It is noteworthy that nonmineralized cultures contain high levels of the uncleaved, full-length protein in the media (Fig. 8, A–D). Taken together, the blotting data indicate that mineralization occurs coincident with uptake and/or cleavage of BAG-75 and BSP by BMF.
Blockage of this cleavage by AEBSF leads to complete inhibition of mineral nucleation within BMF.

Two-dimensional SDS-PAGE Reveals That AEBSF Blocks the Cleavage and Uptake of Other Mineralization-related Proteins by the Cell Layer—Comparative analyses of EDTA extracts by SDS-PAGE (Fig. 8) prompted us to look more extensively at whether proteins other than BAG-75 and BSP have their cleavage inhibited by AEBSF. Cells were grown under serum-depleted conditions, and resultant cell layer fractions were extracted with the two-step protocol using 0.05M EDTA and then 8 M urea, 2% CHAPS. Preparations from each cell layer extract and media fraction were subjected to two-dimensional SDS-PAGE. Gels were stained with colloidal Coomassie Blue and aligned using the PD-Quest program (Bio-Rad) to identify differences in the staining patterns for the +BGP condition compared with that for the +BGP + AEBSF condition (supplemental Figs. 1–3).

There were no major differences detected between the +BGP and the +BGP + AEBSF-treated cultures for either the urea/CHAPS extract or the media fraction (supplemental Figs. 2 and 3). However, the differences detected between the two EDTA extracts were dramatic (supplemental Fig. 1). Gel spots were selected for mass spectral peptide mapping and liquid chromatography-tandem mass spectrometry identification if at least a 2-fold difference existed in staining intensity between the two culture conditions. Over 50 protein spots in EDTA fractions from AEBSF-treated and untreated control cultures were identified (results not shown). Application of the following criteria to this list identified three additional AEBSF-sensitive cleavages. 1) Spot present in EDTA extract was absent in urea extract and in media fraction. 2) Spot exhibits substantially higher staining intensity in the +BGP condition as compared with that in +BGP + AEBSF condition. 3) Size of protein based on second dimension SDS-PAGE is at least 10% smaller than expected. 4) Apparent isoelectric point is inconsistent with that expected for full-length protein.

Table 2 provides a summary list of the five proteins whose cleavage is blocked by treatment with AEBSF. These proteins are procollagen C proteinase enhancer protein (37), bone sialoprotein, 1,25-vitamin D₃ membrane-associated rapid-response steroid-binding protein, nascent polypeptide-associated complex α chain, and bone acidic glycoprotein-75.

DISCUSSION

The data presented here support the following conclusions about the mechanism of mineral crystal nucleation within spherical extracellular BMF structures. First, UMR cells mineralize equally well in the presence or absence of fetal bovine serum. Second, glycophosphoproteins BAG-75 and BSP are specifically enriched in LCM-captured mineralized BMF as compared with the total cell layer. Fragments of each protein (45–50 kDa in apparent size) were also substantially enriched within BMF. Third, a functional survey of different protease inhibitors showed that AEBSF, a covalent serine protease inhib-
Selective proteolysis or fragmentation plays a critical role in many biological processes, e.g., blood coagulation, fertilization, and complement activation, where it represents a means to regulate protein function through activation or inactivation. Serine proteases play a key role in tumor-induced bone formation and tooth mineralization. Enamel mineralization requires serine proteases enamelysin (MMP-20) and kallikrein 4 (38). Enamelysin catalyzes the cleavage of amelogenin, one of the main structural proteins forming enamel. Kallikrein 4 is responsible for degradation of enamel proteins, which results in their removal from the matrix allowing the enamel layer to fully mineralize. Previously, we showed stomelysin cleaved BAG-75 into a 50-kDa fragment in vitro (39); a similar 50-kDa fragment is found in calcified tissue and in serum (24). Preliminary data show that the level of 50-kDa fragment in serum correlates directly with bone formation following ovariectomy of rats (40). BAgS has also been shown to undergo proteolysis by an endogenous protease in UMR 106 cells yielding a fragment of ~47 kDa (41). This fragment is similar in size to that produced here by the AEBSF-sensitive protease. Although cleavage of these phosphoproteins has been noted previously (above), our findings are the first to link BSP and BAG-75 fragments specifically to the site of mineral nucleation.

Because the identity of the protease responsible for endogenous fragmentation of BSP and BAG-75 in bone is presently unknown, we surveyed a wide range of competitive and non-competitive inhibitors against serine and cysteine proteases and matrix metalloproteinases. Of all the inhibitors tested, AEBSF was the only one that had a specific effect on mineralization. Some like Pefablocurokinase-type plasminogen activator were found to block mineralization nonspecifically because of their associated toxicity. This was identified by separate, parallel assays of cell viability. For AEBSF, toxicity was observed only at concentrations 100-fold above those found to completely block mineral nucleation in BMF, e.g. 0.01 mM.

In addition to full-length forms, EDTA extracts of the cell layer contained a 50-kDa fragment of BAG-75 and a 45-kDa fragment of BAgS under mineralizing conditions only. A major source of BAG-75 and BSP was the conditioned media, because in the absence of BGP, full-length proteins were localized predominantly to the media. We have previously shown that addition of a phosphate source to 64-h cultures (mineralization-competent) is necessary to induce BSP uptake by BMF and their subsequent mineralization (12, 22); in the absence of phosphatase, BAG-75 enriched BMF remain unmineralized. The 10-fold greater effectiveness of AEBSF when added to 64-h versus 44-h UMR cultures suggests that the inhibitor acts late in the differentiation phase just prior to mineral nucleation. Similarly, in primary calvarial cultures, late addition of AEBSF on day 9 proved just as effective as on days 3 or 6 during the differentiation phase (5, 25, 26, 42) suggesting that AEBSF acts preferentially during the period immediately before mineral crystal nucleation in nodules. In this way, both the UMR 106 and primary osteoblast culture models exhibited similar sensitivities to AEBSF.

**TABLE 2**

Proteins in EDTA extract whose fragmentation is blocked by AEBSF

| Spot no. | Protein identification | Observed mass | Apparent mass | Expected pl | Method(s) for identification | Peptides identified (Mascot score) |
|----------|-----------------------|---------------|---------------|-------------|-----------------------------|----------------------------------|
| 1        | Procollagen C proteinase enhancer protein | 48,000 | 55,000 (Ref. 37) | 8.5 | Differential staining after two-dimensional SDS-PAGE and mass spectroscopy | PDEFVPGYACR (59) |
| 2        | 1,25-D3-MARRS receptor protein (ERp57) | 50,000 | 57,079 | 5.4 | Differential staining after two-dimensional SDS-PAGE and mass spectroscopy | TDDLDPSPAPGTSKL (49) |
| 3        | Nascent polypeptide associated complex, α chain | 31,000 | 221,512 | 9.4 | Differential staining after two-dimensional SDS-PAGE and mass spectroscopy | SGTLQSSPCSSLLVVTWTVK (75) |
| Bone acidic glycoprotein-75 | 50,000 | 75–80,000 | 4.5–5.0 | One-dimensional SDS-PAGE immunoblotting | LNFAVASR (63) |
| Bone sialoprotein | 45–50,000 | 75–80,000 | 6.0 | One-dimensional SDS-PAGE immunoblotting | LAPEYEAAAATR (88) |

NA* indicates not applicable.
Proteolytic Processing Is Essential for Mineralization of BMF

Protein uptake into the cell layer (and BMF) is a selective process because comparative one-dimensional gel analyses revealed +BGP versus +BGP + AEBSF cultures differed primarily in their content of a single 75-kDa glycoprophosphoprotein band shown later by immunoblotting to contain BAG-75 and BSP. Interestingly, formation of both of the 45–50-kDa fragments was inhibited, and their incorporation into the cell layer was blocked by inclusion of AEBSF. However, a portion of each full-length protein remained in the EDTA extract in +BGP + AEBSF cultures, suggesting that their incorporation into the cell layer required the presence of BGP and can occur prior to cleavage. Although mineral crystals are formed within BMF in the presence of BGP, no crystals could be detected in AEBSF-treated cultures. Thus, initial binding of full-length BAG-75 and BSP likely depends upon protein-protein interactions within the BMF complex; however, their fragmentation correlates with mineral crystal nucleation. This suggests that these fragments may participate in mineral nucleation within BMF. AEBSF may be directly inhibiting a protease that is capable of cleaving BAG-75 and BSP, or alternatively, the inhibition may be indirect, involving multiple proteases. Further work will be necessary to identify the AEBSF-sensitive protease and to determine whether it acts directly.

This is the first report of a serine protease requirement for bone mineral nucleation. Capable of diffusing through bilayer membranes, AEBSF is a covalent serine protease inhibitor. It has been used previously to block trypsin activation of protease-activated receptor in A-549 epithelial cultures (43) and in monocytic cultures to inhibit superoxide release following tumor necrosis factor-α or platelet-activating factor stimulation (44). AEBSF inactivates a wide variety of serine proteases, including chymotrypsin, urokinase plasminogen activator, kallikrein, plasmin, thrombin, furin, and trypsin (45–47). However, more specific inhibitors against most of these proteases were unable to block mineralization. Two possible explanations are that the effect of AEBSF may not be due to a proteolytic enzyme (AEBSF has also been identified as an inhibitor of phospholipase D (48)) or that a more specific inhibitor for the AEBSF-sensitive protease may not be available. In view of the identification of five proteins whose cleavage is inhibited by AEBSF, the evidence strongly supports a role for a serine protease in mineralization.

All five proteins whose cleavage is inhibited by AEBSF are associated directly or indirectly in the process of bone mineralization. BSP is associated with mineralization in bone, teeth, and breast cancer (49–51). Both a 38-kDa mid-protein fragment and a 25-kDa N-terminal fragment of this protein have been identified as nucleators of mineralization in vitro (21). Procollagen C proteinase enhancer protein is a secreted protein that enhances the activity of extracellular matrix procollagen C-terminal proteinase (BMP-1), an enzyme that activates fibrillar assembly of type I procollagen. Procollagen C proteinase enhancer protein knock-out mice increase the diameter of their long bones to apparently compensate for diminished mechanical performance (52). In 1997 an “active” fragment of this protein was identified in 3T6 fibroblast cells by Hulmes et al. (53) who suggested that cleavage of this protein was required for the formation of the extracellular matrix that will later support mineralization. The product of procollagen C-terminal proteinase cleavage of procollagen I is type I collagen C-terminal propeptide. Nicolaidou et al. (54) showed type I collagen C-terminal propeptide rose following vitamin K treatment and correlated with an increase in bone mineral density. The use of this by-product of procollagen type I processing as a marker for bone formation (55) suggests that enhancer activation and inhibition in our system may relate to its osteogenic role. Along with collagen type I, other substrates of procollagen C-terminal proteinase (BMP-1) include collagen type VII (55, 56) and collagens type II and type III (57).

1,25D3-MARRS is a membrane-associated vitamin D-binding protein necessary for calcium and phosphate uptake into the cell for support of bone development (58, 59). In 2005, Sterling and Nemere (60) showed that addition of an antibody against 1,25D3-MARRS, or protein kinase C inhibitor calphostin C, inhibited vitamin D-stimulated phosphate uptake in chick intestinal cell cultures. Calcium transport has also been shown to be regulated by vitamin D binding to 1,25D3-MARRS in aged female chicken intestine, as determined by dose-response curves for ion transport and kinetics (61). Because 1,25D3-MARRS is a plasma membrane protein, we speculate that its presence in the EDTA cell layer extract may reflect an association with released membrane-bound vesicles participating in the process of mineral nucleation or proteolytic release from the cell (46).

Finally, nascent polypeptide-associated complex α chain is a large 220-kDa cytosolic protein that translocates newly synthesized polypeptides to the nucleus. A C-terminal fragment of this protein was previously identified in an epithelial cell line (62), and all three peptides identified in our mass spectrometric MS/MS peptide studies were localized to this same fragment (Table 2). Identification of an intracellular protein in the EDTA extract seems counterintuitive (Table 2). However, we hypothesize that intracellular proteins may become entrapped within secretory vesicles contributing to the assembly of BMF. Alternatively, BMF formation may involve the blebbing of the plasma membrane and release of vesicular structures containing selected cytoplasmic contents.

In summary, the results indicate that cleavage of BAG-75, BSP, 1,25D3-MARRS protein, nascent polypeptide associated complex α chain, and procollagen propeptidase enhancer by an unidentified osteoblast-derived serine protease is associated with mineral nucleation. The fact that both BAG-75 and BSP and their fragments are preferentially localized to mineralizing BMF sites and that inhibition of their cleavage blocks mineral nucleation within BMF suggests that each plays a functional role in this process. Future studies will address the identity of the protease and effect of cleavage on the structure/function of these proteins.

Acknowledgments—J. P. G. acknowledges the excellent technical assistance of Sharon Midura and the generous assistance of Dr. William Landis with initial laser capture microscopy.

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