Evidence of ectokinase-mediated phosphorylation of osteopontin and bone sialoprotein by osteoblasts during bone formation *in vitro*

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Osteopontin (OPN) and bone sialoprotein (BSP) are phosphorylated glycoproteins that, together with osteonectin/secreted protein, acidic, rich in cysteine (SPARC) and osteocalcin, comprise the major non-collagen proteins of bone. Although phosphorylation of OPN and BSP, which is known to influence the biological properties of these proteins, has been shown to occur intracellularly, recent studies have demonstrated ectokinase activity in bone cell populations [Mikuni-Takagaki, Kakai, Satoyoshi, Kawano, Suzuki, Kawase and Saito (1995) *J. Bone Miner. Res.* **10**, 231–241]. To determine whether OPN and BSP are phosphorylated by ectokinase activity we have used [γ - 32 P]ATP and [γ - 32 P]GTP as cell-impenetrable phosphate donors to analyse for ectokinase activity in osteoblastic UMR106.06 cells and fetal rat calvarial cells (FRCCs). By pulse-labelling confluent cells with radiolabelled nucleotides, the phosphorylation of endogenous and exogenously added OPN and

BSP was demonstrated together with the labelling of a number of cell surface proteins. These phosphorylation reactions were inhibited by a cell-impermeable ectokinase inhibitor, K_{252b}, and cell surface phosphorylation was also inhibited by exogenously added OPN and BSP substrates, indicating competition for the ectokinase enzyme. However, phosphorylation of OPN and BSP, both of which can mediate cell attachment through Arg-Gly-Asp (RGD) motifs, was not inhibited by an RGD peptide, suggesting that binding of OPN and BSP to cell surface integrins is not required. In similar experiments, ectokinase-mediated phosphorylation of OPN and BSP was demonstrated during mineralized tissue formation by FRCCs *in vitro*. These studies demonstrate that OPN and BSP secreted by bone cells are phosphorylated by a casein kinase II-like ectokinase present on the surface of osteoblastic cells.

INTRODUCTION

The extracellular matrix of bone becomes mineralized by the deposition of crystals of carbonated apatite (dahlite), which form within and between fibrils of type I collagen [1,2], forming the structural architecture of most dense connective tissues. In addition to collagen, a number of acidic proteins, several of which are phosphorylated, are present in the bone matrix [3]. Because phosphate is the major counter-ion for Ca²⁺ in the apatite crystals, the presence of phosphate groups on these matrix proteins has potential importance in the epitactic nucleation and regulated growth of apatite crystals [4], as well as in providing a potential source of phosphate ions that can be released by alkaline phosphatase activity [5]. Osteopontin (OPN) and bone sialoprotein (BSP), which together with osteocalcin comprise the major non-collagenous proteins secreted by osteoblasts, are extensively phosphorylated on serine and some threonine residues [6–8]. BSP, which is expressed in association with the initial mineralization of bone matrix [9,10], can nucleate apatite formation from physiological concentrations of Ca²⁺ and PO₄³⁻ [11] and is therefore a candidate protein for initiating apatite crystal formation during bone formation *de novo*. In comparison, OPN, which is expressed later than BSP, binds rapidly to pre-existing crystals [12,13] and will inhibit crystal growth, the efficacy of the inhibition being dependent on the presence of phosphate groups [14]. Previous studies using 32 PO₄³⁻ labelling have shown that phosphorylation of BSP and OPN occurs intracellularly [12,13,15], primarily through a casein kinase II-like enzyme [16].

In studies of ATP signalling through purinergic receptors in bone cells (L. C. Luo, H. Yu and J. Ferrier, unpublished work)

we found that the ATP was utilized by cell surface kinases (ectokinases) to phosphorylate cell surface proteins and exogenously added casein. Moreover, in recent studies extracellular casein kinase-like activity has been reported in cultures of neonatal rat calvarial cells [18]. To determine whether phosphorylation of OPN and BSP can occur through ectokinase activity, in addition to intracellular kinase activity, we have used [γ - 32 P]ATPg and [γ - 32 P]GTP as cell-impermeable, and 32 PO₄³⁻ as cell-permeable, phosphate donors to analyse intracellular and extracellular kinase activities in osteoblastic UMR 106.06 cells and fetal rat calvarial cells (FRCCs).

MATERIALS AND METHODS

Cell culture

Phosphorylation of proteins through ecto- and endokinase activity was studied in confluent cultures of the osteoblastic cell line UMR 106.06 (kindly provided by Dr. T. J. Martin) and in confluent and bone-forming cultures of fetal rat calvarial cells (FRCCs). FRCCs were prepared from 21-day-old fetal rat calvariae by a timed digestion with a bacterial collagenase preparation to generate five cell populations (I–V), as described in detail previously [19]. Populations II–V were pooled and plated at 5.0×10^4 cells per 35 mm dish in α -minimal essential medium (α MEM) containing 10% (v/v) fetal bovine serum (FBS) and antibiotics (100 μ g/ml penicillin G, 50 μ g/ml gentamycin and 0.3 μ g/ml fungizone), and grown in a humidified air/CO₂ (19:1) mixture at 37 °C. The medium was changed 24 h after plating and subsequently on every second day until cells were confluent (approx. day 6). To produce bone-forming cultures, confluent FRCCs were further incubated in the presence

Abbreviations used: BSP, bone sialoprotein; FBS, fetal bovine serum; FRCCs, fetal rat calvarial cells; α MEM, α -minimal essential medium; OPN, osteopontin; RGD, Arg-Gly-Asp peptide; RGE, Arg-Gly-Glu peptide.

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of 100 µg/ml sodium ascorbate and 10 nM dexamethasone until day 18 at which time 10 mM β-glycerophosphate was added to the cultures to initiate mineralization of the bone nodules that were being formed. Cultures were continued for a further 3 days with media changes each day, after which labelling procedures were initiated.

UMR 106.06 cells were plated at 3.0×10^4 cells per well in 24-well tissue culture plates (Linbro) and grown to confluence in αMEM containing 15 % (v/v) FBS and antibiotics, as described for FRCCs. All experiments were performed with triplicate sets of cultures.

Radiolabelling procedures

To study the phosphorylation by intracellular kinase activity of proteins synthesized and secreted by UMR 106.06 cells and FRCCs, confluent and bone-forming FRCC cultures were washed three times with PBS and then incubated with 70 µCi/ml of Na₃³²PO₄ (NEX 05H, specific radioactivity 8500–9120 Ci/mmol; Mandel Scientific Co., Guelph, Ont., Canada) in fresh, phosphate-deficient Dulbecco's minimal essential medium containing 0.1 % FBS and antibiotics. Confluent cells were labelled for 24 h at 37 °C before analysis, whereas the bone-forming FRCCs were labelled for 1 h at 37 °C and analysed after chase periods of 12 and 24 h in fresh αMEM containing 10 % FBS. Samples were taken from the medium and the cell layers were extracted as described below.

Phosphorylation of proteins by ectokinase activity was also studied in confluent UMR 106.06 cells and FRCCs as well as in the bone-forming FRCC cultures. Confluent cells were washed three times with PBS and the medium was replaced with one of the following: fresh αMEM; fresh αMEM containing approx. 0.1 mg/ml pig OPN and BSP; fresh αMEM containing approx. 0.1 mg/ml pig OPN and BSP, and 10 µM ectokinase inhibitor K_{252b}; 2 h conditioned medium from UMR 106.06 cells; or 2 h conditioned medium from FRCCs. To each culture 70 µCi/ml [γ-³²P]ATP (BLU-NEG-502A, specific radioactivity 3000 Ci/mmol; Mandel) was added and the cells were incubated for 10 min at 22 °C. For bone-forming cultures the FRCCs were incubated for 10 min at 22 °C with [γ-³²P]ATP and after washing the cell layers, the cultures were chased in fresh medium containing 10 % FBS for 1, 3, 9 or 24 h at 37 °C. Samples of media and cell extracts were taken for analysis as described below.

In a separate series of experiments, confluent UMR 106.06 cells were incubated in 2 h-conditioned medium to which 0.1 mg/ml pig OPN and BSP, and casein were added; the cells were then incubated for 10 min at 22 °C with 71 µCi/ml [γ-³²P]GTP (BLU-506H, specific radioactivity 3000 Ci/mmol; Mandel). In addition, either 10 mM Arg-Gly-Asp (RGD) or 10 mM Arg-Gly-Glu (RGE) peptides were added to some of these cultures to determine whether proteins with RGD cell attachment sequences (OPN and BSP) were bound to the cell surface for phosphorylation to occur. After the 10 min labelling period, media were collected and the cells were washed three times in PBS before scraping and resuspending them in SDS/PAGE sample buffer (without dithiothreitol). Samples of media and cell extracts were analysed by SDS/PAGE as described below.

Extraction procedures

The radiolabelled proteins in the bone-forming cultures were extracted from the cell layers by procedures described in detail previously [12] to determine the presence of phosphorylated

proteins in different tissue compartments. The cells were first removed by a short (1–2 min) incubation with 0.5 M NH₄OH (cell extract) and then proteins were extracted from the tissue matrix with 4 M guanidinium chloride (G1 extract), followed by 0.5 M EDTA (E extract) and again with 4 M guanidinium chloride (G2 extract). Finally the tissue residue was digested with purified bacterial collagenase (100 µg/ml *Achromobacter iophagus* collagenase; C1913 Sigma, St. Louis, MO, U.S.A.) in 50 mM Tris/HCl, 5 mM CaCl₂, 5 mM N-ethylmaleimide, 1 mM PMSF (pH 7.4) at 37 °C for 3 h (CD extract). Each extract was desalted by dialysis against 10 mM ammonium bicarbonate containing 0.005 % Brij-35 solution and then changed to distilled water. After dialysis the samples were freeze-dried and redissolved in a small volume of water.

Thrombin digestion

To identify OPN, samples of radiolabelled proteins were digested with 1 unit of human thrombin (T6759; Sigma) in 50 mM Tris/HCl (pH 8.0)/10 mM CaCl₂ at 37 °C for 30 min. The digestion was terminated by the addition of one part of 4 × SDS/PAGE sample buffer containing 25 mg/ml dithiothreitol to three parts digest and heating to at 95 °C for 3 min before analysis by SDS/PAGE.

Immunoabsorptions

OPN and BSP were also identified by immunoabsorption with monoclonal antibodies (MPIIB101 and WVID1; Developmental Studies Hybridoma Bank, Johns Hopkins University, Baltimore, MD, U.S.A.; under contract from NICHD) raised against the rat proteins, and affinity-purified rabbit polyclonal antibodies raised against pig OPN and BSP [20] as described in detail previously [12,13]. Briefly, samples were dissolved in 400 µl of immunoprecipitation buffer [0.1 % BSA/0.3 % Nonidet P40/0.3 % sodium deoxycholate/0.02 % sodium azide/0.15 M NaCl/10 mM Tris/HCl (pH 7.4)] and 5 µl of normal rabbit serum in a 1 ml Microfuge tube. After incubation for 2 h at 4 °C, the sample was mixed with 100 µl of Pansorbin (Sigma), previously washed three times with immunoprecipitation buffer, and incubated for a further 2 h. Non-specifically bound material was removed by brief centrifugation at 10000 g on a Microfuge. Meanwhile 1 µl of primary antibody was bound to 100 µl of Protein A–Sepharose (Pharmacia, Uppsala, Sweden) by incubation at 4 °C for 3 h with gentle shaking. The Protein A–antibody complex was then washed three times with 0.5 % Tween-20/0.1 % BSA in PBS and the supernatant from the Pansorbin-treated sample was added. The mixture was incubated for 16 h at 4 °C and the specific immune complex that formed was separated by brief centrifugation on a Microfuge. The pellet was washed seven times in PBS containing 0.5 % Tween-20 before dissolving the protein in 2 × SDS/PAGE sample buffer containing 25 mg/ml dithiothreitol and heating the sample to 95 °C for 3 min. The sample was then cooled, centrifuged briefly and the radiolabelled proteins in the supernatant were analysed by SDS/PAGE.

SDS/PAGE

Analysis of radiolabelled proteins was performed on the Hoefer Mini-gel system with Tris/Tricine buffers as described previously [13]. Separations were achieved on 10 % (w/v) cross-linked separating gels with 4 % (w/v) stacking gels. Protein samples were dissolved in SDS/PAGE sample buffer [0.5 M Tris/HCl buffer, pH 6.8, containing 2 M urea, 2 % (w/v) SDS, 8 % (w/v) Bromophenol Blue and 25 mg/ml dithiothreitol] and heated at

95 °C for 3 min, cooled and loaded on the gels. Electrophoresis was performed at 100 V with 1 M Tris/1 M Tricine/1 % SDS adjusted to pH 8.2 with HCl in the upper chamber, and 1 M Tris/HCl, pH 9.0, in the lower chamber until the Bromophenol Blue dye reached the bottom of the gel. After separation, the gels were dried and exposed to Kodak X-OMAT AR film at -70 °C to reveal the radiolabelled proteins.

RESULTS

Identification of phosphorylated proteins secreted by UMR 106.06 cells and FRCCs

Samples of medium from confluent cultures of bone cells labelled continuously with $\text{Na}_3^{32}\text{PO}_4$ over a 24 h period were analysed by Tris/Tricine/SDS/PAGE (10 % gel) (Figure 1). In the UMR 106.06 cultures strong radiolabelled bands were observed at the top of the gel and at 88 kDa, with a weaker band at 66 kDa. The 66 kDa band was selectively degraded by thrombin digestion, producing a major band at 35 kDa, and was immunoadsorbed by monoclonal antibodies to OPN (Figure 1). The 88 kDa protein, which showed a slight increase in mobility after thrombin digestion, was immunoadsorbed by monoclonal antibodies to BSP. In the FRCCs the 66 kDa protein was the predominant phosphorylated protein and was identified as OPN by its susceptibility to thrombin digestion and immunoadsorption to OPN antibodies. In contrast with UMR 106.06 cells, phosphorylated BSP was not detectable in the FRCC cultures. These results were consistent with analyses of mRNA levels for OPN and BSP (results not shown), which revealed high levels of BSP and low levels of OPN in UMR cells and OPN, but no BSP in confluent FRCCs.

Phosphorylation of endogenous and exogenous proteins by cell surface ectokinase activity in UMR 106.06 cells

When UMR cells were labelled with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ in fresh medium for 10 min, a strong phosphorylated band was observed at approx. 100 kDa with weaker bands at 55, 30 and 18 kDa, and an even weaker band at 34 kDa (Figure 2A). When labelling was performed in media preconditioned by UMR 106.06 cells for 2 h,

Figure 1 Intracellular phosphorylation of proteins secreted by confluent UMR 106.06 and FRCC cultures

Confluent cells were labelled continuously for 24 h with $\text{Na}_3^{32}\text{PO}_4$, and samples of the medium were analysed by Tris/Tricine/SDS/PAGE (10 % gel) using autoradiography to reveal the radiolabelled proteins. Lane 1, untreated medium; lane TD, duplicate samples of thrombin-digested medium; lanes IP/OPN, immunoprecipitation of OPN from medium; lanes IP/BSP, immunoprecipitation of BSP from medium. The positions of phosphorylated OPN and BSP are indicated, together with the molecular mass markers. A strongly radiolabelled band for BSP with a weaker band for OPN is evident in the medium of UMR 106.06 cells, whereas only OPN is present in the FRCC cultures.

Figure 2 Extracellular phosphorylation of proteins on the surface of confluent UMR 106.06 and FRCCs

UMR 106.06 cells (UMR) and FRCC cells were labelled for 10 min with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ in fresh medium (FM) or 2 h-conditioned medium (CM) as shown, with some cultures supplemented with pig OPN and BSP (OPN + and BSP + respectively), with or without the cell-impermeable ectokinase inhibitor K_{252b} . Duplicate samples (except the medium containing K_{252b}) of media (A) and cell extracts (C) were analysed by SDS/PAGE and autoradiography. (B) Radiolabelled rat OPN and BSP in the UMR conditioned medium (UMR CM) were identified by immunoadsorption with mouse monoclonal antibodies; the identities of the exogenously added pig OPN and BSP were determined by immunoadsorption with affinity-purified rabbit anti-(pig OPN) and anti-(pig BSP) antibodies, which did not cross-react with the rat proteins. The immunoadsorbed pig OPN was also shown to be digested with human thrombin (OPN). The positions of molecular mass markers are indicated at the left of (A). Note the phosphorylation of the exogenously added pig BSP and OPN, as well as the endogenous rat BSP and OPN in the conditioned media, indicative of ectokinase activity, inhibited by K_{252b} (A). In addition, the presence of the pig BSP and OPN abrogated the phosphorylation of cell surface proteins, which was also blocked by K_{252b} (C).

the bands at 100, 55 and 18 kDa were intensified and a strongly radiolabelled 80 kDa band together with a weaker 66 kDa band, which were immunoadsorbed by anti-rat BSP and OPN antibodies respectively (Figure 2B), were apparent. After the addition of purified pig BSP and OPN to fresh medium, strongly radiolabelled bands corresponding to these proteins were generated in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$; their identity was confirmed by immunoadsorption with affinity-purified antibodies to the pig

Figure 3 Labelling of endogenous and exogenous proteins in confluent UMR 106.06 cell culture with $[\gamma\text{-}^{32}\text{P}]\text{GTP}$

Confluent UMR 106.06 cells were pulse-labelled for 10 min with $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ in 2-h-conditioned medium to which pig BSP and OPN, together with casein, had been added. In addition, some cultures were labelled in the presence of an RGD peptide or a control RGE peptide, as shown, to determine whether integrin binding was required for the phosphorylation of proteins by ectokinase. Radiolabelled proteins from media (A) or cells (B) were analysed by SDS/PAGE and autoradiography. The three strongly labelled protein bands in (A) correspond to the exogenous pig BSP and OPN and the casein, as indicated. Phosphorylation of endogenous proteins is more clearly evident in (B), where cell surface proteins are radiolabelled. The similar labelling patterns in the presence and in the absence of RGD or RGE peptide indicate that phosphorylation of the cell attachment proteins OPN and BSP by the cell surface-associated ectokinase is not dependent on integrin binding.

Figure 4 Intracellular phosphorylation and distribution of proteins labelled during bone formation by FRCCs *in vitro*

Bone nodule-forming cultures of FRCCs were labelled for 1 h with $\text{Na}_3^{32}\text{PO}_4$ and, after chase times of 3 and 24 h in fresh medium, samples of medium (A) and tissue extracts were analysed by SDS/PAGE and autoradiography. Radiolabelled proteins were extracted from the cells by treatment with 0.5 M NH_4OH (B) and from the tissue layer with 4 M guanidinium chloride before (C; G1 extract) or after (E; G2 extract) demineralization with 0.5 M EDTA (D; E extract). Phosphorylated BSP was identified in the media and all of the extracts, whereas phosphorylated OPN was present only in the media and E extract.

proteins (Figure 2B) that do not cross-react with the corresponding rat proteins. However, in the additional presence of the cell-impermeable kinase inhibitor K_{252b} [21] the labelling of the exogenously added pig proteins was strongly inhibited. Analysis of 2-h-conditioned medium from confluent FRCCs revealed phosphorylation of the 66 kDa protein (Figure 2), which was identified as OPN by immunoadsorption (results not shown). Collectively these experiments demonstrated that both endogenous and exogenously added BSP and OPN are phosphorylated by an extracellular kinase produced by UMR 106.06 cells.

That the kinase activity was associated with the cell surface was evident from the analysis of total cell extracts obtained from

Figure 5 Ectokinase-mediated phosphorylation and distribution of proteins labelled during bone formation by FRCCs *in vitro*

While forming bone nodules FRCCs were labelled for 10 min in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and the proteins in the medium and tissue extracts were analysed by SDS/PAGE and autoradiography after chase periods of 1, 3, 9 and 24 h. Some cultures were labelled in the presence of K_{252b} (3^k), 2 mM levamisole (3^l) or 10-fold (10 mM) higher levels of $\text{Na}_3^{32}\text{PO}_4$ (3^p) and analysed after a 3 h chase period. BSP phosphorylated by ectokinase activity was identified in the media and all of the extracts, whereas phosphorylated OPN was present only in the media and E extracts.

the experiments described above. In both fresh and conditioned media a broad range of phosphorylated proteins were revealed by autoradiography of SDS/PAGE gels; most labelled proteins migrated above the 49 kDa protein standard (Figure 2C). However, none of these proteins was immunoadsorbed with antibodies to either OPN or BSP. In the presence of the exogenous pig BSP and OPN, radiolabelling was significantly decreased, indicating that the added proteins were competing with the cell surface proteins as substrates for the kinase activity. Addition of K_{252b} to

Figure 6 Identification of OPN phosphorylated by ectokinase in the media and E extracts of bone nodules

Thrombin digestion (T) was used to cleave the phosphorylated OPN selectively in samples of media (A) after chase periods of 3 and 24 h and in E extracts (B) after chase periods of 1, 3, 9 and 24 h. Note that a radiolabelled band, migrating slightly more slowly than OPN, is left with the BSP after the fragmentation of the OPN into two fragments of approx. 32 kDa and 34 kDa (arrowhead) in the media samples. In the E extracts some OPN remains undigested, while smaller radiolabelled bands, presumed to be fragments of OPN, are completely digested.

the cultures completely blocked the kinase activity, confirming that this was an ectokinase that was labelling cell surface proteins.

To determine the specificity of the kinase activity for the phosphate donor, experiments were repeated with $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ as the source of radiolabelled phosphate (Figure 3). The addition of exogenous casein, together with pig BSP and OPN, to cultures incubated for 10 min in the presence of $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ resulted in the phosphorylation of proteins at 88, 66 and 35 kDa, corresponding to the exogenously added proteins in the medium (Figure 3A). The identities of the OPN and BSP were confirmed by immunoadsorption (results not shown). Notably, the labelling intensities of the OPN and BSP, which are integrin-binding cell attachment proteins, were not significantly altered when labelling was performed in the additional presence of 10 mM RGD or RGE peptides. Similarly, cell surface proteins radiolabelled with the $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ substrate were not affected by these peptides (Figure 3B), indicating that binding to integrins is not a prerequisite for ectokinase-mediated phosphorylation of OPN and BSP.

Ectokinase-mediated phosphorylation of BSP and OPN during bone formation *in vitro*

To study the phosphorylation of bone proteins in mineralizing cultures of FRCCs, bone nodule-forming cultures were first labelled for 60 min with $^{32}\text{PO}_4^{3-}$ and the radiolabelled proteins were analysed in the medium and in tissue extracts after chase

Figure 7 Demonstration of phosphorylated BSP in 4 M guanidinium chloride extracts of newly forming bone nodules

Samples of G1 and G2 extracts from bone nodules, formed by FRCCs and labelled for 1 h with $\text{Na}_3^{32}\text{PO}_4$ or $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, were immunoadsorbed with monoclonal antibody on rat BSP and the immune complexes were analysed by SDS/PAGE and autoradiography. The immunoadsorbed (IP) BSP, migrating at approx. 80 kDa, is shown for 3 and 24 h chase times with an equivalent amount of the original extract shown in the lanes immediately to the left. (A) G1 extract from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ -labelled cultures; (B) G1 extract from $\text{Na}_3^{32}\text{PO}_4$ -labelled cultures; (C) G2 extract from $\text{Na}_3^{32}\text{PO}_4$ -labelled cultures. The immunoadsorbed bands for the G2 extracts of the $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ -labelled cultures were not clear in photographic reproductions of the radioautographs.

times of 12 and 24 h. The accumulation of two prominent radiolabelled proteins, migrating at 88 and 66 kDa on SDS/PAGE, was observed in the chase media over a 24 h period (Figure 4A). The identities of these proteins as BSP and OPN were confirmed from thrombin susceptibility and immunoadsorption. An analysis of phosphorylated proteins in the various tissue extracts revealed a large number of labelled proteins in the cell and 4 M guanidinium chloride (G1 and G2) extracts, whereas OPN and BSP were the predominant proteins phosphorylated in the EDTA (E) extract (Figures 4B to 4E). Although OPN was always labelled more strongly than BSP in the E extracts (Figure 4C), its presence could not be detected in the guanidinium chloride extracts by immunoadsorption. In contrast, BSP was revealed in both the G1 extract and more weakly in the G2 extract, as observed in studies described below.

Extracellular kinase activity was determined in comparable cultures incubated for 10 min in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and chased for various periods up to 24 h. Radiolabelled proteins corresponding to OPN and BSP accumulated in the culture medium, as indicated by the increase in radioactivity over the 24 h period (Figure 5). Several phosphorylated proteins, including bands corresponding to BSP and OPN, were present in cell extracts and declined with increase in chase time, indicating their release from the cell surface. In contrast, in the demineralizing EDTA extracts, BSP and especially OPN were heavily labelled together with some smaller proteins. These smaller proteins seem to be fragments of OPN because they were, together with the OPN, selectively degraded by thrombin (Figure 6). The radiolabelled proteins in the mineral fraction increased with time, indicating a steady accumulation of the pulse-labelled proteins. Analysis of the G1 and G2 extracts revealed an increase in the intensity of radiolabelled proteins including BSP, which was identified by immunoadsorption (Figure 7). However, no radiolabelled OPN was immunoadsorbed from either the G1 or G2 extracts (G), as observed for the proteins radiolabelled with $^{32}\text{PO}_4^{3-}$.

To evaluate the effects of alkaline phosphatase on the phosphorylation, some FRCC cultures in the same experiment

were incubated with 2 mM levamisole, an alkaline phosphatase inhibitor, and other cultures were incubated in the presence of K_{252b} . In a third series of cultures the PO_4^{3-} in the culture medium was increased from 1 to 10 mM to prevent radiolabelled $^{32}PO_4^{3-}$, generated from the hydrolysis of $[\gamma-^{32}P]ATP$ by extracellular phosphatases, from entering the cells and being utilized by intracellular kinases. These cultures were analysed after a 3 h chase period and compared with the corresponding controls (Figure 5). The addition of the kinase inhibitor K_{252b} was observed to decrease the phosphorylation of proteins in the cell extract, but not in the media or in the tissue extracts. In comparison, levamisole decreased phosphorylation in the first 4 M guanidinium chloride extract but not in the other tissue compartments. Importantly, increasing $[PO_4^{3-}]$ in the labelling medium did not affect phosphorylation in any of the fractions analysed, indicating that incorporation of radiolabelled phosphate into proteins in these experiments was occurring outside the cell.

DISCUSSION

The phosphorylation of proteins is a post-translational event mediated by kinase enzymes that can have a profound influence on biological activity. Thus protein phosphorylation is an integral part of regulatory mechanisms in cells, including receptor-mediated signal transduction, second messenger signalling and modulation of gene transcription. Although these phosphorylation reactions occur primarily within the cell, the activity of extracellular kinases (ectokinases) has also been implicated in cell-cell communication [22–24], cell adhesion and long-term control of cell function [25]. Similarly to the intracellular kinases, ectokinase enzymes utilize nucleoside triphosphates as phosphate donors. However, to have a significant effect on extracellular phosphorylation reactions ectokinase activity is dependent on the availability of a suitable extracellular phosphate donor. Although the extracellular concentrations of nucleoside triphosphates such as ATP are generally low in comparison with their intracellular concentrations, many cell types can release ATP in a controlled fashion through exocytosis or via cell membrane transport, generating extracellular concentrations in excess of 1 mM [26–29]. Notably, extracellular nucleoside triphosphates at micromolar concentrations are ligands for cell surface P2 purinergic receptors that have been implicated in a variety of biological processes including neuronal [30,31] and smooth muscle activity [32], platelet aggregation [25] and bone cell regulation [33–37]. Although the extracellular ATP concentration near active osteoblasts *in vivo* is not known, it is conceivable that sufficiently high levels of extracellular ATP can be produced to provide substrate for ectokinases that have K_m values that range from 10 mM down to 0.04 mM [32].

Interest in the phosphorylation of extracellular matrix proteins of mineralized connective tissues is based on the potential of such proteins to initiate the calcification process through heterogeneous nucleation (epitaxy), because the phosphate group forms the major counter-ion of Ca^{2+} in hydroxyapatite crystals. Thus phosphophoryn, a tissue-specific highly phosphorylated protein in dentine, is a putative nucleator of hydroxyapatite in dentine [38], whereas BSP and OPN are prominent phosphorylated proteins in bone and cementum [39–41]. Whereas BSP is essentially unique to these tissues [40,41] and has been suggested to be a nucleator of hydroxyapatite in bone formation *de novo* [11,42], OPN is expressed in various tissues in different forms [43]. In the mineralized bone matrix OPN can exist in forms of low and high phosphorylation [14,44] and has been implicated in both the formation and resorption of bone [4,45–48]. Notably,

the ability of OPN to mediate osteoclast attachment has been shown to be dependent on its phosphorylation state [49].

Although phosphorylation of OPN and BSP is believed to occur intracellularly, as demonstrated previously in bone cell cultures with $^{32}PO_4^{3-}$ as a substrate [12,15], our previous studies of purinergic receptors in bone cells, including the UMR 106.06 cell line (L. C. Luo, H. Yu and J. Ferrier, unpublished work), have indicated the presence of ectokinase activity. Similarly, ectokinase activity has been demonstrated in neonatal rat calvarial bone cell populations [18], confirming an earlier observation of an extracellular enzyme in bone that could catalyse the transfer of phosphoryl groups from ATP to protein within the bone matrix [50]. In the present studies we first used $^{32}PO_4^{3-}$ to confirm that OPN synthesized by confluent FRCCs, and both OPN and BSP synthesized by UMR cells, are phosphorylated intracellularly (Figure 1). To demonstrate that these proteins could also be phosphorylated extracellularly we incubated the same cells over a short (10 min) pulse period with $[\gamma-^{32}P]ATP$. That radiolabelling of endogenous and exogenous OPN and BSP reflects ectokinase activity is evident from the following: the high negative charge of ATP, which limits its uptake by the cells, its low concentration relative to intracellular ATP, and the short labelling time preclude its utilization by intracellular kinases. Although it is conceivable that $^{32}PO_4^{3-}$ released by extracellular ATPases could enter the cell, the specific activity would be markedly suppressed by the presence of 1 mM PO_4^{3-} in the labelling medium. Indeed, increasing $[PO_4^{3-}]$ to 10 mM (Figure 5) did not influence ectokinase activity, whereas intracellular protein phosphorylation was barely detectable when cells were incubated with $^{32}PO_4^{3-}$ for the same period (10 min). Further, exogenously added proteins, including casein, that do not enter the cell were phosphorylated. Finally, the phosphorylation reaction was abrogated by the presence of the cell-impermeable ectokinase inhibitor K_{252b} [21] in the culture media of the UMR and confluent FRCCs. That the same inhibitor was effective on the phosphorylation of cell-associated proteins only in the mineralized tissue-forming FRCCs might reflect its limited access to the multi-layered osteoblastic cells that synthesize the OPN and BSP in these cultures. Therefore these experiments show unequivocally that both endogenous and exogenous OPN and BSP are phosphorylated by ectokinase activity.

Because the exogenously added pig OPN and BSP had not been dephosphorylated, whereas the endogenous proteins would be phosphorylated intracellularly before their secretion, it is apparent that the ectokinase activity performs additional phosphorylation reactions. Thus OPN, which is synthesized in different tissues [43], could be more highly phosphorylated in bone through the osteoblast ectokinase activity. Although a detailed analysis of the sites of ectokinase phosphorylation has not been performed, similar patterns of radiolabelled OPN peptides generated by thrombin and trypsin digestion of endokinase- and ectokinase-labelled proteins (results not shown) would suggest that the ectokinase targets the same site as the intracellular enzyme, or similar sites. This is supported by the demonstration that most phosphorylation sites in OPN and BSP are targets of mammary gland casein kinase II [16], which has been characterized as the principal enzyme involved in the intracellular phosphorylation of OPN [51], and that the ectokinase has, through its effective utilization of GTP and its inhibition by K_{252b} , characteristics of a casein kinase II enzyme. Although at least 58 consensus phosphorylation sites for different kinases have been estimated [51], rat bone OPN contains an average of 12 phosphoserines and one phosphothreonine [6], indicating that there is considerable potential for additional

phosphorylation and that the degree of phosphorylation is in part dependent on the availability and time of exposure of the OPN to the kinases.

That the ectokinase is a cell surface-associated enzyme is indicated by experiments in which phosphorylation was studied in fresh media. Although it is conceivable that intracellular kinase could be released during the 10 min labelling period, it is clearly evident that exogenously added protein substrates inhibited the phosphorylation of the cell surface proteins (Figure 2), indicating that they compete for the same enzyme. Although both OPN and BSP can mediate cell attachment through RGD motifs [52,53], the binding of these proteins to integrins on the cell surface does not seem to be a prerequisite for ectokinase phosphorylation because the phosphorylation was not changed in the presence of RGD peptide, which blocks the cell attachment activity of these proteins.

Using dissociative extraction procedures to isolate OPN and BSP from mineralized bone tissue formed by FRCCs, we have shown that the phosphorylation of these proteins by extracellular kinase occurs during bone formation *in vitro*. Moreover, consistent with previous studies [12,13] the phosphorylated OPN is rapidly bound to the mineral phase, whereas some of the phosphorylated BSP is extracted from the G1 and G2 extracts, indicative of its binding to the organic matrix before the mineralization of the matrix. In the studies by Mikuni-Takagaki et al. [18], using cell populations derived from neonatal rat calvariae, ectokinase activity was highest in more osteoblastic and osteocytic cell populations in which alkaline phosphatase activity was decreased, but only a 53 kDa fragment of BSP seemed to be phosphorylated. On the basis of these results it was suggested that extracellular phosphorylation is prevented by alkaline phosphatase expressed by osteoblasts during osteoid formation and that fragmentation of the BSP might be necessary for extracellular phosphorylation. However, our studies show a significant phosphorylation of intact OPN and BSP by ectokinase in cells that express alkaline phosphatase, and although levamisole, which inhibits alkaline phosphatase activity, seemed to decrease the phosphorylation of proteins in the G1 extract of the mineralizing FRCC cultures, no effect was observed on the phosphorylation of proteins in the other extracts.

In summary, these studies demonstrate that an ectokinase enzyme with characteristics of casein kinase II can utilize nucleoside triphosphates as phosphate donors to catalyse the transfer of phosphoryl groups to newly synthesized bone proteins. Because protein-bound phosphate groups have been shown to be important for cell signalling mechanisms and also for hydroxyapatite crystal nucleation and growth, the ectokinase activity might be important for regulating the mineralization of bone by influencing bone cell activity or by directly influencing mineral formation through the phosphorylation of OPN and BSP.

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