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# Biosynthesis of bone proteins [SPP-1 (secreted phosphoprotein-1, osteopontin), BSP (bone sialoprotein) and SPARC (osteonectin)] in association with mineralized-tissue formation by fetal-rat calvarial cells in culture

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To determine the relationship between the expression of bone proteins and the formation of mineralized-tissue matrix, the biosynthesis of non-collagenous bone proteins was studied in cultures of fetal-rat calvarial cells, which form mineralized nodules of bone-like tissue in the presence of β-glycerophosphate. The temporal pattern of protein synthesis in both mineralizing and non-mineralizing cultures was studied by metabolic labelling with [$^{35}$S]methionine, $^{35}SO_4^{2-}$ or $^{32}PO_4^{3-}$ over a 5-day period. After a 24 h labelling period, the culture media were harvested and the cell layers extracted sequentially with aq. 0.5 M-$NH_3$, followed by 4 M-guanidinium chloride (GdmCl), 0.5 M-EDTA and a second extraction with 4 M-GdmCl. Protein associated with collagenous bone matrix was analysed after digestion with bacterial collagenase. On the basis of [$^{35}$S]methionine labelling, the major proteins extracted from the mineralizing matrix were secreted phosphoprotein-1 (SPP-1; osteopontin), bone sialoprotein (BSP) and a 14 kDa phosphoprotein. The presence of SPP-1 and BSP in the conditioned media of both mineralizing and non-mineralizing cultures and their incorporation into the mineralizing nodules indicated that these proteins associate with preformed mineral crystals. However, some BSP was also present in GdmCl extracts and, together with a 35 kDa sulphated protein, was released from a bacterial-collagenase digestion of the tissue residue in both non-mineralizing and mineralizing cultures. Two forms of sulphated SPP-1 were identified, a highly phosphorylated 44 kDa species being the predominant form in the mineralized matrix. The BSP was more highly sulphated but less phosphorylated than SPP-1. Bone SPARC (secreted protein, acid and rich in cysteine) protein (osteonectin) was present almost entirely in the conditioned media and did not incorporate $^{32}PO_4^{3-}$ or $^{35}SO_4^{2-}$. The SPP-1 and the 14 kDa protein were susceptible to thrombin digestion, the 44 kDa SPP-1 being specifically cleaved into 28 and 26 kDa fragments. The fragments were labelled uniformly with [$^{35}$S]methionine, but the 28 kDa fragment incorporated more $^{35}SO_4^{2-}$, but less $^{32}PO_4^{3-}$, than the 26 kDa fragment. These studies demonstrate that SPP-1 and BSP are the major osteoblast-derived bone proteins to bind to the bone mineral. That BSP also binds to the collagenous bone matrix indicates a potential role for this protein in linking the hydroxyapatite with collagen.

## INTRODUCTION

A number of proteins present in demineralizing extracts of rat bone have been described and the major proteins produced by osteoblastic cells have been characterized. These include two classes of small proteoglycan (Franzén & Heinegård, 1985*a*,*b*; Sato *et al.*, 1985), two sialoproteins (Oldberg *et al.*, 1986, 1988; Prince *et al.*, 1986), SPARC (secreted protein, acidic and rich in cysteine) protein (Zung *et al.*, 1986) and osteocalcin (Hauschka *et al.*, 1975; Price *et al.*, 1976). Despite extensive studies on the non-collagenous proteins from several species, their precise function in bone formation and remodelling is unknown. Since these proteins bind $Ca^{2+}$ and $PO_4^{3-}$, in addition to having a high affinity for hydroxyapatite, they have the potential to act as both nucleators of hydroxyapatite crystal formation as well as regulators of crystal growth and dissolution, these activities being dependent upon the physical state of the protein (Linde & Lussi, 1989). Thus, osteocalcin (Hauschka *et al.*, 1975) and especially SPARC/osteonectin in solution are potent inhibitors of crystal growth (Menanteau *et al.*, 1982; Romberg *et al.*, 1985). In contrast, SPARC protein has been reported to promote hydroxyapatite crystal formation when bound to collagen (Termine *et al.*, 1981*a*,*b*). A specific association with collagen might be anticipated for a potential nucleator, since hydroxyapatite crystals in bone are spatially oriented along the long axis of collagen fibrils (Glimcher, 1984). However, many proteins identified in demineralizing extracts of bone are not produced by osteoblasts and, although they have an affinity for the hydroxyapatite, are unlikely to be involved in mineral formation or metabolism.

The ability of rodent cells with osteogenic potential to form bone-like tissue when cultured in the presence of ascorbic acid and organic phosphate (Ecarot-Charrier *et al.*, 1983; Nefussi *et al.*, 1985; Bellows *et al.*, 1986; Maniatopoulos *et al.*, 1988) provides a unique means of studying the synthesis of those proteins produced by osteoblastic cells that become deposited into a mineralizing matrix. Moreover, since the mineralization of

Abbreviations used: SPP-1, secreted phosphoprotein-1 (osteopontin); BSP, bone sialoprotein; SPARC, secreted protein, acidic and rich in cysteine (osteonectin); GdmCl, guanidinium chloride; DTT, dithiothreitol; β-GP, (sodium) β-glycerophosphate; α-MEM, α-minimal essential medium; PBS, phosphate-buffered saline [$KH_2PO_4$ (0.20 g/l)/$Na_2HPO_4$,$7H_2O$ (2.16 g/l)/NaCl (8.00 g/l)/KCl (0.2 g/l), pH 7.2].

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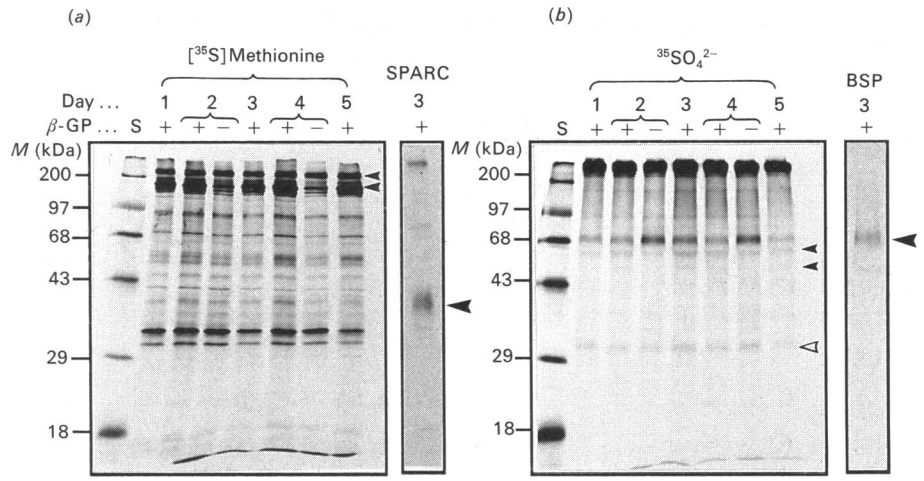


**Fig. 1. Analysis of radiolabelled proteins in the conditioned media of rat bone-cell cultures and immunoprecipitation of SPARC and BSP**

Rat bone cells were cultured for 14 days in 35 mm-diameter tissue-culture dishes in the presence of $10^{-8}$ M-dexamethasone, and 10 mM-β-GP was then added to one group of cultures at the start of day 1 of the experiment to initiate mineralization of the preformed tissue nodules. Radiolabelling with (*a*) [$^{35}$S]methionine and (*b*) $^{35}SO_4^{2-}$ was performed for a 24 h period in triplicate dishes of both mineralizing (+β-GP) and non-mineralizing (−β-GP) cultures on the days shown. Aliquots (10 μl) of conditioned medium from single cultures were analysed by SDS/PAGE on 10% cross-linked gels, the radioactive protein bands being revealed by fluorography. Radiolabelled proteins from 100 μl of culture media were immunoprecipitated with antibodies to SPARC and BSP (large arrowheads). The position of fibronectin (small arrowhead) and procollagens (open arrowhead) are indicated on the [$^{35}$S]methionine-labelled gel, and the SPP-1 (small arrowheads) and 32 kDa protein (open arrowhead) are indicated on the gel of $^{35}SO_4^{2-}$-labelled proteins. The radiolabelled protein standards (S) are shown in the first lane of each gel (*M*, molecular mass).

the connective tissue formed by these cells can be regulated through the availability of an organic-phosphate source (Tenenbaum & Heersche, 1982; Bellows *et al.*, 1986), the relationship between the synthesis and deposition of these proteins into a bone matrix and tissue mineralization can be assessed. Previous studies have characterized the bone-forming potential of bone-cell populations derived from fetal-rat calvariae and have shown that the osteogenic potential is increased in the presence of dexamethasone (Bellows *et al.*, 1987; Bellows & Aubin, 1989; Bellows *et al.*, 1990*a*).

As the major non-collagenous proteins from rat bone, with the exception of osteocalcin, contain methionine, we have used [$^{35}$S]methionine to study the temporal aspects of the synthesis and deposition of these proteins in both mineralizing and non-mineralizing tissues formed *in vitro*. In addition, since SPARC (Termine *et al.*, 1981*a*; Engel *et al.*, 1987) has been reported to be phosphorylated, and secreted phosphoprotein-1 (SPP-1; osteopontin) and bone sialoprotein (BSP) are both sulphated and phosphorylated (Ecarot-Charrier *et al.*, 1989; Nagata *et al.*, 1989; Prince *et al.*, 1986; Kubota *et al.*, 1989), additional cultures were also labelled with $^{35}SO_4^{2-}$ and others with $^{32}PO_4^{3-}$. The synthesis of bone proteins was determined from the analysis of culture media, and incorporation of the radiolabelled proteins into the tissue matrix was examined using a dissociative extraction procedure designed to identify proteins in the different tissue compartments of bone (Domenicucci *et al.*, 1988; Goldberg *et al.*, 1988*a*,*b*).

## MATERIALS AND METHODS

### Cell cultures

Fetal-rat calvarial cells were prepared and cultured as described previously (Rao *et al.*, 1977; Bellows *et al.*, 1986; Antosz *et al.*, 1989). Briefly, calvariae from 21-day fetal Wistar rats were dissected and loosely adherent soft connective tissues removed. Cells were isolated by sequential digestion in a collagenase-containing enzyme mixture (Rao *et al.*, 1977). The cells obtained from the last four of the five-step digestion sequence (populations II–V) were pooled, plated in an α-minimal essential medium (α-MEM) containing 10% heat-inactivated fetal-bovine serum and antibiotics [100 μg of penicillin G (Sigma)/ml, 50 μg of gentamycin sulphate (Sigma)/ml and 0.3 μg of fungizone (Flow Laboratories)/ml] in T-75 flasks. Cells were maintained for 24 h at 37 °C in a humidified air/$CO_2$ (19:1) atmosphere, after which viable adherent cells were collected by trypsin treatment (0.01% trypsin in citrate/saline buffer), counted, and plated at $3 \times 10^4$ cells/35 mm dish. After 24 h, fresh α-MEM supplemented as above and containing 50 μg of ascorbic acid/ml and $10^{-8}$ M-dexamethasone was added. Medium was changed routinely every second day for 14 days, after which numerous unmineralized nodules were present. To initiate mineralization of the nodules, 10 mM-sodium β-glycerophosphate (β-GP) was added to the culture medium and medium was changed daily during mineralization of nodules.

### Radiolabelling procedures

To study the biosynthesis of bone proteins in 14-day cultures, metabolic labelling with (100 μCi/ml of) [$^{35}$S]methionine (R51006; 600 mCi/mmol; ICN Radiochemicals, Irvine, CA, U.S.A.), $Na_3{}^{32}PO_4$ (64052; 1000 mCi/mmol; ICN) or $Na_2{}^{35}SO_4$ (NEX 041; 500 mCi/mmol; New England Nuclear, Boston, MA, U.S.A.) was performed. When labelling with [$^{32}PO_4$], phosphate-deficient Dulbecco's MEM was used instead of α-MEM. In each case, duplicate 35 mm dishes of cells were first washed twice with phosphate-buffered saline (PBS) containing antibiotics and the radioisotope added in fresh medium containing 0.5% serum and antibiotics. Labelling was initiated at days 1–5 after the addition of β-GP and was continued for 24 h under the usual culture conditions. Two series of dishes that did not receive β-GP were labelled at days 2 and 4. After labelling, the media were collected and the tissue layers fractionated as described below.

### Extraction procedures

A procedure adapted from Domenicucci *et al.* (1988) was used

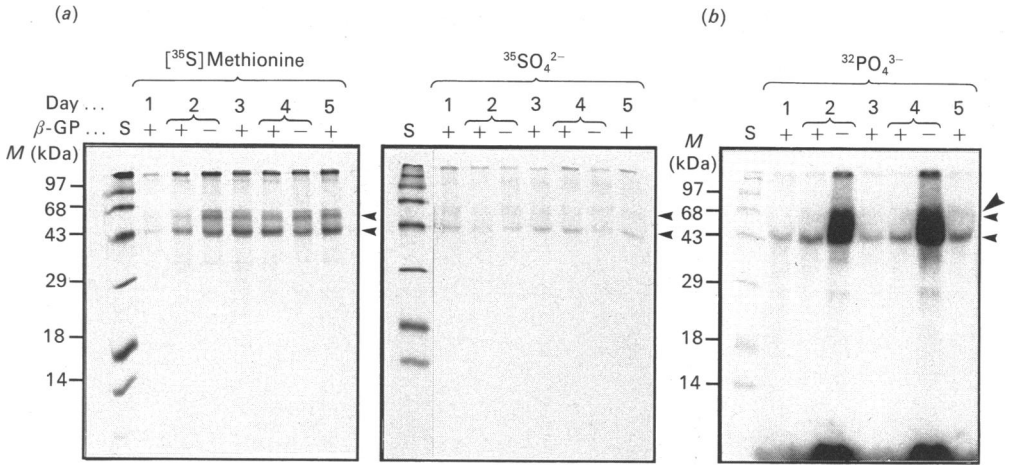


**Fig. 2. Identification of SPP-1 in conditioned media**

(*a*) The radiolabelled culture media shown in Fig. 1 were used for immunoprecipitations with antibody to SPP-1. The immunoprecipitated proteins obtained from 100 $\mu$l of culture media were analysed by SDS/PAGE on 15% cross-linked gels. Notably, the two major SPP-1 forms migrate faster (at 44 kDa and 55 kDa, shown by small arrowheads) than observed on 10% gels (56 kDa and 60 kDa). (*b*) Also shown is the analysis of $^{32}PO_4^{3-}$-labelled proteins corresponding to the two forms of SPP-1 (small arrowhead), with some radioactivity associated with the BSP (large arrowhead) co-migrating with the 55 kDa SPP-1. Standards (S) are shown in the first lane (*M*, molecular mass).

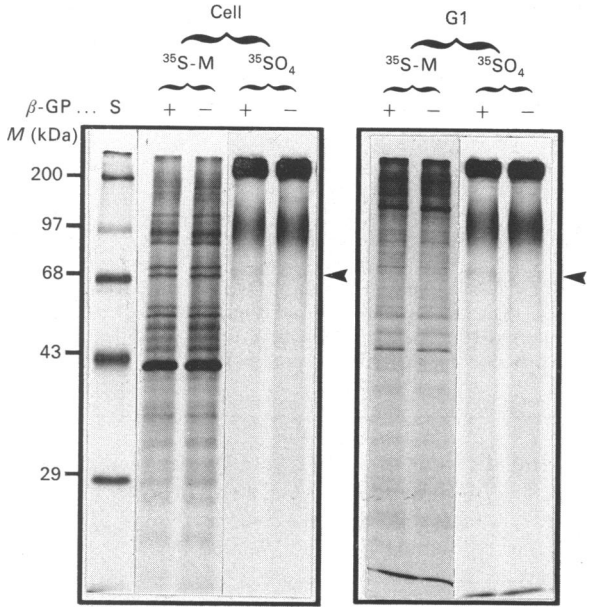


**Fig. 3. Analysis of radiolabelled proteins extracted from cells and non-mineralized matrix**

Representative samples of [$^{35}$S]methionine ($^{35}$S-M)-labelled and $^{35}SO_4^{2-}$ ($^{35}SO_4$)-labelled proteins extracted from cells with aq. 0.5 M-$NH_3$ and from the tissue matrix with 4 M-GdmCl (G1-extract) of mineralizing (+$\beta$-GP) and non-mineralizing (−$\beta$-GP) on day-4 are shown after separation by SDS/PAGE on 10% cross-linked gels. The position of the $^{35}SO_4^{2-}$-labelled BSP is indicated by the arrowhead below a broad band characteristic of a small proteoglycan. The radiolabelled protein standards (S) are shown in the first lane.

to extract the radiolabelled proteins from the tissue layer. All procedures were carried out at 4 °C, and all buffers contained proteinase inhibitors (100 mM-hexanoic acid, 5 mM-benzamidine hydrochloride and 1 mM-phenylmethanesulphonyl fluoride). Briefly, the tissue layer was first washed three times in 3 ml of PBS and the cells extracted for 40 s with 1 ml of aq. 0.5 M-$NH_3$ (Nagata *et al.*, 1989). This was followed by two extractions with each of the following solutions: 4 M-GdmCl (G1-extract) and 0.5 M-EDTA (E-extract), each prepared in 50 mM-Tris/HCl buffer, pH 7.4. After a further wash in PBS, 1.0 ml of 4 M-GdmCl was added to the cell layer, which was scraped off the dish with a rubber policeman and transferred to a 1.5 ml Microfuge tube. Each extraction was continued for 24 h with gentle shaking, and three 30 min washes with PBS were included before the subsequent extractant was used. A brief 1 min centrifugation at 10000 *g* on an Eppendorf Microfuge was used to separate the tissue residue from the supernatant. After each series of extractions, the two supernatants were pooled and dialysed against two changes of 0.1 M-$(NH_4)_2SO_4$ containing 0.05% Brij-35, followed by a final dialysis against one-tenth strength buffer and kept at −20 °C. Some extracts were desalted by chromatography on a 1 cm × 20 cm column of Sephadex G-50. The tissue residue was then washed three times in PBS followed by three washes with 50 mM-Tris/HCl buffer, pH 7.6, containing 5 mM-$CaCl_2$ and 25 nM-*N*-ethylmaleimide and incubated with 100 $\mu$g of highly purified bacterial collagenase (Worthington CLSPA collagenase, further purified by chromatography on Sephadex S-200) for 3 h at 37 °C. The radiolabelled proteins released by the degradation of the collagenous matrix were then analysed by SDS/PAGE.

### Thrombin digestion

Digestions with thrombin were carried out on freeze-dried aliquots of medium, extracts and tissue residue. The digestions were performed at 37 °C for 30 min in 10 $\mu$l of 10 mM-Tris/HCl buffer, pH 8.0, containing 10 mM-$CaCl_2$ and 1 unit of thrombin (T-7513, Sigma Chemical Co., St. Louis, MO, U.S.A.). Digestions were terminated by the addition of 0.25 vol. of 4-fold-concentrated SDS/PAGE sample buffer containing 60 mg of DTT/ml and heating to 56 °C for 25 min.

### PAGE

SDS/PAGE electrophoresis of proteins on mini-slab gels (Hoefer Scientific Instruments, San Fransisco, CA, U.S.A.) using the discontinuous Tris/glycine buffer system with 10 and 15% cross-linked linear gels was performed as described previously (Overall *et al.*, 1989). Samples were dissolved in 10 $\mu$l sample buffer containing 1% SDS, 2.0 M-urea and Bromophenol Blue

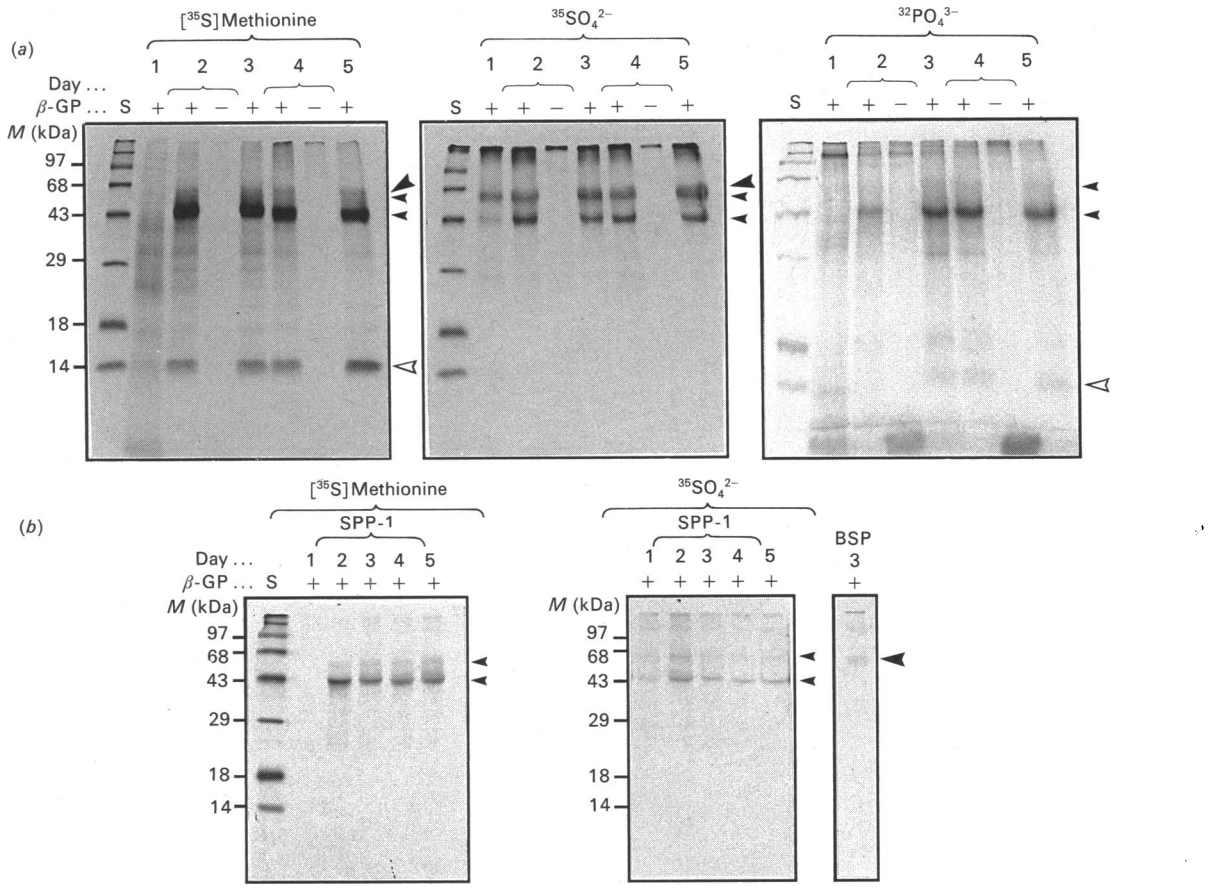


**Fig. 4. Analysis of radiolabelled proteins extracted from the tissue matrix with EDTA**

(*a*) Tissues from mineralizing (+β-GP) and non-mineralizing (−β-GP) cultures, radiolabelled with [$^{35}$S]methionine $^{35}SO_4^{2-}$ or $^{32}PO_4^{3-}$ on the days indicated were extracted with 0.5 M-EDTA, following prior extractions with aq. 0.5 M-$NH_3$ and 4 M-GdmCl, and the proteins analysed by SDS/PAGE on 15% cross-linked gels. The $^{35}$S-labelled proteins were revealed by fluorography and the $^{32}PO_4^{3-}$-labelled proteins by autoradiography. The positions of two forms of SPP-1 are shown by small arrowheads. The large arrowheads indicate the position of BSP. In addition, a prominent methionine-labelled phosphoprotein is indicated (open arrowhead) at 14 kDa. (*b*) The identity of the radiolabelled SPP-1 forms and BSP was confirmed by immunoprecipitation of SDS/15%-PAGE gels. In each case the radiolabelled protein standards (S) are shown in the first lane (*M*, molecular mass).

marker. For the analysis of proteins under reducing conditions, 150 μg of DTT was included. Samples were heated to 56 °C for 20 min and cooled immediately before application to individual wells; radiolabelled protein standards were run with each gel. Electrophoresis was carried out for 1 h at 150 V. After separation, proteins were detected by fluorography, or by autoradiography for $^{32}PO_4^{3-}$, on Kodak SB-5 film.

### Immunoprecipitations

Radiolabelled proteins were immunoprecipitated with specific antibodies to SPARC (Domenicucci *et al.*, 1988), SPP-1 (Prince *et al.*, 1986) and BSP [monoclonal hybridoma MPIIIBIO$_1$ (VD12) obtained from the Developmental Studies Hybridoma Bank, Johns Hopkins University, Baltimore, MD, U.S.A., under contract from the NICHD] using procedures described previously (Otsuka *et al.*, 1984, 1988; Wrana *et al.*, 1988). Briefly, the freeze-dried sample was dissolved in 200 μl of immunoprecipitation buffer (0.3% Nonidet P40/0.3% sodium deoxycholate/0.1% BSA in Tris/HCl-buffered saline/0.02% $NaN_3$) in a 1.5 ml Microfuge tube and incubated with 100 μl of pre-washed Pansorbin for 2 h. Simultaneously, the primary antibody, equivalent to 1.5 μl of the original antiserum or ascites fluid, was coupled to 100 μl of Protein A–Sepharose. After the removal of the Pansorbin the radiolabelled proteins were incubated with the primary antibody for 16 h at 4 °C with gentle shaking. The radiolabelled proteins bound to the antibodies were washed four times with PBS/Tween (PBS containing 0.5% Tween-20 and 0.1% BSA in the first three washes) and then dissolved in 15 μl of SDS/PAGE sample buffer containing DTT and heated at 56 °C for 30 min before analysis.

## RESULTS

### Analysis of culture media

Radiolabelled proteins from the media of non-mineralized and mineralizing cultures were analysed by SDS/PAGE on 10% and 15% cross-linked gels as shown in Figs. 1 and 2. The patterns of [$^{35}$S]methionine-labelled proteins were similar for each condition and revealed no apparent qualitative differences. In each case, procollagens and fibronectin, migrating between 160–220 kDa, were the predominant proteins secreted into the culture medium. Immunoprecipitation with specific antibodies revealed $^{35}$S-labelled SPP-1, BSP and SPARC protein. SPARC was immunoprecipitated as a 40 kDa protein (Fig. 1), whereas the SPP-1 was resolved into two bands migrating at 44 kDa and 55 kDa on 15% cross-linked gels (Fig. 2*a*). After radiolabelling with $^{35}SO_4^{2-}$, four different radiolabelled proteins could be recognized: proteoglycans, which were at the top of the gels, a 68 kDa protein identified by immunoprecipitation as BSP (Fig. 1), 44 and 55 kDa proteins (migrating at 56 and 60 kDa on 10% gels) that were

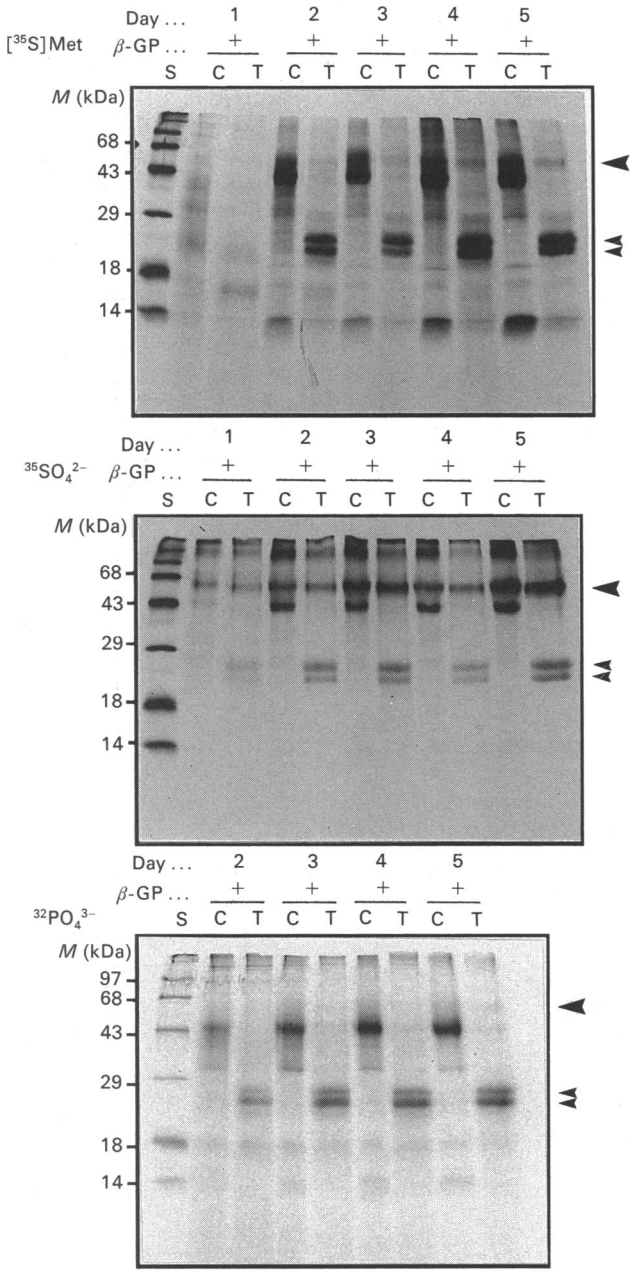


**Fig. 5. Thrombin digestion of proteins in the E-extract**

Aliquots of radiolabelled proteins extracted from mineralizing (+β-GP) cultures labelled with [$^{35}$S]methionine, $^{35}SO_4^{2-}$ and $^{32}PO_4^{3-}$, on the days shown were digested with 1 unit of thrombin and the digestion products analysed by SDS/PAGE on 15% cross-linked gels. The positions of the BSP (large arrowhead) just below the 68 kDa protein marker and the 28/26 kDa fragments (small arrowheads) produced from the SPP-1 are indicated. Note the [$^{35}$S]-methionine-labelled 14 kDa phosphoprotein in the control (C) undigested lanes that is digested by the thrombin (T). Also note the relative intensities of different radiolabels in the 28/26 kDa fragments. S, molecular mass (*M*) standards.

immunoprecipitated with antibodies to rat SPP-1 (Fig. 2*a*) and an unidentified protein that migrated at 32 kDa. The radiolabelled bands corresponding to BSP were noticeably stronger in the media of the non-mineralizing cultures. The labelling with $^{32}PO_4^{3-}$ was essentially restricted to a strong 44 kDa band and a more diffuse band extending above the 44 kDa band to 68 kDa (Fig. 2*b*). The weaker labelling of the protein in the mineralizing cultures was due to the dilution of the $^{32}PO_4^{3-}$ with phosphate derived from the breakdown of the β-GP. Almost all of the $^{32}PO_4^{3-}$-labelled protein was immunoprecipitated with antibodies to SPP-1, with a small amount remaining in the position of BSP (results not shown). The SPP-1 radioactivity was in two bands corresponding to the 44 kDa and 55 kDa forms. Notably, the 44 kDa SPP-1 was labelled more strongly with $^{32}PO_4^{3-}$ than the 55 kDa form, when compared with the [$^{35}$S]methionine labelling, demonstrating greater phosphorylation of the 44 kDa form.

### Cell and G1-extracts

After the extraction of cells with aq. 0.5 M-$NH_3$, the tissue residue was extracted with 4 M-GdmCl to isolate the non-covalently bound matrix proteins (Fig. 3). Analysis of [$^{35}$S]-methionine-labelled proteins revealed a broad range of proteins which were clearly different in the two extracts. The $^{35}SO_4^{2-}$-labelled material revealed proteoglycan-like material migrating at the 97 kDa marker in both extracts. A weak protein band at 68 kDa, which was stronger in the mineralizing cultures, was immunoprecipitated with antibodies to BSP. However, no $^{32}PO_4^{3-}$-labelled material could be resolved, nor was any radiolabelled SPP-1 immunoprecipitated (results not shown).

### E-extracts

After extraction with 4 M-GdmCl, radiolabelled proteins could be extracted with 0.5 M-EDTA, but only from the mineralizing cultures. The 44 kDa SPP-1 was the major [$^{35}$S]methionine-labelled protein, followed by a 14 kDa protein with lesser amounts of BSP at 60 kDa on 15% gels (Fig. 4*a*). The identity of the SPP-1 and BSP was confirmed by immunoprecipitation (Fig. 4*b*) and from thrombin digestions (Fig. 5). Small amounts of the 55 kDa SPP-1 were evident in the immunoprecipitates obtained with the SPP-1 antibodies (Fig. 4*b*), but no radiolabelled protein was immunoprecipitated with antibodies to SPARC protein (results not shown). Incorporation of $^{35}SO_4^{2-}$ was evident in proteoglycan, but, in addition, more discrete protein bands that were immunoprecipitated with antibodies to BSP and SPP-1 (Fig. 4*b*) were also observed (Fig. 4*a*). Labelling with $^{32}PO_4^{3-}$ produced a major band corresponding to the 44 kDa SPP-1, with minor bands reflecting the presence of the 56 kDa SPP-1 and the BSP. Thrombin digestion of the [$^{35}$S]methionine-labelled proteins resulted in the conversion of SPP-1s into 28/26 kDa fragments of comparable intensity, whereas the 14 kDa band was degraded to fragments that were too small to be resolved on the gel (Fig. 5). Thrombin digestion of the $^{35}SO_4^{2-}$-labelled proteins produced a 28/26 kDa doublet for the SPP-1s, with the 28 kDa band being more intense than the 26 kDa band. Notably, the mobility of the BSP band increased marginally, but consistently, in thrombin digestions of both [$^{35}$S]methionine-labelled and $^{35}SO_4^{2-}$-labelled protein. The $^{32}PO_4^{3-}$-labelled SPP-1s were also degraded to a 28/26 kDa doublet but, in contrast with the $^{35}SO_4^{2-}$-labelled fragments, the 26 kDa band was more intense than the 28 kDa band. Since the 44 kDa SPP-1 was clearly the predominant form of SPP-1 in the E-extract, the 28 and 26 kDa fragments must have been generated from the 44 kDa SPP-1. A $^{32}PO_4^{3-}$-labelled 14 kDa protein corresponding to the [$^{35}$S]methionine-labelled protein was also digested by thrombin, indicating that the 14 kDa protein was also a phosphoprotein.

From the relative intensity of the radiolabelled bands obtained with the different precursors, it was evident that the BSP incorporated more $^{35}SO_4^{2-}$, but less $^{32}PO_4^{3-}$, than the 44 kDa SPP-1, whereas the 44 kDa SPP-1 appeared to be more heavily sulphated and phosphorylated than the 55 kDa SPP-1. Further, the incorporation of radiolabelled SPP-1 and BSP into the mineralized matrix appeared to increase from day 1 to day 3, in accordance with the increase in mineralized tissue.

### G2-extract

Extraction of the tissue residue with 4 M-GdmCl or SDS/PAGE sample buffer revealed a series of [$^{35}$S]methionine-labelled proteins similar in pattern to those observed with the initial cell extracts. However, the $^{35}SO_4^{2-}$-labelled proteins were mostly proteoglycan-like in their behaviour and included a prominent BSP band which was markedly reduced in the non-mineralizing cultures, similar to the pattern observed in the G1-extracts (Fig. 6). Digestion of the sulphated proteins with thrombin revealed a 35 kDa fragment derived from an unidentified thrombin-sensitive protein. Of note, neither this fragment nor any other $^{35}SO_4^{2-}$-labelled protein could be immunoprecipitated from the extract with antibodies to SPP-1.

### Bacterial-collagenase digestion

To determine whether some non-collagenous proteins might be associated with the collagenous matrix remaining after the various extractions, the tissue residue, after exhaustive extraction with 4 M-GdmCl, was digested with highly-purified bacterial collagenase and the solubilized proteins analysed by SDS/PAGE. In addition to a strong band of proteoglycan-like material at the top of the gel, a 60 kDa band (on 15% gels) corresponding to BSP was observed together with a more prominent ~ 38 kDa doublet band of [$^{35}$S]methionine and $^{35}SO_4^{2-}$-labelled protein (Fig. 7). Although the $^{35}SO_4^{2-}$-labelled proteins were seen in both mineralized and non-mineralized matrices, their intensities were greater in the mineralized cultures. No discrete $^{32}PO_4^{3-}$-labelled proteins could be resolved. Also, whereas the 60 kDa protein was immunoprecipitated with antibodies to BSP, neither these antibodies nor SPP-1 antibodies immunoprecipitated the 38 kDa proteins, which were also resistant to thrombin digestion.

## DISCUSSION

In the present study we have used a well-characterized culture system (Nefussi *et al.*, 1985; Bellows *et al.*, 1986; Bhargava *et al.*, 1988) to study the synthesis of bone proteins and their distribution in both mineralizing and non-mineralizing bone matrices. Of the major non-collagenous proteins synthesized by rodent bone cells SPP-1 (Oldberg *et al.*, 1986) and BSP (Oldberg *et al.*, 1988), each has two methionine residues per molecule, and SPARC protein has four (Mason *et al.*, 1986). Thus metabolic labelling with [$^{35}$S]methionine provides a realistic comparison of the relative amounts of these proteins that are synthesized. Analysis of the culture medium revealed the presence of SPARC, SPP-1 and BSP in both mineralizing and non-mineralizing cultures. Since the labelling time was the same for each day studied, the low amounts of SPP-1 and BSP in the mineralizing cultures on days 1, compared with the non-mineralizing cultures on days 2–5, indicate that the synthesis of these proteins may be affected by the depleted $Ca^{2+}$ levels resulting from the deposition of $Ca^{2+}$ in the nodules (Bellows *et al.*, 1990*b*). In contrast, the low amounts of SPP-1 and BSP in the culture medium of mineralizing cultures was due to the association of the proteins with the preformed mineral, as judged from the amounts of these proteins in the E-extract.

Surprisingly, radiolabelled SPARC protein was not detected in the mineralized tissue, which explains the similar amounts of this protein in the conditioned media of both mineralizing and non-mineralizing cultures. The apparent lack of SPARC incorporation into the newly forming bone has also been observed in osteogenic rat bone-marrow-cell cultures (Kasugai *et al.*, 1991) and in studies of bone formation by fetal porcine calvarial bone fragments maintained *in vitro* (Nagata *et al.*, 1991). Since SPARC is present, albeit in relatively low concentration (Zung *et al.*, 1986), in rat bone and has been demonstrated immunohistochemically in the mineralized tissue formed in the same culture system used here (Bellows *et al.*, 1986), SPARC must be incorporated very slowly compared with SPP-1 and BSP. Notably, studies *in vivo* have revealed incorporation of radiolabelled sialoprotein, but not SPARC, into rat bone within 24 h (Zung *et al.*, 1986). Consistent with previous studies on rat bone cells and fibroblasts (Zung *et al.*, 1986) and pig bone (Nagata *et al.*, 1991), we were unable to detect any incorporation of $^{32}PO_4^{3-}$ into SPARC. These results contrast with the incorporation of $^{32}PO_4^{3-}$ into SPARC synthesized by PYS cells (Engel *et al.*, 1987) and with the report that SPARC extracted from bone is a phosphoprotein (Termine *et al.*, 1981*a*).

Although the expression of BSP appears to be essentially restricted to differentiated osteoblastic cells (Oldberg *et al.*, 1988), SPP-1 and SPARC are also synthesized in low amounts by less-differentiated bone cells and, in the case of SPARC, fibroblasts (Mark *et al.*, 1987; Kubota *et al.*, 1989), which are present in these cultures. Consequently, some of the SPARC in the conditioned media may be derived from cells in the non-osteogenic tissue layer. For SPP-1, previous studies have shown that several forms, which differ as a result of post-translational modifications, are produced by rat bone cells (Kubota *et al.*, 1989; Nagata *et al.*, 1989). The two major forms identified show the anomalous behaviour of SPP-1 on SDS/PAGE, migrating at 44 kDa and 55 kDa on 15% gels and 56 kDa and 60 kDa on 10% gels. The slower-migrating form is prominent in confluent cultures of rat bone cells (Nagata *et al.*, 1989). However, in the multilayered cultures used in the present studies, the faster-migrating SPP-1 is the predominant species. Moreover, the predominance of this 44 kDa form was even more pronounced in the mineralized matrix, indicating that it is preferentially synthesized by differentiated osteogenic cells, whereas the 55 kDa form may be preferentially synthesized by less differentiated bone cells. Further, on the basis of the incorporation of $^{35}SO_4^{2-}$ and $^{32}PO_4^{3-}$, it is apparent that the 44 kDa SPP-1 is more highly phosphorylated, and also more highly sulphated, than the 55 kDa form, features that may increase the affinity of the 44 kDa SPP-1 for both $Ca^{2+}$ and hydroxyapatite.

The presence of BSP in the G-extracts of non-mineralizing, as well as mineralizing, cultures indicates that some of this protein becomes associated with collagenous matrix and can affect its subsequent mineralization on the addition of $\beta$-GP. That some of the BSP might be bound to the collagen fibres is also indicated by the release of some BSP by collagenase digestion of the exhaustively extracted demineralized tissue. Thus BSP could provide a link between the hydroxyapatite crystals and the organic bone matrix. In comparison, this study has shown that SPP-1, which by virtue of its poly(aspartic acid) sequence (Oldberg *et al.*, 1986; Fisher *et al.*, 1990; Zhang *et al.*, 1990), its sulphation (Nagata *et al.*, 1989; Zhang *et al.*, 1990) and high degree of phosphorylation (Prince *et al.*, 1986; Butler, 1989; Kubota *et al.*, 1989) has many of the features expected of a nucleator of mineral-crystal formation (Glimcher, 1984; Addadi *et al.*, 1987), does not bind to collagen, either directly or indirectly. That SPP-1 is unlikely to be a primary nucleator of hydroxyapatite is also indicated by its absence from the matrix of non-mineralizing tissues. However, it could conceivably be involved in secondary nucleation or in the regulation of crystal growth, since it associates rapidly with preformed mineral.

Two apparently novel proteins have been identified in the course of this study. The phosphorylated 14 kDa protein could be a degradation product of SPP-1, which has been shown to fragment during bone formation (Zhang *et al.*, 1990). However,

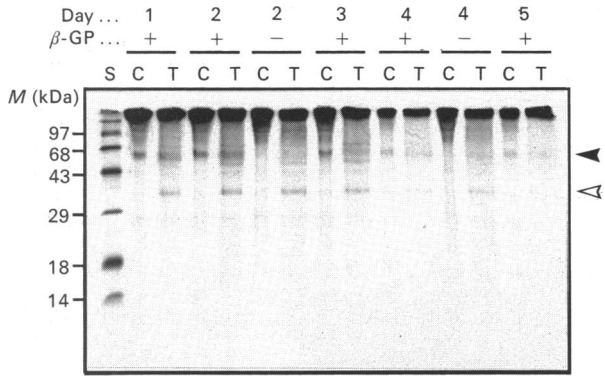


**Fig. 6. Analysis of radiolabelled proteins associated non-covalently with the tissue residue**

The radiolabelled tissue from mineralizing (+β-GP) and non-mineralizing (−β-GP) cultures remaining after extraction with EDTA was extracted with 500 μl of twice-concentrated electrophoresis sample buffer and the proteins analysed by SDS/PAGE on 15% cross-linked gels. Samples from tissues radiolabelled with $^{35}SO_4^{2-}$ on the different days (C) are shown together with identical samples that were digested with thrombin (T). The radiolabelled BSP which is stronger in the mineralizing cultures is indicated (closed arrowhead) together with a 35 kDa fragment (open arrowhead) produced by thrombin digestion. S, molecular-mass (*M*) markers.

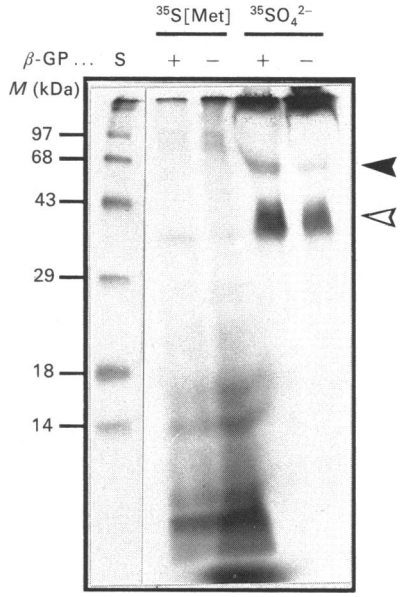


**Fig. 7. Analysis of radiolabelled proteins released from the tissue residue by bacterial-collagenase digestion**

The tissue residue remaining after exhaustive extraction with 0.5 M-EDTA and 4 M-GdmCl was digested with 100 μg of highly purified bacterial collagenase and the radiolabelled proteins released analysed by SDS/PAGE on 15% cross-linked gels for each time point in both mineralizing (+β-GP) and non-mineralizing (−β-GP) cultures on day-4. The positions of the BSP (arrowhead) and the protein doublet at 35 kDa (open arrowhead) are shown. S, molecular-mass (*M*) markers.

a single fragment of this prominence has not been observed in similar studies of bone formation by rat bone-marrow cells (Kasugai *et al.*, 1991) nor fetal porcine calvaria (Nagata *et al.*, 1991). Consequently, the significance of this protein in bone formation remains to be evaluated. The $^{35}SO_4^{2-}$-labelled protein doublet that appears to be bound to collagen could also be a fragment of a larger molecule. However, it could not be immunoprecipitated with antibodies to either SPP-1 or BSP. Notably, a sulphated protein with similar properties has been observed in bone formed by rat bone-marrow cells in culture (Kasugai *et al.*, 1991).

This study has also revealed that the major proteins synthesized by bone cells in bone-forming cultures are typically phosphorylated, sulphated and susceptible to thrombin. Thrombin digestion produces only a single 28–30 kDa band for the 55 kDa form of SPP-1 (Wrana *et al.*, 1990) and the single form of SPP-1 synthesized by porcine (Zhang *et al.*, 1990) and human tissues (Senger *et al.*, 1989). Since the thrombin-susceptible site has been identified (Senger *et al.*, 1989) in a highly conserved sequence (Zhang *et al.*, 1990) in the middle of the molecule, two fragments would be expected, as found with the 44 kDa SPP-1. The presence of single methionine residue in each half of the rat SPP-1 is consistent with the comparable labelling intensity observed for the two fragments from the 44 kDa SPP-1. The differential intensity of these bands when labelled with $^{35}SO_4^{2-}$ and $^{32}PO_4^{3-}$ demonstrates that one half of the molecule is more heavily sulphated and the other more heavily phosphorylated. However, because the location of the sulphate and phosphate groups in the SPP-1 is not known, the arrangement of the fragments in the intact SPP-1 molecule cannot be resolved at this time.

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