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SULPHATION OF SECRETED PHOSPHOPROTEIN I (SPPI, OSTEOPONTIN) IS ASSOCIATED WITH MINERALIZED TISSUE FORMATION

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Secreted phosphoprotein I (SPPI) is a prominent structural protein in mineralized connective tissues. Rat bone cells in culture produce several forms of SPPI that differ in post-translational modifications such as phosphorylation and sulphation. To determine the significance of protein sulphation in bone formation, the synthesis of SPPI was studied in vitro using rat bone marrow cells (RBMC) which form bone-like tissue when grown in the presence of dexamethasone (Dex) and ß-glycerophosphate (ß-GP). In the presence of 10^{-7} M Dex SPPI expression was stimulated 4-5-fold. Radiolabelling multilayered RBMCs for 48 h with [^{35}S]-methionine, $Na_2[^{35}SO_4]$, or $Na_3[^{32}PO_4]$ revealed that two major phosphorylated forms of SPPI were secreted into the culture medium: a highly phosphorylated form migrating at 44 kDa on 15% SDS-PAGE and a less phosphorylated 55 kDa form. In the mineralized tissue formed in the presence of Dex and ß-GP, both forms of SPPI, in addition to proteoglycans and a 67 kDa protein, incorporated significant amounts of $[^{35}SO_4]$. Sulphation of SPPI was not observed in the absence of mineral formation, indicating that the sulphation of SPP I is closely associated with mineralization and that it can be used as a sensitive and specific marker for the osteoblastic phenotype.

Secreted phosphoprotein I has been characterized as a 44 kDa phosphorylated glycoprotein which contains 12 P-ser, 1 P-thr, 5-6 O-linked, and 1 N-linked oligosaccharides (1). Although the protein is expressed in several tissues, it is a prominent component of mineralizing connective tissues such as bone and dentine (2). Previous studies have revealed that rat bone cells in culture produce several forms of SPPI (3). The major phosphorylated forms of the protein correspond to the transformation-associated proteins, pp69 and pp62 (4). The most highly phosphorylated form, equivalent to pp62, migrates at 44 kDa on 15% cross-linked SDS-PAGE gels, and appears to be similar to the 44 kDa SPPI extracted from rat bone. However, based on [^{35}S]-methionine labelling of fetal rat bone cells it is synthesized in significantly lower amounts than the less phosphorylated 55 kDa form (3,5).

RBMC prepared from the long bones of young adult rats have been shown to form mineralized tissue nodules with properties of bone when grown in the presence of Dex and ß-GP (6). We have used this system to study the relationship between SPPI synthesis and tissue mineralization. These studies have revealed that SPPI, and a second protein, tentatively identified as BSP, are sulphated when mineralization of the tissue culture matrix occurs.

Abbreviations: SPPI, secreted phosphoprotein I; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; RBMC, rat bone marrow cells; BSA, bovine serum albumin; FBS, fetal bovine serum; α-MEM, α-minimal essential medium; DMEM, Dulbecco's minimal essential medium; Dex, dexamethasone; ß-GP, ß-glycerophosphate; DTT, dithiothreitol.

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MATERIALS AND METHODS

Materials: The antibiotics, penicillin G and gentamycin sulphate were from Sigma Chemical Co. (St. Louis, MO.). Fungizone and fetal bovine serum were obtained from Flow Laboratories (McLean, VA). Ascorbic acid, dexamethasone, Na-ß-glycerophosphate, and bovine thrombin (EC 3.4.21.5), ethylenediamine tetraacetic acid, tetrasodium salt (EDTA), Tris base, and guanidine hydrochloride (Ultrapure) were from Sigma. The radioisotopes, [^{35}S]-methionine, $Na_2[^{35}SO_4]$ and $Na_3[^{32}PO_4]$ were from ICN and Amersham Corp. Antibodies to SPPI (osteopontin) were a generous gift from Dr. W.T. Butler.

Rat Bone Marrow Cells were cultured essentially as described by Maniatopoulos et al. (6). In brief, young adult male rats (CBL Wistar), of 120 g were killed, the femora removed aseptically, cleaned of adherent soft tissues and washed 4x for 10 min each wash, in culture medium; α–minimal essential medium (α-MEM) containing 15% fetal bovine serum, 50 µg/ml ascorbic acid, and 10 x concentrated antibiotics (100 µg/ml penicillin G, 50 µg/ml gentamycin sulphate, 0.3 µg/ml Fungizone). The distal portions of the bone were removed and marrow flushed out with 5 ml of culture medium, and the cells suspended by repeated aspiration through a 20 gauge needle. After 1 week in culture, the cells were subcultured, counted, and seeded into 60 mm culture dishes at a density of 4 x 10^3 cells/cm^2, in culture medium as described above. The medium was supplemented with 10 mM ß-GP and/or 10^{-7} M Dex, or neither, as indicated in the 'Results'. The culture medium was changed every other day, and the cultures maintained in a humidified atmosphere consisting of 95% air and 5% CO_2 at 37°C for 14 days at which time the cells had multilayered and, in the presence of Dex, formed tissue nodules that mineralized in the additional presence of ß-GP.

Metabolic Labelling: To study the biosynthesis of SPPI, 14-day, multilayered cultures of RBMC were first washed twice with PBS containing antibiotics, and replaced with culture medium containing 0.5% FBS and antibiotics, supplemented with or without ß-GP as indicated in the 'Results', and either 100 µCi/ml [^{35}S]-methionine or 100 µCi/ml $Na_2[^{35}SO_4]$ added. For labelling with $Na_3[^{32}PO_4]$, phosphate-deficient DMEM was used instead of the α-MEM, and 100 µCi/ml $Na_3[^{32}PO_4]$ was added. The RBMCs were cultured in the presence of the radioisotopes continuously for 48 h, and the newly synthesized proteins extracted as described below.

Extraction of Radiolabelled Proteins: The extraction of the proteins was performed by a protocol modified from Domenicucci et al.(7). After labelling, the medium was removed and the cell layer washed twice with 3 ml of PBS. All buffers contained protease inhibitors and all procedures were performed at 4°C (7). The cellular proteins were removed by extraction in 0.5 M NH_4OH (2 ml, 40 sec), the culture dishes washed once with PBS (3 ml), and then 1.0 ml of 0.05 M Tris/HCl, pH 7.4, containing 4 M GuHCl (G1 extract) was added and the extracellular matrix scraped off the dishes with a rubber policeman (twice). The 2.0 ml of suspension in 5 ml polypropylene tubes were sonicated (10 sec, twice), aliquoted (1.0 ml) into 2 microcentrifuge tubes and extracted for 24 h on a vertical shaker. The suspension was centrifuged at 10,000 x g for 5 min, and the supernatants removed and combined with two additional extractions (G1-extract). The residue after guanidine extraction was washed 3 times, for 1 h each, in PBS, and then further extracted in 0.05 M Tris/HCl, pH 7.4, containing 0.5 M EDTA. Two, 24-h extractions at 4°C were combined (E-extract). The residue was then washed with PBS as above and extracted with a small volume (50-100 µl) of 2x concentrated electrophoresis sample buffer (see below), at 56°C for 30 min.

Immunoprecipitation and Thrombin Cleavage: The radiolabelled proteins from the various extracts were desalted either on columns (0.7 x 25 cm) of Sephadex G-50 using 0.01 M ammonium bicarbonate containing 0.005% Brij, or by dialysis using Spectropor 4 membranes against 0.1M ammonium bicarbonate, containing 0.05% Brij, for the first 3 buffer changes over 40 h, followed by dialysis against 1/10th strength buffer containing 0.005% Brij for 6 h. The radioactivity was measured and aliquots freeze-dried and redissolved in 2 x immunoprecipitation buffer (0.3% v/v NP-40, 0.3% v/v sodium deoxycholate, 0.15% w/v BSA in Tris-buffered saline, 0.02% w/v sodium azide). The radiolabelled proteins were immunoprecipitated as described previously (3,5). Polyclonal antibodies to rat osteopontin-SPPI (1), and porcine SPARC protein (7) were used. The radiolabelled proteins in the specific immune complex were dissolved in 20 µl electrophoresis sample buffer containing DTT, and heated for 25 min at 56°C, and then electrophoresed (see below). For thrombin (EC 3.4.21.5) digestions, freeze-dried aliquots of the medium and extracts were dissolved in 10 µl of 10 mM Tris/HCl buffer, pH 8.0, containing 10 mM $CaCl_2$, to which was added 1 unit of thrombin, and incubated at 37°C for 30 min. The reaction was stopped by the addition of 4 µl of 4x electrophoresis sample buffer containing DTT, heated to 56°C for 25 min, and analyzed by SDS-PAGE (8) on 10% or 15% cross-linked mini-gels.

Slot Blot Hybridization. Four groups of cells, each with or without Dex and ß-GP or both, were grown for 14 days in 60 mm dishes and, after washing in PBS, the cells were scraped into 2 ml guanidine thiocyanate and sonicated as described in detail previously (9) to extract the RNA (10). The total RNA was isolated by precipitating in phenol/chloroform. Aliquots containing 10 µg RNA were applied in triplicate to a Biotrans nitrocellulose membrane using a slot blot apparatus. Measurement of

SPPI mRNA was achieved by hybridization with a porcine SPPI cDNA labelled with [^{32}P]-dCTP, using stringency conditions of 42°C for hybridization, and 60°C for washes, determined from Northern hybridizations (3,5). The hybridization was visualized by autoradiography at -70°C, using Kodak X-OMAT film and intensifying screens, and quantitated by scanning laser densitometry on an LKB Ultroscan.

RESULTS

In preliminary studies, 50g rats were given 10 μCi/g body weight $Na_2[^{35}SO_4]$ and radiolabelled proteins extracted from calvariae and long bones were analyzed by SDS-PAGE on 15% cross-linked gels. In demineralizing extracts of bone, radiolabelled proteins were observed at the top of the gel, corresponding to proteoglycans, and at ~44 kDa and ~67 kDa. Although thrombin susceptibility, a characteristic of SPPI (11), could be observed, the low specific radioactivity precluded the photographic reproduction of the 26 and 28 kDa thrombolytic fragments. Consequently, the synthesis of these sulphated proteins was studied *in vitro* using RBMCs which form nodules of mineralized bone-like tissue when grown in the presence of sodium ascorbate together with Dex and ß-GP (6).

To determine the effects of Dex and ß-GP on the expression of SPPI the levels of SPPI mRNA were measured in four groups of cells cultured for 14 days in the presence or absence of Dex and ß-GP. Compared to control cells, the mRNA was increased from 4-5-fold in cultures grown in the presence of Dex; the presence of ß-GP not affecting SPPI expression significantly in either the presence or absence of Dex (Fig. 1). The form of SPPI and its distribution in the culture was then analyzed in RBMCs grown in the presence of Dex and in the presence or absence of ß-GP by metabolically radiolabelling with several different precursors. When 14-day cultures were labelled with $Na_2[^{35}SO_4]$, the incorporation of radiolabel into proteins found in the medium or tissue fractions of the minus ß-GP cultures, was confined essentially to proteoglycan that migrated as a broad band at the top of the 10% cross-linked SDS-PAGE gels (Fig. 2A). In comparison, cultures with added ß-GP synthesized two prominent [$^{35}SO_4$]-labelled 56 kDa and 67 kDa proteins (on 15% gels the 56 kDa SPPI migrates at 44 kDa) recovered in 0.5 M EDTA extracts of the tissue, and at 67 kDa in the tissue residue left after the EDTA extraction. Analyses of [$^{32}PO_4$]-labelled proteins on 15% cross-

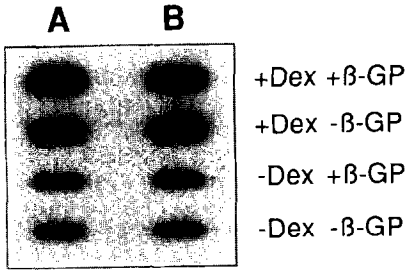

Fig 1. Slot Blot Analysis of SPPI mRNA from RBMC. Cells were grown in the presence (+) and absence (-) of 10^{-7}M Dex and/or 10 mM ß-GP. After 14 days, total RNA was extracted from the cell layers and 10 μg applied onto a Biotrans nitrocellulose membrane using a slot blot apparatus. The SPPI mRNA was hybridized with [^{32}P]-dCTP-labelled porcine SPPI cDNA at 42°C and, after washing at 62°C, the membrane subjected to radioautography. The degree of hybridization was measured by laser densitometry. **A** and **B** represent duplicate sets of samples.

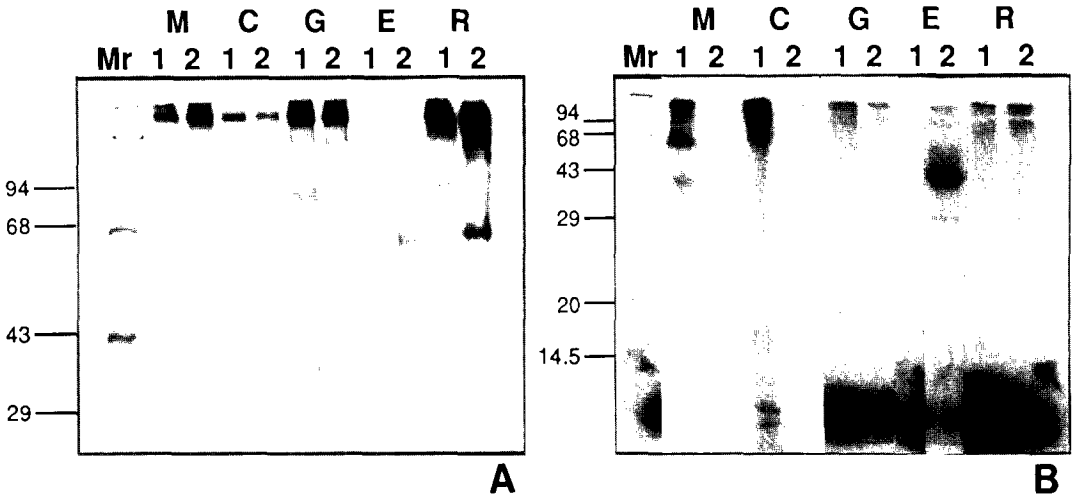

Fig 2. Distribution of the Newly Synthesized Radiolabelled Proteins in RBMC Cultures. RBMCs were cultured in the presence of 10^{-7}M Dex with or without 10mM ß-GP. Radioisotope (see below) was added and cultures continued for 48 h. After sequential extractions, aliquots of the extracts were analyzed by SDS-PAGE under reducing conditions and the gels processed for fluorography or radioautography. $Na_2[^{35}SO_4]$-labelled proteins on 10% cross-linked mini-gels (**A**); $Na_3[^{32}PO_4]$-labelled proteins on a 15% cross-linked gel (**B**). M_r, molecular weight markers; M, culture medium; C, NH_4OH extract of cells; G, GuHCl extract; E, EDTA extract; R, cell-layer residue. Lanes; 1, - ß-GP ; 2, + ß-GP. Note the different migration of the SPPIs on 10% cross-linked gels compared to the 15% cross-linked SDS-PAGE gels.

linked SDS-PAGE gels revealed two bands (44 kDa and ~55 kDa) in the media of cultures incubated with or without ß-GP. A strong band at 44 kDa with an indication of a weaker, diffuse band at 67 kDa, was evident in the demineralizing extracts of the ß-GP containing cultures (Fig. 2B). Notably, there was less of the $[^{32}PO_4]$-labelled proteins in the media of mineralizing cultures indicating that these proteins were associating with the hydroxyapatite. Metabolically labelling of the mineralizing cultures with $[^{35}S]$-methionine revealed numerous bands in the different fractions analyzed. The results of these analyses, carried out under reducing and non-reducing conditions on SDS-PAGE using 10% cross-linked gels were compared with replicate cultures labelled concomitantly with $[^{35}SO_4]$ in Fig 3A and 3B. A major unidentified band of $[^{35}S]$-methionine-labelled material was observed at 40 kDa, which was not immunoprecipitated with either SPPI or SPARC antibodies. With longer exposures of the fluorographs a band could be observed at 67 kDa but the intensity of the 40 kDa band precluded the identification of a 44 kDa SPPI, which on 10% gels migrates more slowly, as observed previously (1), closer to 56 kDa. As anticipated, in view of the absence of cysteines in SPPI (12), the mobility of the $[^{35}SO_4]$-labelled proteins was not altered by the presence or absence of DTT

Thrombin digestion of the $[^{32}PO_4]$-labelled and $[^{35}SO_4]$-labelled proteins in the demineralizing extracts of the RBMC cell layers revealed a complete digestion of the $[^{32}PO_4]$-labelled proteins and a partial digestion of the $[^{35}SO_4]$-labelled proteins producing, in each case, characteristic fragments at 26 kDa and at 28 kDa (Fig.4A and 4B). Extended digestions with thrombin did not produce any further degradation of the remaining $[^{35}SO_4]$-labelled protein bands. Of note, although both fragments were labelled with each precursor, the 28 kDa fragment was more strongly labelled

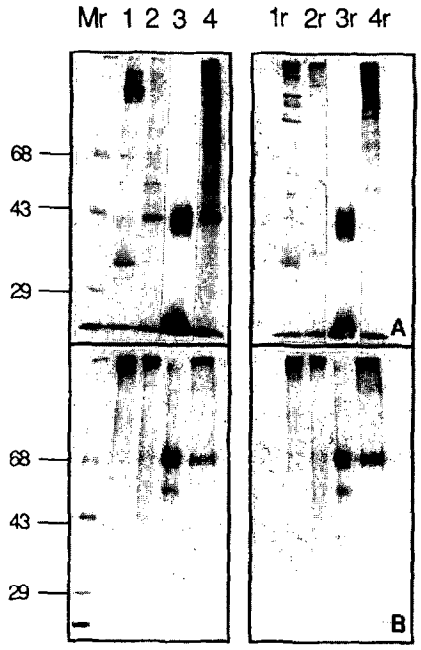

Fig 3. Analysis of [^{35}S]-Met and [$^{35}SO_4$]-Labelled Proteins on SDS-PAGE. RBMC were incubated for 48 h in the presence of Dex, ß-GP and radioisotope. The medium and extracts were analyzed by SDS-PAGE and fluorography of the radiolabelled proteins separated on 10% cross-linked gels under non-reducing and reducing (r) conditions. (**A**) [^{35}S]-methionine-labelled proteins ; (**B**) [$^{35}SO_4$]-labelled proteins. M_r, molecular weight markers; lanes 1, medium; 2, GuHCl extract; 3, EDTA extract; 4, cell residue.

with [$^{35}SO_4$] whereas the 26 kDa fragment was more strongly labelled with [$^{32}PO_4$]. To demonstrate that sulphated SPPI was present in both of the original 44 kDa and the 67 kDa bands, the [$^{35}SO_4$]-labelled proteins were specifically immunoprecipitated with rabbit antibodies to rat SPPI and the

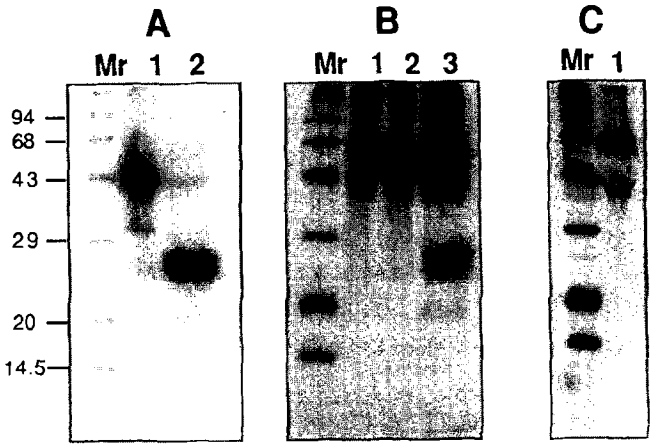

Fig 4. Thrombin Digestion and Imunoprecipitation of SPPI. EDTA extracts of RBMC cultures incubated in the presence of both Dex and ß-GP, and labelled with [$^{32}PO_4$] or [$^{35}SO_4$] and analyzed by SDS-PAGE on 15% cross-linked gels, and fluorography. **Panel A**: [$^{32}PO_4$]-labelled proteins before (lane 1) and after (lane 2) digestion with thrombin **Panel B**: [$^{35}SO_4$]-labelled proteins before digestion (lane 1), incubated in digestion buffer (lane 2), and digested with thrombin (lane 3). **Panel C**: Specific immunoprecipitation of [$^{35}SO_4$]-labelled proteins (lane 1) using antiserum to rat SPPI. M_r, molecular weight markers.

proteins analyzed on SDS-PAGE (Fig. 4C). Although the migration of the radiolabelled proteins was slightly distorted because of antiserum proteins eluting from the protein A-Sepharose, radiolabelled bands approximating the 44 kDa and 67 kDa positions could be resolved.

DISCUSSION

Using a well-characterized system for bone tissue formation in vitro, we have shown that RBMCs grown in the presence of Dex and ß-GP produce 44 kDa and 67 kDa sulphated forms of SPPI which are specifically associated with the formation of mineralized tissue matrix. There was no evidence of SPPI sulphation in any of the fractions analyzed in non-mineralizing cultures grown under identical conditions but without ß-GP. Moreover, the sulphated SPPI was present only in the mineralized tissue extracts and could not be identified in the cell-layer extracts or medium. In contrast, [$^{32}PO_4$]-labelled forms of SPPI were present in the medium of both culture systems and in the mineralized tissue matrix. Whereas the level of radioactivity associated with the two forms of SPPI was similar in the culture medium, the 44 kDa form was predominant in the mineralized tissue, indicating a preferential association of the 44 kDa form with the mineralized tissue. In previous studies we have shown that confluent cultures of fetal rat calvarial cells produce two major forms of [$^{32}PO_4$]-labelled SPPI, that migrate at 44 kDa and 55 kDa on 15% SDS-PAGE gels and at 56 kDa and 60 kDa on 10% gels (3). The [$^{32}PO_4$]-labelled SPPIs in the medium of RBMCs behave in a similar manner. However, the form corresponding to the 55 kDa SPPI in the mineralized matrix appears to migrate closer to the 67 kDa marker.

The sulphated SPPI forms in the mineralized tissue were identified at 67 kDa and at 44 kDa by immunoprecipitation with specific antibodies and by their susceptibility to thrombin (11), which cleaves the SPPI in the central region of the molecule, after the (G)RGD(S) cell-binding site. The complete digestion of the [$^{32}PO_4$]-labelled protein with thrombin demonstrates that SPPI is the major phosphorylated protein in bone. However, the incomplete digestion of the [$^{35}SO_4$]-labelled proteins appears to be due to the sulphation of a second protein, tentatively identified as BSP, which is sulphated in mineralized tissue formed by mouse osteoblasts *in vitro* (13). That the sulphation of SPPI and BSP is important in bone formation is apparent from the results of the *in vivo* studies, and also from our current *in vitro* studies. We have observed sulphation of proteins with the characteristics of SPPI and BSP in organ cultures of fetal porcine calvarial bone chips and in fetal rat calvarial cell cultures producing mineralized tissue nodules. In the porcine system only a 67 kDa SPPI exists and this has been separated from the sulphated BSP (Nagata, Goldberg, Zhang, Domenicucci and Sodek, in preparation).

Protein sulphation can occur as sulphated tyrosine or as sulphated sugars in glycosaminoglycan chains of proteoglycans, or on oligosaccharides (13). Although the site of sulphation of the SPPI has not been determined unequivocally, deglycosylation did not remove the [$^{35}SO_4$]-label from the protein. In the mouse bone cell cultures tyrosine sulphation accounts for all the [$^{35}SO_4$] in BSP (13). However, there are only 5 tyrosines in rat SPPI (12) compared to 23 in rat BSP (14). Therefore, it is possible that sulphated O-linked sugars may exist in SPPI. Of note, sulphated O-linked oligosaccharides occur in hydroxyapatite-binding mucins and salivary proteins. From the present studies it is not possible to determine whether the formation of sulphated SPPI precedes or follows the formation of hydroxyapatite crystal formation. However, it is of interest to note that sulphate groups have been shown to act co-operatively with polyaspartic acid peptides in a

ß-sheet structure in promoting hydroxyapatite formation (15). Rat SPPI has a polyaspartic acid sequence that is conserved in in human (16) and porcine (Wrana, Zhang and Sodek, in preparation) SPPI. Moreover, sulphate has been shown to be concentrated at sites of early mineral formation (17). Although this sulphate has been attributed to proteoglycans, at least some is likely due to SPPI, since SPPI has been localized to such sites by immunogold labelling (18). These observations indicate that the sulphation of SPPI and perhaps also BSP may be important in the early formation of hydroxyapatite crystals in bone. Further, the sulphation of these proteins provides a valuable indicator of bone formation and as such provides a unique marker for the osteoblastic phenotype.

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