A Rat Osteogenic Cell Line (UMR 106-01) Synthesizes a Highly Sulfated Form of Bone Sialoprotein*

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The rat osteosarcoma cell line (UMR 106-01) synthesizes and secretes relatively large amounts of a sulfated glycoprotein into its culture medium (~240 ng/10^6 cells/day). This glycoprotein was purified, and amino-terminal sequence analysis identified it as bone sialoprotein (BSP). [35S]Sulfate, [3H]glucosamine, and [3H]tyrosine were used as metabolic precursors to label the BSP. Sulfate esters were found on N- and O-linked oligosaccharides and on tyrosine residues, with about half of the total tyrosines in the BSP being sulfated. The proportion of 35S activity in tyrosine-O-sulfate (~70%) was greater than that in N-linked (~20%) and O-linked (~10%) oligosaccharides. From the deduced amino acid sequence, rat BSP from UMR 106-01 cells would contain a total of ~3 N-linked and ~25 of the above O-linked oligosaccharides. This large number of oligosaccharides is in agreement with the known carbohydrate content (~50%) of the BSP.

The osteosarcoma cell lines UMR 106 and UMR 106-01 have been used to study various aspects of osteoblast physiology and bone formation in vitro (1–3). Recently, the proteoglycans synthesized by UMR 106-01 cells have been characterized and shown to be similar to those synthesized by cultured human osteoblasts (4). While studying the UMR osteoblasts, we observed that a substantial amount of 35S glycans synthesized by cultured human osteoblasts (4) was incorporated into a 75-kDa glycoprotein when UMR 106-01 cells would contain a total of ~3 N-linked and ~25 of the above O-linked oligosaccharides. This large number of oligosaccharides is in agreement with the known carbohydrate content (~50%) of the BSP.

The osteosarcoma cell lines UMR 106 and UMR 106-01 have been used to study various aspects of osteoblast physiology and bone formation in vitro (1–3). Recently, the proteoglycans synthesized by UMR 106-01 cells have been characterized and shown to be similar to those synthesized by cultured human osteoblasts (4). While studying the UMR osteoblasts, we observed that a substantial amount of 35S glycans synthesized by cultured human osteoblasts (4) was incorporated into a 75-kDa glycoprotein when UMR 106-01 cells would contain a total of ~3 N-linked and ~25 of the above O-linked oligosaccharides. This large number of oligosaccharides is in agreement with the known carbohydrate content (~50%) of the BSP.

**EXPERIMENTAL PROCEDURES**

**Materials—**All reagents were of the highest purity commercially available. Neuraminidase (Clostridium perfringens) and N-acetyl-neuraminic acid were obtained from Boehringer Mannheim. Tunicamycin and glycosaminoglycan standards were from Calbiochem. Sephadex G-50 (fine), Q-Sepharose, and Superose 6 HR 10/30 were from Pharmacia LKB Biotechnology Inc. CarboPac PA1 (4 × 250 mm) and AminoPac PA1 (4 × 250 mm) columns were from Dionex Corp. Bio-Gel P-10 (200–400 mesh), a column of Aminex A9 (4 × 250 mm), and SDS-PAGE protein standards were from Bio-Rad. TBE, a gel buffer, and PolyPrep gel was from Tosol-Haas through Thomson Instrument Co. Radioisotopes were from Du Pont-New England Nuclear. [35S]Sulfuric acid (carrier free), D- [3H]glucosamine HCI (30 Ci/mmol), L-[ring-3,5-3H]tyrosine (50 Ci/mmol), and L-[3H(G)]serine (14 Ci/mmol). Radioactive protein standards (14C-methylated) for fluorography were from Bethesda Research Laboratories. Eagle’s minimum essential medium (Earle’s salts) and a 100 x solution of nonessential amino acids were from Mediatech (Herdon, VA). All

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other culture fluids were from Gibco, including sulfate-free medium 199. Tissue culture flasks and dishes were from Falcon, Centricon centrifugal filters (10-kDa cutoff) were from Polysciences, Inc. Centricon 10 microconcentrators were from Amicon Corp. Stains-All was from Eastman Kodak, and 50% (w/v) NaOH was from Fisher. Radioactivity was measured in a Beckman LS8501 spectrometer using a 10:1 ratio of Ready Safe (Beckman Instruments). The clonal cell line UMR 106-01 was kindly provided by Dr. T. J. Martin through personal communication.

Isolation of the UMR Sialoprotein—The medium from labeled cultures was collected and made 4 M with solid guanidine HCl and 0.5% with 20% (v/v) Triton X-100. Labeled macromolecules from the medium samples were isolated from unincorporated radioisotopic precursors by Sephadex G-50 chromatography (17) followed by Lohmander (25). N-Acetylneuraminic acid (sialic acid) was also quantified by neuraminidase digestion of the oligosaccharides (25) followed by CarboPac PA1 chromatography as described above. Standard N-acetylneuraminic acid elutes at ~25 min in the above gradient elution program.

Tyrosine-O-sulfate Analyses—Portions of sialoprotein preparations were exchanged into water by ultrafiltration and then vacuum dried. Samples were hydrolyzed in 100 µl of either 1 M NaOH or 0.2 M Na2O2O at 110 °C for 24 h under nitrogen (14). Hydrolyses in 0.5 M NaOH were not as complete, and digestions in 2

Tyrosine-O-sulfate and serine-O-sulfate standards were prepared by a modification of a procedure described by Reitz et al. (26). Briefly, 200 µCi of [3H]tyrosine or [3H]serine was vacuum dried in a 250-µl microcentrifuge tube and reconstituted on dry ice with 5 µl of a 5 mM sodium acetate-acetic acid (pH 5.0) stock solution containing 0.2% (w/v) sodium bicarbonate and 0.2% (v/v) sodium dodecyl sulfate. After 30 min, 18 µl of 0.01 M NaOH was added to bring the pH to ~8. Sulfated amino acids were separated from their nonsulfated forms by AminoPac PA1 chromatography and desalted as described below; typically, 20–30% of the labeled amino acids were converted to their sulfated forms by this procedure. A TSK HW-40S column (1 x 30 cm) in 0.5 M pyridinium acetate (pH 5.0) was used instead of the Bio-Gel P-10 column. The flow rate was 0.5 ml/min, and 15-s fractions were collected.
fractons were collected. Repeated application of the samples to the column was necessary for complete desalting.

Tyrosine sulfation was confirmed by mild acid treatment. Portions of the desalted pools described above were vacuum dried, resuspended in 20 μl of 10 mM \( \text{H}_2\text{SO}_4 \), and then vacuum dried again. Such samples were immediately reapplied to the AminoPac PA1 column as described above. The removal of \(^3\text{H}\) label from tyrosine or tyrosine sulfate was assessed as follows. Desalted and dried samples of the 2 labeled amino acids (including 10 ng of unlabeled tyrosine) were immediately reapplied to the AminoPac PA1 column as described above. The removal of \(^3\text{H}\) label from tyrosine or tyrosine sulfate was assessed as follows. Desalted and dried samples of the 2 labeled amino acids (including 10 ng of unlabeled tyrosine) were reconstituted in 100 μl of 1 M \( \text{NaOH} \) and heated at 110°C for 24 h under nitrogen. After cooling and centrifuging, the samples were then chromatographed on an AminoPac PA1 column and fractions counted as described above.

RESULTS

Identification of BSP—Studies to be reported elsewhere\(^1\) revealed that UMR 106-01 cells synthesize a sulfated glycoprotein, most of which is secreted into the culture medium. We prepared chemical quantities of this protein from medium harvested after culturing ~3 × 10^6 cells for 3 days. The \([\text{35S}]\) sulfate-labeled macromolecules from the medium of ~4.7 × 10^6 cells were added to this sample to allow detection of the sulfated glycoprotein during purification (see next section). About 250 μg (dry weight) of the purified sulfated glycoprotein was isolated, and a portion was analyzed by SDS-PAGE (Fig. 1). A single band at an apparent mass of 75 kDa was observed when the gel was stained with Stains-All. Coomassie Blue did not stain this band, nor did it reveal any other bands in the gel (data not shown). A fluorogram of this gel (Fig. 1) showed a single \(^3\text{H}\)-labeled band at the same position as for the stained band. About 46% of the mass of this purified sample was protein as determined by amino acid analysis (data not shown). A portion of this glycoprotein (100 μg) was sequenced under nitrogen. After cooling and centrifuging, the samples were then chromatographed on an AminoPac PA1 column and fractions counted as described above.

Purification of BSP from UMR Cells— Cultures were labeled for 24 h in the presence of tunicamycin with \([\text{35S}]\) sulfate or \([\text{1}^\text{H}]\) glucosamine or in the absence of tunicamycin with \([\text{35S}]\) sulfate, \([\text{1}^\text{H}]\) glucosamine, or \([\text{1}^\text{H}]\) tyrosine. The media were made 4 M with solid guanidine \( \text{HCl} \) and then eluted on Sephadex G-50 in a 10 M formamide, 0.3 M \( \text{NaCl} \) solvent to remove unincorporated precursors. The excluded fractions were applied to Q-Sepharose to bind the proteoglycans and the sulfated BSP while removing other labeled proteins (Table I). Since the sulfated BSP and proteoglycans co-eluted from Q-Sepharose, the bound molecules were step eluted from the column with 4 M guanidine \( \text{HCl} \), 0.5% CHAPS and concent-

![Fig. 1. SDS-PAGE and amino-terminal sequence analysis of the sulfated glycoprotein from UMR culture medium. Lanes 1 and 2 (1 and 4 μg, respectively) were stained with Stains-All. Lanes 3 and 4 are fluorograms of lanes 1 and 2, respectively.](image)

TABLE I

<table>
<thead>
<tr>
<th>Precursor</th>
<th>Unbound</th>
<th>Bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>[^{35S}]\text{Sulfate}*</td>
<td>13</td>
<td>87</td>
</tr>
<tr>
<td>[^{1}H]\text{Glucosamine}*</td>
<td>41</td>
<td>59</td>
</tr>
<tr>
<td>[^{1}H]\text{Tyrosine}*</td>
<td>84</td>
<td>16</td>
</tr>
</tbody>
</table>

\* 100% = 38.5 × 10^6 cpm/culture.
\* 100% = 62.6 × 10^6 cpm/culture.
\* 100% = 15.2 × 10^6 cpm/culture.

Macromolecules from the medium of cultures (150-mm dishes) labeled with the indicated radioisotopic precursors for 24 h were isolated by Sephadex G-50 chromatography. Samples were then applied to a column of Q-Sepharose, and the nonbinding molecules were collected as described under "Experimental Procedures." Bound material was recovered by step eluting the column with 4 M guanidine \( \text{HCl} \), 0.5% CHAPS, and 50 mM sodium acetate (pH 6.0). Shown are the results from control cultures, but tunicamycin-treated cultures yielded similar percentages. The table represents an average of two experiments; standard errors were <5% of the means.

Identification of Sulfated N-Linked Oligosaccharides—Por-
Fig. 3. SDS-PAGE analysis of purified BSP recovered from Superose 6 in Fig. 2E. Shown are fluorograms of samples labeled with either [35S]sulfate (lane 1), [3H]glucosamine (lane 2), or [3H]tyrosine (lane 3) as precursor.

Fig. 4. Isolation of oligosaccharides from alkaline borohydride-treated BSP. Samples were labeled with [35S]sulfate and [3H]glucosamine as precursors. Arrows in A and B indicate the position of N-linked glycopeptides. Bar in A indicates the fractions recovered for analysis on CarboPac PA1 (C). In C, the [3S]profile is in expanded scale, and the programmed sodium acetate gradient appears as a dashed line. Numbered peaks (C) were pooled for further analysis.

peptides with attached N-linked oligosaccharides (27, 28). The treated samples were eluted on Bio-Gel P-10 (Fig. 4, A and B). Neither control nor tunicamycin-treated samples contained appreciable amounts of labeled material in the excluded volume, indicating the absence of glycosaminoglycans, and hence of proteoglycans, in the sialoprotein preparations. In the control, a prominent broad peak with ~50% of the [3H] and ~20% of the [3S] eluted at a K_d of 0.2 (Fig. 4A, arrow) where N-linked glycopeptides have been shown to elute (27, 28). Hexosamine analysis of this peak revealed an absence of hexosaminol, ~7% glucosamine, ~10% sialic acid, and ~13% galactosamine, thus confirming its N-linked nature. The presence of a significant amount of galactosamine indicates a potential for sulfation of these oligosaccharides (10). This peak was virtually absent in the samples treated with tunicamycin (Fig. 4B, arrow). These results indicate: (a) that the UMR BSP contains N-linked oligosaccharides; and (b) that some of these N-linked oligosaccharides contain sulfate bound to carbohydrate, since there are no tyrosine residues near the sites of N-glycosylation (9) (see analyses below).

Identification of Sulfated O-Linked Oligosaccharides—In Fig. 4A, two major [3H] peaks (K_d values of 0.51 and 0.58 containing ~12 and ~24% of the total [3H], respectively) and a minor peak (K_d = 0.70 containing ~6% of the total [3H]) eluted in the region where O-linked hexa-, tetra-, and trisaccharides elute on this column (28). These were present in samples from both the control and tunicamycin-treated cultures, and they appeared also to contain [35S]activity (Fig. 4, A and B). Another minor peak (K_d = 0.89, ~5% of the total [3H]) was observed but not analyzed further; it might represent unsubstituted N-acetylgalactosamine residues on the protein (28). The fractions from the control were recovered as indicated (Fig. 4A, bar) and eluted on a CarboPac PA1 column with a sodium acetate gradient (Fig. 4C). A series of three prominent [3H] peaks (peaks 1, 2, and 3) eluted early in the gradient, whereas two additional peaks (peaks 4 and 5) that eluted later contained almost all of the [3S] and a small proportion (~7%) of the [3H].

Each of these peaks was recovered and rechromatographed on Bio-Gel P-10 (Fig. 5, A–E). Portions were acid hydrolyzed and analyzed for hexosamine and [3S]activity.

Fig. 5. Bio-Gel P-10 profiles and hexosamine analysis of oligosaccharide peaks from Fig. 4C. NANA, N-acetyleneuraminic acid (sialic acid); GlcN, glucosamine; GalNol, galactosaminol.
and analyzed for labeled hexosamines, hexosaminitols, and sialic acid; neuraminidase digestions were performed to confirm the sialic acid quantitation. The data are presented in tabular form for each oligosaccharide in Fig. 5, A–E. Peak 2 (∼10% of the total 3H) eluted in the position of a hexasaccharide (Kd = 0.51), contained equimolar amounts of glucosamine and galactosaminitol, and contained a labeling ratio of 1.3 for sialic acid to galactosaminitol. The metabolic pathway for labeling sialic acid from a hexosamine precursor probably involves some loss of label (29) and hence cannot be compared directly with hexosamines on a molar basis. However, the results strongly suggest that peak 2 contains a hexasaccharide, similar or identical to that found on the proteoglycan from rat chondrosarcoma (28), which contains 2 sialic acid residues. Peak 3 (∼24% of the total 3H) eluted in a position near to that of a tetrasaccharide (Kd = 0.58). Labeled glucosamine was essentially absent, and the labeling ratio of sialic acid to galactosaminitol was 1.2. This O-linked oligosaccharide, then, resembles the disialylated tetrasaccharide found on the rat chondrosarcoma proteoglycan (28). Peak 1 (∼5 of the total 3H) eluted in what appears to be a trisaccharide position (Kd = 0.70), contained little if any labeled glucosamine, and had a labeling ratio of sialic acid to galactosaminitol of 0.66. This O-linked oligosaccharide resembles the monosialylated trisaccharide found on the rat chondrosarcoma proteoglycan (28).

The major sulfated O-linked oligosaccharide, peak 5 (∼2% of the total 3H) eluted from Bio-Gel P-10 at a Kd of 0.50 and had essentially the same amino sugar labeling pattern as for the nonsulfated hexasaccharide (peak 2). Thus, it is likely to be a sulfated form of the peak 2 oligosaccharide. The other prominent sulfated O-linked oligosaccharide, peak 4 (∼1% of the total 3H), eluted between tetra- and trisaccharide positions (Kd = 0.66) and had a different amino sugar labeling pattern than that of peaks 1 or 3. Peak 4 contained equimolar amounts of glucosamine and galactosaminitol and had a labeling ratio of 0.66 for sialic acid to galactosaminitol. Therefore, it is likely to be a monosialylated sulfated tetra- or trisaccharide. Overall, ∼10% of the total 35S label in the UMR BSP is bound in two O-linked oligosaccharides, with their probable structures being a hexasaccharide and a tetra- or trisaccharide.

Identification of Tyrosine-O-sulfate—From the above analyses, only ∼30% of the total 35S label from sulfate in the UMR BSP is bound to oligosaccharides. The remaining 70% is relatively small in size after alkaline borohydride treatment and did not co-elute with 3H-labeled oligosaccharides on Bio-Gel P-10 (Fig. 4A). These data suggest that the majority of the 35S label in the sialoglycoprotein is bound to the polypeptide, although it is possible that some may represent alcali-labile sulfate esters. Portions of BSP labeled with [35S]sulfate or [3H]tyrosine as precursors (Fig. 3) were hydrolyzed in 0.2 M Ba(OH)2 or in 1 M NaOH under conditions designed for recovering tyrosine sulfate (14). After removing insoluble material, the hydrolysates were applied to an AminoPac PA1 column to separate free sulfate, tyrosine, and tyrosine sulfate. Hydrolysis in 1 M NaOH yielded a slightly better solubilization of both the 35S and 3H label than did 0.2 M Ba(OH)2 and allowed a direct quantification of free [35S]sulfate, which precipitates in the presence of barium (Table II).

Fig. 6A shows the AminoPac PA1 profile of a mixture of [35S]sulfate- and [3H]tyrosine labeled BSP hydrolyzed in 1 M NaOH. Three 3H peaks were observed: an unbound peak that was the result of exchange of 3H from tyrosine into solvent during the hydrolysis (see below), a second peak at the position of tyrosine, and a third peak that eluted at the position of tyrosine sulfate. Two 35S peaks were detected, with one at the position of free sulfate and the other at the position of tyrosine sulfate. Table II summarizes the results of both hydrolysis procedures for the BSP. The identity of tyrosine sulfate was further confirmed by recovering the 35S- and 3H-labeled peak at the position of tyrosine sulfate (Fig. 6A, bar) and hydrolyzing a portion by mild acid treatment followed by vacuum drying. The resultant digest, when rechromatographed on the AminoPac PA1 column (Fig. 6A, inset), yielded only two labeled products: ∼70% of the 3H label as tyrosine and all of the 35S label as free sulfate. The remaining 30% of the 3H label was apparently exchanged into the solvent and then lost during the vacuum drying step.

Table II summarizes the results of both hydrolysis procedures for the BSP. The identity of tyrosine sulfate was further confirmed by recovering the 35S- and 3H-labeled peak at the position of tyrosine sulfate (Fig. 6A, bar) and hydrolyzing a portion by mild acid treatment followed by vacuum drying. The resultant digest, when rechromatographed on the AminoPac PA1 column (Fig. 6A, inset), yielded only two labeled products: ∼70% of the 3H label as tyrosine and all of the 35S label as free sulfate. The remaining 30% of the 3H label was apparently exchanged into the solvent and then lost during the vacuum drying step.

Table III shows that the appearance of 3H label in the unbound fraction from the AminoPac PA1 column was the result of "H exchange from unmodified tyrosine during alkaline hydrolysis. This label in the unbound fraction was also found to be lost if the sample was vacuum dried prior to chromatography. In contrast, the 3H label in tyrosine sulfate was stable during the hydrolysis procedures (Table III). Thus, the estimate of unsubstituted tyrosine in the intact BSP is the sum of the "H label in the unbound fraction and in the
position of tyrosine after alkaline hydrolysis and AminoPac PA1 chromatography. For the sialoprotein, then, 49% of the radioactivity was detected as free sulfate and 69% as tyrosine sulfate. The amount of radioactivity in the label in 5 positions of tyrosine after alkaline hydrolysis and AminoPac PA1 chromatography, of which -70% is secreted into the medium. This also demonstrates that the UMR BSP contains sulfated oligosaccharides (-30% as discussed above) is equivalent to the observed amount of free sulfate released during alkaline hydrolysis. Fig. 6B shows that under such hydrolysis conditions virtually all of the oligosaccharides were hydrolyzed sufficiently to release their sulfate. Taken together, the above results indicate that the majority of the radioactivity label (-70%) in the BSP is tyrosine sulfate and that about half of the tyrosine residues in the polypeptide are sulfated.

**DISCUSSION**

The rat osteogenic cell line UMR 106-01 synthesizes and secretes a relatively large amount of BSP (~240 ng/10^6 cells/day). It constitutes ~1% of the total H from tyrosine, ~12% of the total H from glucosamine, and ~15% of the total H from sulfate incorporated by UMR cells in a 24-h labeling period, of which ~70% is secreted into the medium. This study also demonstrates that the UMR BSP contains sulfated oligosaccharides and tyrosine-O-sulfate.

Oligosaccharide analyses after alkaline borohydride treatment of the BSP isolated from cultures labeled with [3H]glucosamine in the presence or absence of tunicamycin indicate that both N- and O-linked oligosaccharides are present on the polypeptide, with 50% of the H label in O-linked glycoconjugates. Assuming that these N-linked oligosaccharides are complex-type biantennary structures, the ratio of N- to O-linked oligosaccharides is 5:1.

Rabbit BSP isolated from tunicamycin-treated cultures bound as effectively to Q-Sepharose as that from control cultures. Fisher et al. (6) demonstrated that the BSP from fetal bovine bone could be stained in SDS-PAGE gels with Alcian blue. Much of this staining was eliminated by neuraminidase treatment, although not all of it, suggesting a presence of other anionic groups such as phosphate or sulfate esters. While our studies were in progress, Ecarot-Charric et al. (31) reported that mouse osteoblasts synthesize a sulfated BSP in vitro. Therefore, sulfation of BSP may be a common post-translational modification, perhaps with the sulfation of the UMR BSP being greater than other sources of BSP.

The BSP from rat (7) and human (30) bone has been reported to elute earlier in a salt gradient than proteoglycans from anion-exchange columns. In contrast, UMR BSP co-elutes with proteoglycans (which are sulfated normally) on Q-Sepharose.1 This tight binding is presumably due to the presence of a relatively large number of sulfate esters on tyrosine since UMR BSP isolated from tunicamycin-treated cultures is secreted into the medium. This region also contains many acidic amino acids (aspartic acid, glutamic acid) as well as "turn-inducing" amino acids (glycine, proline, aspartic acid, serine, asparagine) surrounding the tyrosine residues, both of which are now considered as requirements for tyrosine sulfation (15). The 6 tyrosine residues in the amino-terminal portion of the polypeptide lack these properties, so it is likely that most, if not all, of the sulfated tyrosines in the UMR BSP reside in its carboxyl-terminal region.

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Rabbit BSP has been reported to contain short O-linked keratan sulfate chains (8). Presumably, these chains would have a linkage oligosaccharide structure similar to that of keratan sulfate chains and O-linked hexaasaccharides from the rat chondrosarcoma proteoglycan (27, 28). Using sequential endo-β-N-acetylglucosaminidase digestion, data have been obtained to suggest that a likely site of sulfation on the O-linked hexaasaccharide from the UMR sialoprotein would be N-acetylgalactosamine.4 Thus, a structural relationship might exist between the sulfation of the O-linked hexaasaccharide in the UMR BSP and the keratan sulfate chains (an elongation of this core oligosaccharide) in the rabbit BSP.

4 Using [14C]glucosamine as precursor, the number of O-linked oligosaccharides/N-linked oligosaccharide was calculated using the following formula: no. of O-linked oligosaccharides/N-linked oligosaccharide = (radioactivity in galactosaminotol for a particular O-linked oligosaccharide)/(radioactivity in hexose equivalents for an N-linked oligosaccharide of known structure) x (no. of hexosamine equivalents in this N-linked oligosaccharide).
The functions of sulfate groups on oligosaccharides and tyrosine residues are not yet clear. Tyrosine sulfation is generally considered to be linked to secretion transport (15) although not absolutely (16). Also, tyrosine sulfation has been shown to be essential for the biological activity of some peptides and inhibitory for others (15). Perhaps tyrosine sulfation near the Arg-Gly-Asp tripeptide (32) site in the BSP could act to modulate its cell-binding potential. Sulfation of oligosaccharides is thought to lengthen the half-life of hormones in serum (11). Perhaps this may function to increase the half-life of the BSP in bone tissue. The sulfation of the BSP may also promote an ionic interaction with bone mineral. Together, these may explain the accumulation of partially degraded products of the BSP in bone matrix (5, 6).

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