Biosynthesis of *O*-phosphoserine-containing phosphoproteins by isolated bone cells of mouse calvaria

Y. Gotoh[+], M. Sakamoto, S. Sakamoto and M.J. Glimcher*

*Department of Orthopaedic Surgery, Laboratory for the Study of Skeletal Disorders and Rehabilitation, Harvard Medical School, *Children's Hospital Medical Center, 300 Longwood Avenue, Boston, MA 02115* and *Harvard School of Dental Medicine, Boston, MA 02115, USA*

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Five groups of isolated bone cells from mouse calvaria were incubated with [^{3}H]serine and the presence and amount of *O*-[^{3}H]phosphoserine used as an indication of phosphoprotein synthesis. Cells in the osteoblastic fraction were the most active in synthesizing phosphoproteins, and unlike the other cell groups, released most of the phosphoproteins into the tissue culture medium. When subjected to molecular sieving and ion-exchange chromatography, the phosphoproteins synthesized by the bone cells of the osteoblastic group behaved like the phosphoproteins extracted from mouse calvaria by EDTA.

Bone cell *Phosphoprotein synthesis* *Phosphoserine* *Mouse calvaria*

1. INTRODUCTION

Phosphoproteins of calcified vertebrate tissues have been postulated to play a key role in mineralization [1–4]. Although recent experiments have demonstrated the synthesis of matrix phosphoproteins by explants of chick bone in vitro [5], osteoblasts, osteocytes and osteoclasts of bone substance per se constitute only a small fraction of the cells resident in bone as a tissue and organ, especially in the young growing skeleton. Therefore, to explore whether the phosphoproteins of bone are synthesized by bone substance cells per se, it was necessary to isolate and characterize 5 groups of bone cells isolated from young mouse calvaria. Using the incorporation of [^{3}H]serine into [^{3}H]Ser(P) as an indication of phosphoprotein synthesis [6–9], it was found that the osteoblastic group of isolated bone cells was most active in synthesizing phosphoproteins containing Ser(P). The behavior of these phosphoproteins on molecular sieving and ion-exchange chromatography was similar to that of phosphoproteins isolated from young mouse calvaria.

2. MATERIALS AND METHODS

2.1. *Extraction and purification of phosphoproteins of mouse calvaria*

The calvaria of 1000 6–9-day-old Swiss albino mice were dissected immediately after death and frozen at −20°C. The pooled mouse calvaria were washed 3 times with phosphate-buffered saline containing the protease inhibitors phenylmethyl sulfonyl fluoride (1 mM), ϵ-aminocaproic acid (50 mM) and benzamidine hydrochloride (5 mM), and homogenized and extracted in 0.5 M EDTA (0.5 M Tris–HCl, pH 7.4) for 2 weeks at 4°C. The EDTA extract was centrifuged, the supernatant concentrated with a YM-10 membrane (Amicon

[+] Present address: Department of Oral Biochemistry, School of Dentistry, Nagasaki University, Nagasaki, Japan

* To whom reprint requests should be addressed

Abbreviations: PTH, parathyroid hormone; MEM, minimum essential medium (Eagle); Ser(P), *O*-phosphoserine; Gdn–HCl, guanidine hydrochloride

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Corp., Lexington MA), dialyzed against water, and lyophilized.

The EDTA-soluble, non-diffusible phosphoproteins were chromatographed on a 1.5 × 12 cm column of DEAE–Sephacel (Pharmacia Fine Chemicals, Piscataway NJ) in 0.05 M Tris–HCl buffer (pH 8.3) containing 6 M urea. Adsorbed proteins were eluted with a linear gradient of NaCl from 0–0.8 M in the same buffer.

2.2. *Preparation and characterization of bone cells*

The calvaria of 2-day-old Swiss Albino mice were dissected immediately after sacrifice and digested with collagenase (180 units/ml) and trypsin (0.5 mg/ml) (Worthington Biochemicals, Freehold NJ) [10]. Five separate populations of cells were isolated [10] and subsequently cultured in MEM (Grand Island Biological, Grand Island NY) containing 10% fetal calf serum (Flow Labs., McLean VA) for 7 days. The tissue culture medium contained no serine. At that time, aliquots of each cell population were characterized by biochemical parameters established in [10]. The production of cAMP in response to parathyroid hormone (PTH) by each of the separate cell populations was measured as in [11] using a ^{125}I-radioimmunoassay kit (New England Nuclear, Boston MA). Changes in alkaline and acid phosphatase activities of each cell population in response to the addition of PTH was determined as in [12] and cell protein measured as in [13]. Small aliquots of bone cells were cultured in Leighton tubes containing glass coverslips. After 7 days of culture they were stained with hematoxylin and eosin and examined by light microscopy.

2.3. *Radioactive labeling of bone cells with [^{3}H]serine*

Each population of bone cells cultured for 7 days to confluence in 75 cm^3 flasks containing 10 ml tissue culture medium was incubated with [^{3}H]serine (5 μCi/mM, spec. act. 16.8 Ci/mM, New England Nuclear, Boston MA). After 24 h the culture medium was collected by centrifugation, dialyzed against a phosphate-buffered saline solution containing protease inhibitors (phenylmethylsulfonyl fluoride, 1 mM; ϵ-aminocaproic acid, 50 mM; benzamidine–HCl, 5 mM) for 2 days at 4°C, and lyophilized (medium fraction). Cell layers were frozen and thawed 3 times and then extracted in phosphate-buffered saline containing 0.1% Triton X-100. This is referred to as the cell phosphoprotein fraction.

2.4. *Purification and characterization of the [^{3}H]Ser(P) containing phosphoproteins*

The media from cell populations 4 and 5 were pooled and serially subjected to ion-exchange chromatography as described and molecular sieving through a 1.6 × 90 cm column of Sephacryl S-300 (Pharmacia Fine Chemicals) in 5 M Gdn–HCl at pH 7.4.

2.5. *Analytical determinations*

Samples of protein were hydrolyzed in HCl for both total amino acid analyses and for the detection of Ser(P) and Thr(P) [9,14]. [^{3}H]Ser(P) was detected in 4 N HCl, 105°C, 6 h hydrolysates by two-dimensional, high-voltage paper electrophoresis (first dimension, 2400 V, pyridine/acetic acid (pH 3.5); second dimension, 2000 V, acetic acid/formic acid (pH 1.9) as in [15]. Spots corresponding to [^{3}H]Ser(P) were cut out, extracted with water and counted in a liquid scintillation spectrophotometer (LS-150, Beckman Instruments, Palo Alto CA).

3. RESULTS

As reported for 8 other mammalian species [9], phosphoproteins extracted from mouse calvaria in EDTA contained both Ser(P) and Thr(P). The result of ion-exchange chromatography of the EDTA-soluble extract was very similar to that obtained from chicken bone: most of the phosphoprotein was eluted in the last peak from DEAE–Sephacel (peak III, phosphorus content: 3%, fig.1). In addition to Ser(P) and Thr(P), amino acid analyses of the partially purified phosphoproteins (DEAE–Sephacel peak III) revealed the characteristic high concentrations of glutamic and aspartic acids and glycine similar to the composition of other species [8,9]. The one difference noted in mouse bone phosphoproteins was the relatively high concentration of Thr(P) compared to Ser(P) [Ser(P)/Thr(P)] of 1.2 in mouse, compared with ~3–5 in other species] [9].

The production of cAMP in response to the addition of PTH by various cell populations is shown

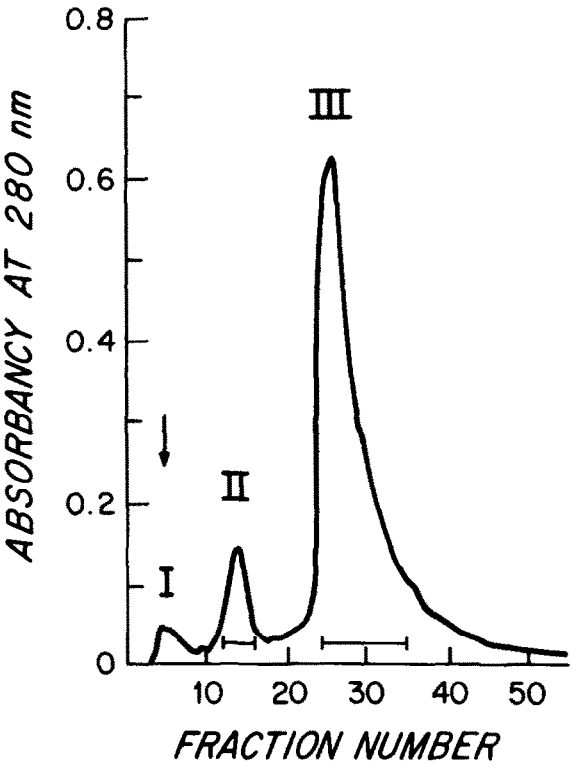

Fig.1. DEAE–Sephacel ion-exchange chromatography of phosphoproteins extracted in EDTA from mouse calvaria. About 40 mg in 10 ml of an EDTA extract was applied to a 1.5 × 12 cm column equilibrated with 0.05 M Tris buffer (pH 8.3) containing 6 M urea. Fractions of 4 ml were collected at 12 ml/h. The column was eluted with 25 ml of this buffer before a linear gradient was started (⟶) which consisted of 100 ml of the equilibration buffer and 100 ml of the limiting buffer containing 0.8 M NaCl. Aliquots of the fractions were hydrolyzed in 6 N HCl at 105°C for 24 h and their phosphorus contents and amino acid compositions were determined. Peak fractions II and III (indicated by bars) were hydrolyzed in 4 N HCl at 105°C for 6 h and chromatographed on the Beckman 121-M automatic amino acid analyzer to detect Ser(P) and Thr(P) [9,14].

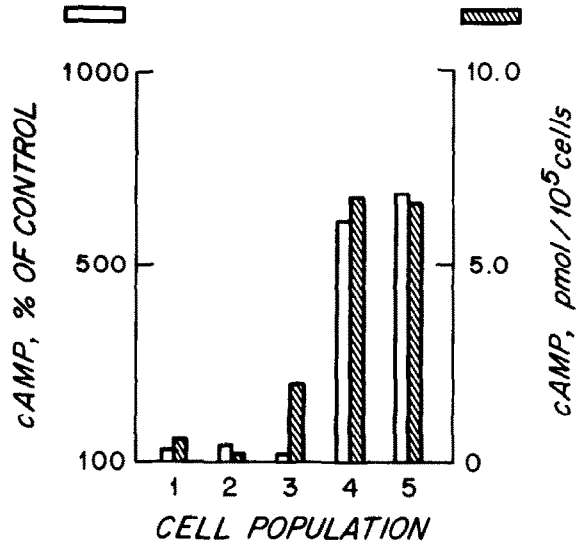

Fig.2. The effect of parathyroid hormone on cAMP synthesis and release by isolated bone cell populations.

in fig.2. Cells of populations 1 and 2 did not increase their production of cAMP, whereas the cells in populations 4 and 5 increased cAMP production nearly 7-fold. There was also a very marked increase in acid phosphatase activity in response to PTH in cell populations 1 and 2, but none in cell populations 4 and 5. In contrast, alkaline phosphatase activity of cell populations 4 and 5 decreased in response to PTH, while no change was noted in alkaline phosphatase activity in cell populations 1–3 (not shown). The biochemical parameters indicate that cell populations 1 and 2 have the characteristics of osteoclastic cells (OC cells), while cell populations 4 and 5 have the characteristics of osteoblastic cells (OB cells) [12]. Cell population 3 appears to contain a mixture of both cell types.

Analysis of the nondialyzable [^{3}H]Ser(P) contents in the medium and in the cells of the 5 populations of cells (fig.3) clearly indicate that not only do the OB cells synthesize more phosphoprotein than the other cell groups, but most importantly they secrete most of the phosphoprotein into the culture medium as one would expect for an extracellular, matrix protein. OC cells, on the other hand, synthesize less phosphoprotein and release very little of the phosphoprotein into the culture media (fig.3A,B), a property more like that of a cellular protein.

When the media from cell populations 4 and 5 were pooled and chromatographed on a DEAE-Sephacel column, the radioactivity emerged in 4 peaks (I–IV) (fig.4). Peak III contained the largest amount of [^{3}H]Ser(P). Peak II also contained a small amount of [^{3}H]Ser(P) (3.3%) but none of the other fractions eluted from DEAE–Sephacel contained significant amounts of [^{3}H]Ser(P). When peak III was molecularly sieved through Sephacryl

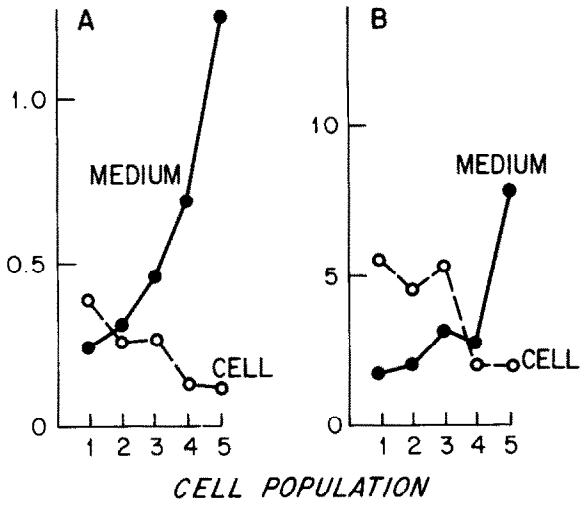

Fig.3. Biosynthesis of phosphoprotein by isolated bone cell populations. Distribution of *O*-[^{3}H]phosphoserine recovered in medium (●) and cell (○) fractions from the 5 bone cell populations: (A) *O*-[^{3}H]phosphoserine content expressed as % of the total radioactivity of each sample; (B) specific radioactivity of *O*-[^{3}H]phosphoserine (cpm/mg protein, $\times 10^{-3}$).

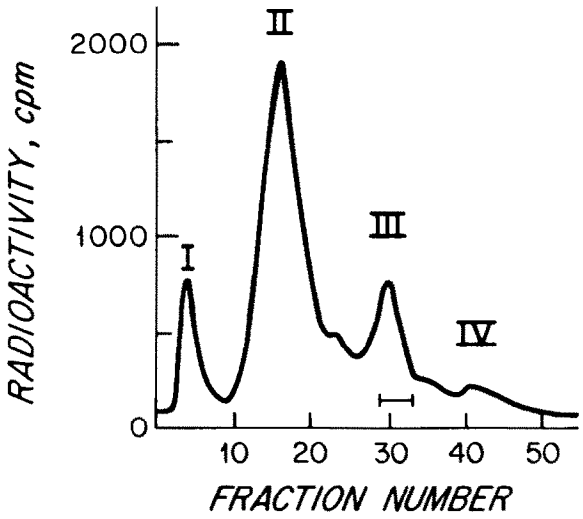

Fig.4. DEAE–Sephacel ion-exchange chromatography of the phosphoproteins synthesized by isolated bone cells. The medium of cell populations 4 and 5 were combined and applied (~20 mg in 10 ml) to a 1.5 × 12 cm column as in fig.1. Radioactivity was measured in 0.1 ml aliquots of each fraction dissolved in 12 ml Instagel. Fractions 29–33 were pooled for further purification.

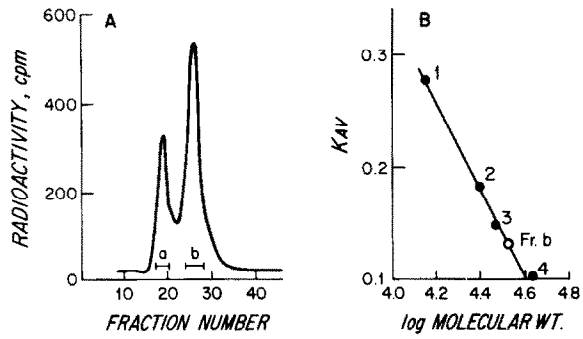

Fig.5. (A) Molecular sieve filtration of fraction III from DEAE–Sephacel (fig.4) applied to a 1.6 × 90 cm column of Sephacryl S-300 equilibrated with 0.05 M Tris buffer (pH 7.4) containing 4 M guanidine–HCl. Radioactivity was measured as in fig.4. (B) Determination of the molecular weight of fraction b from Sephacryl S-300 molecular sieving. Calibration with following standard proteins of known molecular weight: (1) lysozyme (14000); (2) chymotrypsinogen (25000); (3) carbonic anhydrase (30000); (4) ovalbumin (43000).

S-300 resin, two peaks emerged, both of which contained [^{3}H]Ser(P) (fig.5). Since peak *a* from Sephacryl S-300 eluted at and just after the void volume its molecular weight could not be accurately estimated. Calculation of the molecular weight of peak *b* from both the unlabelled protein of mouse bone calvaria and the phosphoprotein synthesized by isolated bone cells gave values of ~33000–34000.

4. DISCUSSION

Previous work using bone in organ culture [5,16] has established that unlike other bone matrix proteins such as albumin and α_2HS-glycoproteins, which are synthesized in the liver and subsequently transported and concentrated in bone [17–19], the non-collagenous phosphoproteins of bone are synthesized in bone as an organ. However, because the vast majority of cells in bone as an organ or tissue are non-osseous (viz., are not osteoblasts, osteoclasts nor osteocytes), it was not heretofore possible to say that the phosphoproteins were synthesized by bone cells per se. This study, however, has unequivocally established that bone cells, and in all probability osteoblasts, synthesize phosphoproteins, which are then released into the tissue culture medium as one would expect of pro-

teins destined to be incorporated into the extracellular matrix. This conclusion is also supported by the findings that the phosphoproteins synthesized by the cells behave identically on ion-exchange chromatography with the bone matrix phosphoproteins of the same species and indeed of the same bone from which the cells were isolated.

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