Proc. Natl. Acad. Sci. USA
Vol. 77, No. 4, pp. 2234–2238, April 1980
Medical Sciences

Radioimmunoassay for the vitamin K-dependent protein of bone and its discovery in plasma

(γ-carboxyglutamate/γ-carboxyglutamate-containing protein/calcified tissues/serum protein/antibody specificity)

PAUL A. PRICE AND SATORU K. NISHIMOTO

Department of Biology, University of California at San Diego, La Jolla, California 92093

Communicated by Andrew A. Benson, December 10, 1979

ABSTRACT **The vitamin K-dependent protein of bone has been detected in human plasma by radioimmunoassay at 4.5 ng per ml. The plasma protein has the same apparent molecular weight as the pure bone Gla protein (BGP) and other studies indicate the plasma protein is probably the intact bone protein. BGP also has been detected in bovine serum by radioimmunoassay. The bovine serum levels of BGP decrease with developmental age from 200 ng per ml in fetal calves to 26 ng per ml in adult cows. The implications of the discovery of BGP in plasma to the function of this unique protein are discussed. This assay employs rabbit antibody directed against calf BGP and has a sensitivity of 0.1 ng. The antibody crossreacts with purified human BGP but not with BGP from rat or rabbit bone. Studies with peptides of known structure derived from enzymatic digests of BGP indicate that the rabbit antibody recognizes the COOH-terminal region of the 49-residue calf bone protein.**

Bone has an abundant protein of unknown function which contains the vitamin K-dependent amino acid γ-carboxyglutamic acid (Gla) (1, 2). We have named this protein "bone Gla protein" (BGP) and have determined the complete amino acid sequence of the 49-residue calf protein and the 47-residue swordfish protein (3–5). Comparison of these two structures reveals approximately 50% sequence homology. Both proteins have three residues of Gla and a single disulfide bond in identical sequence positions, indicating the importance of these structures to the biological function of BGP (4).

We have previously proven that BGP is synthesized in calf cortical and cancellous bone culture (6). This conclusion is based upon the incorporation of ^3H into a protein that was identified as BGP by comigration with pure calf BGP in acrylamide gel electrophoresis and isoelectric focusing (6). Trypsin digestion of the newly synthesized BGP also generated [^3H]proline-labeled peptides that cochromatographed on DEAE-Sephadex with the tryptic peptides from pure calf BGP. Other experiments demonstrated that newly synthesized BGP is fully γ-carboxylated in calf bone culture and that it is synthesized at a rate of about one BGP molecule per molecule of tropocollagen. The cells that synthesize BGP in bone have not been identified. However, the presence of 4-hydroxyproline at position 9 in the calf BGP sequence (3) shows that the protein has been modified by prolyl hydroxylase, an enzymatic marker used widely to distinguish osteoblasts from osteoclasts in mixed cell populations. (7).

The abundance of BGP in calf bone suggests that BGP is located in the extracellular bone matrix (6). This matrix BGP is probably bound to hydroxyapatite in an interaction that involves γ-carboxyglutamate side chains. *In vitro* studies show that BGP binds strongly to hydroxyapatite with a simple logarithmic binding isotherm, whereas protein in which γ-carboxyglutamate has been converted to glutamate by a specific thermal decarboxylation procedure binds to hydroxyapatite less strongly (4, 8). Further evidence that γ-carboxyglutamate coordinates native BGP to hydroxyapatite is provided by the observation that γ-carboxyglutamate is completely protected against thermal decarboxylation when BGP is bound to hydroxyapatite (8).

In this paper, we present a specific and sensitive radioimmunoassay for the detection of BGP and the application of this procedure in the discovery of the BGP in human and bovine plasma.

MATERIALS AND METHODS

Preparation of BGPs. BGP was purified from the proteins released by demineralization of calf, rat, rabbit, human, and swordfish bone by gel filtration over Sephadex G-100 and subsequent gradient elution from DEAE-Sephadex A-25 as described (2–5). An additional affinity chromatography step with purified rabbit antibody against calf BGP as the specific adsorbent was used in the purification of the human protein. Partial sequence analysis of purified human BGP gave tyrosine and leucine as the first two NH_2-terminal amino acids, the same NH_2-terminal amino acids found in calf BGP (3). The DEAE-Sephadex-purified calf BGP used for immunizations, iodinations, and standards gave a single band on electrophoresis in 20% acrylamide gels and a single band on isoelectric focusing gels by using described methods (6). Thermal decarboxylation of the three Gla residues to glutamic acid and the reduction and carboxamidomethylation of the disulfide bond have been described elsewhere (8). Tryptic peptides corresponding to residues 1–19, 20–43, and 45–49 and the carboxypeptidase Y digestion product corresponding to residues 1–40 were prepared as described (3).

Preparation of Radioiodinated Calf BGP. Purified calf BGP was labeled with ^{125}I (4×10^{18} cpm per mol of I, Amersham) by the solid state lactoperoxidase method by incubating 10 μg of BGP with 1 mCi of ^{125}I (9) (1 Ci = 3.7×10^{10} becquerels). The labeled BGP was separated from unincorporated ^{125}I by gel filtration on a Sephadex G-25 column equilibrated with assay diluent (0.14 M NaCl/0.01 M phosphate/25 mM EDTA/0.1% gelatin/0.1% Tween-20 at pH 7.4).

Preparation of Antibodies. Ten rabbits were immunized by monthly multiple site intradermal injection of purified calf BGP adsorbed to polyvinyl pyrrolidone (PVP-40) (10). Each injection was made with 0.5–1.0 mg of purified BGP emulsifed

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: Gla, γ-carboxyglutamic acid; BGP, bone Gla protein.

in either complete (initial challenge) or incomplete Freund's adjuvant. Serum samples were withdrawn at regular intervals and tested for the titer of antibody to BGP by radioimmunoassay.

Radioimmunoassay. All assays contained (in order of addition) 0.2 ml of assay diluent, either a known amount of unlabeled BGP in 0.1 ml of assay diluent or 0.1 ml of heparinized plasma sample, 0.125 μl of antiserum R397 (final 1:4000 dilution) and 2.5 μl of normal rabbit serum in 0.1 ml of assay diluent, and 15,000 cpm of ^{125}I-labeled BGP in 0.1 ml of assay diluent. For the equilibrium radioimmunoassay all components were combined and incubated for 20 hr at 25°C. For the nonequilibrium assay all components except ^{125}I-labeled BGP were combined and incubated for 24 hr at 4°C followed by addition of ^{125}I-labeled BGP and a second 24-hr incubation at 4°C. Assays were terminated by precipitation of rabbit antibody with the addition of 1.9 units of goat antiserum to rabbit γ-globulin (Calbiochem, lot 860217) in 0.1 ml of assay diluent. After 1.5 hr at 25°C reaction mixtures were centrifuged to sediment ^{125}I-labeled BGP bound to rabbit antibody and the supernatant was discarded. Background ^{125}I label that nonspecifically adhered to the precipitate or to the glass reaction tube was measured by incubating ^{125}I-labeled BGP and normal rabbit serum without specific antiserum followed by the usual second antibody precipitation. Total and antibody-bound ^{125}I-labeled BGP were determined by assay in a Nuclear Chicago gamma counter for times sufficient to achieve a 2% counting accuracy. The fraction of ^{125}I-labeled BGP bound to antiserum, B, is defined as cpm in precipitate minus cpm in background divided by total cpm in assay; B_0 is the value of B when no unlabeled BGP is present. The B_0 values for each radioimmunoassay reported here are the average of nine independent determinations and the B values for all standards and unknowns are the average of three independent measurements.

Preparations of purified BGP from human, rat, rabbit, and swordfish bone were tested for radioimmunoassay crossreactivity by measuring the effect of different amounts of the purified BGP preparations on binding of ^{125}I-labeled calf BGP to antibody. Serum samples from rat, rabbit, and calf and human plasma samples were also tested for crossreactivity in this way, and the nonspecific effect of plasma or serum samples on the radioimmunoassay was tested by adding known quantities of calf BGP to the samples. The apparent molecular weight of BGP in circulation was investigated by comparing the Sephadex G-100 elution position of heparinized human plasma and calf serum BGP as determined by radioimmunoassay with the elution position of purified calf or human BGP. In order to test the stability of plasma BGP, heparinized human plasma samples were assayed after incubation at 25°C for 24 hr or at 4°C for 72 hr, after repeated freeze-thawing, and after lyophilization. Human plasma and serum samples from the same subjects were also compared.

RESULTS

Preparation of Radioiodinated Calf BGP. Iodination of pure calf BGP by the lactoperoxidase method gives an ^{125}I-labeled protein with a specific radioactivity of 40–50 mCi per mg. Over 90% of this labeled protein binds to an excess (1:100 dilution) of antibody raised to purified calf BGP. Labeled BGP is stable when stored in assay diluent, retaining over 90% of its initial immunologic activity after 8 weeks at −20°C. Repeated freezing and thawing has no effect on the immunologic activity of iodinated BGP.

Preparation of Antisera to Calf BGP. All ten rabbits produced antibody to BGP within 4 weeks after the initial injection, whereas no antibody could be detected in sera from 10 control rabbits. The antiserum (batch R397) that bound 20% of the iodinated BGP at the highest dilution was used for all radioimmunoassays reported here.

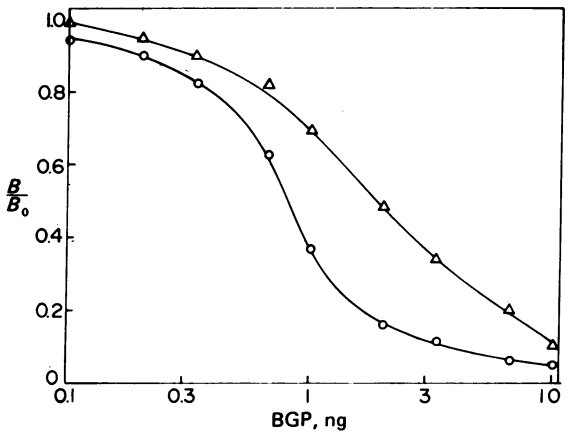

FIG. 1. Radioimmunoassay for calf BGP. Relative fraction of ^{125}I-labeled BGP bound to antibody (B/B_0) at increasing levels of BGP. △, Equilibrium radioimmunoassay; ○, nonequilibrium radioimmunoassay.

Radioimmunoassay for BGP. Rabbit antiserum to calf BGP was used in both equilibrium and nonequilibrium radioimmunoassays. As can be seen in Fig. 1, standard curves can be constructed for both assays by plotting the relative fraction of labeled BGP bound to antibody (B/B_0) against increasing amounts of unlabeled calf BGP. The nonequilibrium assay is somewhat more sensitive than the equilibrium assay, with a detection limit of 0.1 ng compared with 0.3 ng. However, the more rapid equilibrium assay has an adequate sensitivity and was used in all further experiments. The intraassay variation of the equilibrium radioimmunoassay is typically less than 10%. Interassay variation was evaluated by repeated measurement of calf and human serum standards and was less than 15% in 20 assays performed over a 6-month period.

The radioimmunoassay for calf BGP was tested for crossreactivity with BGP purified from human, rat, rabbit, and swordfish bone. As can be seen in Fig. 2, human BGP completely displaced labeled calf BGP from antibody and the standard curve generated by dilutions of human BGP parallels the calf BGP standard curve. One nanogram of human BGP

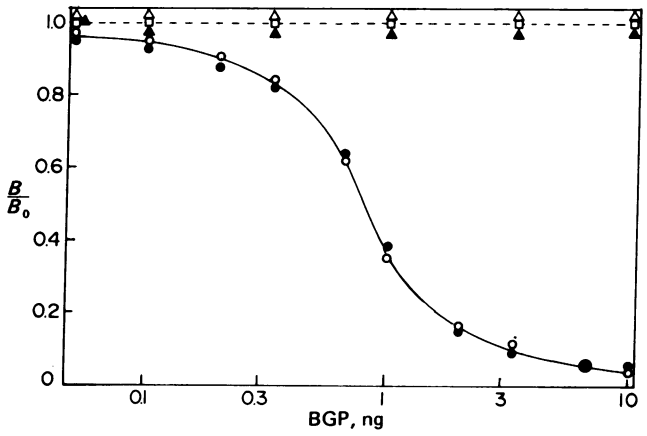

FIG. 2. Radioimmunoassay crossreactivity. Relative fraction of ^{125}I-labeled calf BGP bound to antibody (B/B_0) at increasing levels of purified BGP from calf (○), human (●), rat (△), rabbit (□), and swordfish (▲).

is equivalent to 1 ng of calf BGP in the radioimmunoassay and so dilutions of purified calf BGP were used as standards in radioimmunoassay for the calf and human proteins. BGPs from rat, rabbit, and swordfish bone did not displace labeled calf BGP from antibody, even at 1 μg of added protein. Thus, in the radioimmunoassay, calf BGP crossreacts with the human BGP but not with BGP of other species tested. BGPs from rat, rabbit, and swordfish bone did not displace labeled calf BGP from antibody even at 1 μg of added protein. Thus, the radioimmunoassay for the calf BGP crossreacts with the human BGP but not with BGP of other species tested.

Location of the Antigenic Site. The location of the region of calf BGP recognized by the rabbit antibody was determined with peptides derived from enzymic digests of BGP and with chemically modified forms of BGP. As can be seen in Fig. 3, specific thermal decarboxylation of γ-carboxyglutamate to glutamate (8) has no effect on antigenicity. Thus, the unique vitamin K-dependent region of BGP is not part of the antigenic determinant. This result also shows that the radioimmunoassay detects non-γ-carboxylated BGP such as might be present in coumadin-treated animals. Reduction and carboxamidomethylation of the single disulfide bond had no effect on antigenicity (Fig. 3), which suggests that antigenicity is not dependent on the native conformation of BGP. Because the three γ-carboxyglutamate residues are at positions 17, 21, and 24 and the disulfide bond joins half-cystine residues at positions 23 and 28, the region between 17 and 28 in the 49-residue sequence is probably not involved in antibody recognition of BGP. Specific enzymatic cleavage of BGP with trypsin (3) produces three peptides (1–19, 20–43, and 45–49), none of which is antigenic (Fig. 3). Thus, the antigenic site may involve either of the pairs of basic residues at sequence positions 19 and 20 or 43 and 44. Because the 1–40 peptide derived from a carboxypeptidase Y digestion of BGP is not antigenic (Fig. 3), the COOH-terminal region of BGP is required for antigenicity.

Detection of BGP in Blood Plasma. Both calf and human plasma displace labeled calf BGP from antibody (Fig. 4). The results of several experiments demonstrate that this effect is due to the presence of the BGP in plasma. First, dose dilution curves for human and calf plasma paralleled the radioimmunoassay standard curve (Fig. 4). Second, up to 0.1 ml of rat and rabbit plasma failed to displace any labeled calf BGP from antibody,

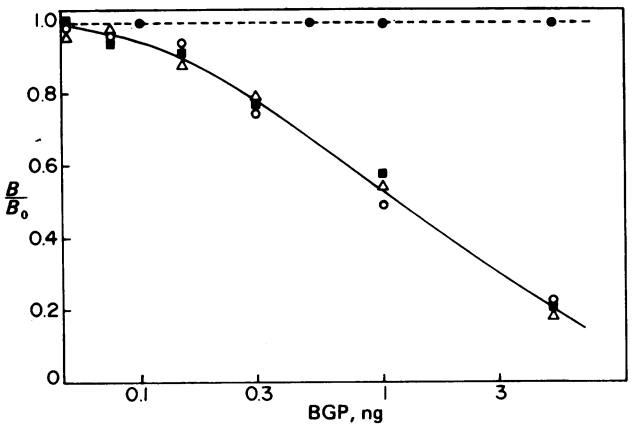

FIG. 3. Location of antigenic determinant in calf BGP. Relative fraction of ^{125}I-labeled calf BGP bound to antibody (B/B_0) at increasing levels of the following forms of calf BGP: ○, native; ■, with γ-carboxyglutamate thermally decarboxylated; △, with disulfide bond reduced and carboxamidomethylated; ●, tryptic peptides corresponding to residues 1–19, 20–43, and 45–49, chymotryptic peptide 6–38, and carboxypeptidase Y digestion product corresponding to 1–40.

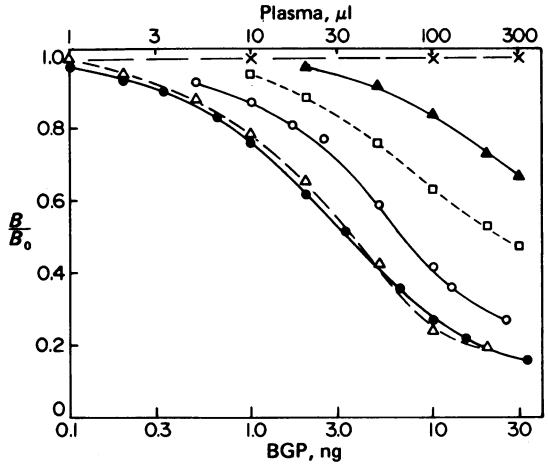

FIG. 4. Dose dilution radioimmunoassay of plasma. Relative fraction of ^{125}I-labeled calf BGP bound to antibody (B/B_0) at increasing amounts of calf BGP (●), human plasma from patient with Paget disease (○), two normal human plasmas (□ and ▲), calf serum (△), and rat and rabbit serum (×).

a result that is consistent with the absence of antibody crossreactivity with purified BGP from these species. Finally, experiments in which purified calf or human BGP was added to the respective plasma samples demonstrated that plasma does not interfere with the quantitative detection of the added BGP by radioimmunoassay.

The form of BGP found in plasma is probably the same as the intact 49-residue protein found in bone. The gel filtration of fetal calf and human plasma on Sephadex G-100 demonstrates that over 90% of the antigenic material elutes in the exact

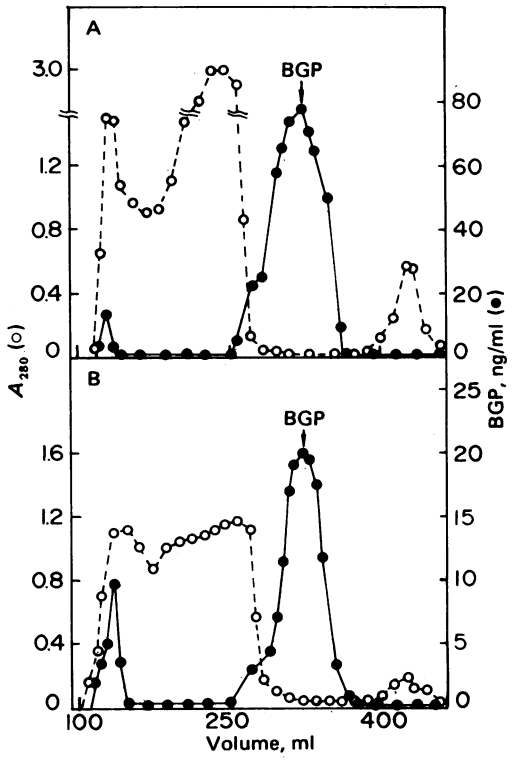

FIG. 5. Gel filtration of fetal calf serum (*A*) and human plasma (*B*) samples (10 ml) on Sephadex G-100. Column, 2 × 150 cm; eluent, 5 mM NH_4HCO_3; 4°C. ○, A_{280}; ●, BGP determined by radioimmunoassay on 0.1 ml of effluent.

Table 1. BGP level in normal plasma

Type of sample	Number of samples	BGP, ng per ml
Human plasma	30	4.5 ± 2
Fetal calf serum	5	200 ± 20
Calf serum	5	50 ± 6
Adult cow serum	5	26 ± 3

BGP levels were determined by radioimmunoassay of plasma from normal humans and of sera from fetal calves, 6-month-old calves, and 5-year-old cows. The level of BGP in human plasma is given in nanograms of calf BGP. Values are means ± SD.

position found for pure calf or human BGP on the same filtration column (Fig. 5). The small amount of higher molecular weight immunogen was completely dissociated into BGP by incubation with 6 M guanidine·HCl for 1 hr at 25°C prior to a second filtration. Thus, plasma BGP is not a proteolytic degradation product of bone BGP. Further indication that plasma BGP is not degraded is the fact that it is antigenic at all, because tryptic, chymotryptic, or carboxypeptidase digestion destroys antigenicity (Fig. 3). Lastly, because $BaSO_4$ removes immunoreactive BGP from plasma (data not shown), the plasma protein probably contains the region of BGP with the three γ-carboxyglutamate residues that are involved in mineral binding (8, 11).

Stability of Plasma BGP. The stability of BGP in plasma was investigated in order to facilitate clinical studies of BGP levels in human plasma samples. In a study of 30 human plasma samples, BGP levels were not affected by four freeze–thaw cycles or by lyophilization followed by reconstitution with either water or radioimmunoassay diluent. The level of BGP in five human plasma samples stored at −20°C remained constant over a period of 14 months, and BGP levels in 10 human plasma samples were unchanged after 24 hr at 25°C and after 72 hr at 4°C. No differences in BGP levels could be detected between heparinized plasma and serum samples obtained at the same time from 30 normal individuals. However, serum BGP levels fell 19% after 8 hr at 25°C, indicating that BGP may be less stable in serum than in plasma.

BGP Levels in Human and Bovine Plasma. The human plasma levels of BGP were determined by radioimmunoassay of heparinized plasma from 30 normal individuals ranging in age from 18 to 82 years. The average BGP level found in plasma is 4.5 ± (SD) 2 ng per ml (Table 1). The BGP level was also determined in fetal calf serum and in serum obtained from 5-year-old cows. As can be seen in Table 1, the BGP level is high in fetal calf serum and is lower in calf and adult cow sera. The elevated BGP level in plasma from immature animals may be due to the increased level of bone metabolism in animals with net bone growth.

DISCUSSION

We have developed a radioimmunoassay for quantitative measurement of the low molecular weight, vitamin K-dependent protein from calf and human bone. This radioimmunoassay should be useful in biochemical studies on BGP biosynthesis and function. The availability of an antibody directed against a specific region of BGP should also permit the immunohistological localization of BGP in bone and the identification of the cells that synthesize it. Because the radioimmunoassay crossreacts with human BGP, it can be used in clinical studies to determine BGP levels in bone from patients with bone disease. To facilitate such studies, we have recently developed a rapid method to solubilize all BGP from human bone and have found that we can accurately estimate its level in bone samples as small as 10 mg, an amount easily obtained during routine bone biopsy.

We have used the radioimmunoassay to discover that bovine and human plasma displace iodinated BGP from antisera. Several experiments indicate that this effect is due to the presence of BGP in plasma. The results of dose dilution, addition, and crossreactivity studies rule out a nonspecific effect of bovine or human plasma on the radioimmunoassay, and gel filtration studies indicate that the plasma antigen is probably the intact 49-residue protein found in bone. Further evidence that BGP is in plasma is provided by preliminary observations with another radioimmunoassay we have developed recently for measurement of rat BGP (12). This assay detects BGP levels of 75–150 ng per ml in rat sera but does not detect BGP in human and calf sera, the purified bone proteins of which do not crossreact. In addition, gel filtration studies demonstrate that rat serum BGP has the same molecular weight as BGP purified from rat bone. The presence of high BGP levels in calf, human, and rat plasma suggests that a high level of BGP may be a general characteristic of mammalian plasma.

The exact origin of plasma BGP is not known. One possible source of plasma BGP could be bone matrix BGP, which is released during bone resorption. We have previously shown that BGP is an abundant bone matrix protein (2) and that it is rapidly released during neutral or acid demineralization of bone (2). Another possible source of plasma BGP could be the direct release of newly synthesized BGP into plasma. Because bone is a site of BGP biosynthesis (6), bone cells could release BGP directly into plasma. The assignment of bone itself as the tissue responsible for plasma BGP is strengthened by separate clinical studies in which we find that the plasma BGP levels are markedly elevated in metabolic bone diseases such as Paget disease of bone, renal osteodystrophy, primary hyperparathyroidism, and metastatic bone cancer.*

The presence of high BGP levels in plasma raises the prospect that some bone matrix BGP comes from plasma. BGP does bind strongly to hydroxyapatite (4), and a simple calculation† shows that the quantity of BGP actually present in bone is remarkably close to the amount calculated on the basis of hydroxyapatite affinity and plasma concentration. Plasma BGP could also contribute to the high levels of Gla found in pathological apatitic calcifications, such as atheromatous lesions (13) and ectopic calcifications. In preliminary experiments with the radioimmunoassay described here, we find BGP in the calcified regions of atheromatous plaques.

The function of the vitamin K-dependent BGP is presently unknown. Our studies with vitamin K-deficient animals, however, would appear to preclude a function for BGP in bone matrix structure (ref. 3, unpublished results). The BGP content of the bones of these animals is 5% of normal, yet the bone is normally mineralized and has normal growth morphology and normal strength (ref. 3; unpublished results). The present discovery that BGP circulates in plasma suggests that BGP may play some role in the regulation of skeletal mass and calcium

* Price, P. A., Nishimoto, S. K., Parthmore, J. G. & Deftos, L. J. (1979) *Proceedings of the First Annual Meetings of the American Society for Bone and Mineral Research*, 14A (abstr.). Also, unpublished results.

† The affinity of BGP for apatite is about 0.1 μM in protein binding sites on the crystal surface (4). Because the binding of BGP to apatite shows single-class binding behavior, at 10 nM BGP in plasma, about 9% of the apatite surface binding sites should have the protein bound. Because each 100 g of apatite binds 5.8 g of BGP at saturation, the amount bound to 100 g of apatite at 10 nM BGP should be 0.09 × 5.8 or about 0.5 g. The actual bone level of BGP is 0.4–0.5 g per 100 g of apatite.

homeostasis. An informational function for BGP is supported by structural features such as the pro-lys unit, which is present in many informational proteins (14), and the two pairs of basic residues, which are proteolytic cleavage sites in the activation of prohormones such as proinsulin (15).

We thank Sharon Baukol and Jill Souder for their superb technical assistance. We thank James Poser and Allen Otsuka for several of the purified preparations of BGP used for crossreactivity and antigenic site localization studies. This work was supported in part by U.S. Public Health Service Grants GM 17702 and AM 25921.

1. Hauschka, P. V., Lian, J. B., & Gallop, P. M. (1975) *Proc. Natl. Acad. Sci. USA* **72,** 3925–3929.
2. Price, P. A., Otsuka, A. S., Poser, J. W., Kristaponis, J. & Raman, N. (1976) *Proc. Natl. Acad. Sci. USA* **73,** 1447–1451.
3. Price, P. A., Poser, J. W. & Raman, N. (1976) *Proc. Natl. Acad. Sci. USA* **73,** 3374–3375.
4. Price, P. A., Epstein, D. J., Lothringer, J. W., Nishimoto, S. K., Poser, J. W. & Williamson, M. K. (1979) in *Vitamin K Metabolism and Vitamin K-Dependent Proteins,* ed. Suttie, J. W. (Univ. Park Press, Baltimore, MD) pp. 219–226.
5. Price, P. A., Otsuka, A. S., & Poser, J. W. (1977) in *Calcium Binding Proteins and Calcium Function,* ed. Wasserman, R. H. (Elsevier, Amsterdam) pp. 333–337.
6. Nishimoto, S. K. & Price, P. A. (1979) *J. Biol. Chem.* **254,** 437–441.
7. Luben, R. A., Wong, G. L, & Cohn, D. V. (1976) *Endocrinology* **99,** 526–534.
8. Poser, J. W. & Price, P.A. (1979) *J. Biol. Chem.* **254,** 431–436.
9. David, G. S. & Reisfeld, R. A. (1974) *Biochemistry* **13,** 1014–1021.
10. Worobec, R. B., Wallace, J. H. & Huggins, C. G. (1972) *Immumochemistry* **9,** 229–238.
11. Suttie, J. W. & Jackson, C. M. (1977) *Physiol. Rev.* **57,** 1–70.
12. Price, P. A., Lothringer, J. W. & Nishimoto, S. K. (1980) *J. Biol. Chem.,* in press.
13. Lian, J. B., Skinner, M., Glimcher, M. J. & Gallop, P. M. (1976) *Biochem. Biophys. Res. Commun.* **73,** 349–355.
14. Tzehoval, E., Segal, S., Stabinsky, Y., Fridkin, M., Spirer, Z. & Feldman, M. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 3400–3404.
15. Steiner, F. D., Kemmler, W., Tager, H. S. & Peterson, J. B. (1974) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **33,** 2105–2115.