Secretion of the Vitamin K-dependent Protein of Bone by Rat Osteosarcoma Cells

EVIDENCE FOR AN INTRACELLULAR PRECURSOR

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Four clonal cell lines derived from a rat osteosarcoma were tested for the ability to secrete the γ-carboxyglutamic acid-containing protein of bone (BGP) using a specific radioimmunoassay for this protein. Two cell lines secreted BGP into culture media while the other two did not. Other investigators have shown that these two cell lines are also the only ones with the high parathyroid hormone responsiveness and alkaline phosphatase activity expected for osteoblasts in culture. Both cell lines also form a mineralized sarcoma when implanted in rats. The BGP in culture media is identical in molecular weight and in electrophoretic mobility with the 5800-dalton BGP purified from rat bone. Thus, BGP is probably secreted by osteosarcoma cells directly and not derived from an extracellular precursor by proteolytic cleavage.

There are two immunoreactive components within osteosarcoma cells which secrete BGP. One component is identical in molecular weight and in electrophoretic mobility with BGP from rat bone. The other component has a higher molecular mass (approximately 9000 daltons) and about half the electrophoretic mobility of BGP from bone. The presence of both components within these cells raises the possibility that the larger component may be an intracellular precursor which is processed to BGP prior to secretion.

All bone examined to date contains an abundant low molecular weight protein which has the vitamin K-dependent amino acid, γ-carboxyglutamic acid (Gla) (1, 2). We have named this protein the 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 the two sequences shows that about half of the amino acids in these two proteins are in identical sequence positions, including the 3 residues of γ-carboxyglutamic acid and the single disulfide bond (5). The conservation of these structural features after 400 million years of divergent evolution indicates their importance to the biological function of BGP.

We have proven previously that BGP is synthesized in bone culture (6). Newly synthesized BGP is fully γ-carboxylated and is synthesized at a rate of about 1 BGP molecule/molecule of tropocollagen. The cells which 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).

In the present investigation, we have applied a specific radioimmunoassay for rat BGP (8) to the analysis of BGP synthesized by rat osteosarcoma cells. The four clonal rat osteosarcoma cell lines used in this study have been isolated and characterized recently by Majeska et al. (9). Two of these clonal cell lines have the high parathyroid hormone responsiveness and alkaline phosphatase activity which are associated with the osteoblast phenotype and form ossified sarcomas when implanted into rats. We report here that only these two clonal cell lines produce the bone Gla protein in cell culture.

An important question in the analysis of possible biological functions for the bone Gla protein is the molecular weight of the initially secreted species. One possibility is that BGP is a nonfunctional cleavage peptide released from a larger precursor protein in an extracellular proteolytic cleavage event. This possibility is supported by the fact that the γ-carboxyglutamic acid-containing portion of prothrombin released upon prothrombin activation to thrombin has no further biological function (10). A second possibility is that BGP itself is the secreted protein. To resolve this question, we have characterized the molecular weight and the electrophoretic mobility of BGP synthesized by rat osteosarcoma cells. These studies demonstrate that the secreted protein is identical with BGP from bone. We also present evidence for the existence of an intracellular precursor which is processed to BGP before secretion from the cell.

EXPERIMENTAL PROCEDURES

Materials—Bone Gla protein was purified from the proteins released by demineralization of calf and rat bone by gel filtration over Sephadex G-100 and subsequent gradient elution from DEAE-Sephadex A-25 as described previously (1, 8). L-[4, 5-3H]leucine (110 Ci/mol) and 125I (4 × 10^8 cpm/mol) were purchased from Amersham. Coons F12, fetal calf serum, and antibiotic-antimycotic solution were purchased from Irvine Scientific. Triton X-100, Omnufluor, and Protosol were purchased from New England Nuclear. Sephadex G-25, G-100, and Sephacryl S-200 were purchased from Pharmacia. Rat osteosarcoma cells ROS 2/3, ROS 17/2, ROS 25/1, ROS 25/4, and ROS 25 were generously provided by Dr. Gideon Rodan (9). Ultrapure guanidine HCl was purchased from Bethesda Research.

Radioimmunoassay—The procedures used for radioimmunoassay of rat and calf BGP and the description of the antibody preparation used for each have been previously described (8, 11). Aliquots of 0.1 ml or less were assayed for rat BGP or calf BGP in all experiments except those containing 6 M guanidine HCl in the solution to be assayed. In the latter case, aliquots of 20 μl or less were used in the
rat assay and aliquots of 10 µl or less were used in the calf assay. Control experiments demonstrated that this level of guanidine HCl in the assay had no effect on the ability to quantitate BGP levels. We have previously shown that the antibody for calf BGP does not cross-react with rat BGP (11) and that the antibody for rat BGP does not cross-react with calf BGP (8). Thus, it is possible to employ calf BGP as an internal marker of molecular weight and electrophoretic mobility in the analysis of secreted and intracellular BGP produced by rat osteosarcoma cells. All rat or calf BGP concentrations reported in this paper are the average of triplicate radioimmunoassay. The intra-assay variation is less than 10% for both radioimmunoassays (8, 11).

Osteosarcoma Cell Culture—All osteosarcoma cell lines were grown in Coons F12 medium supplemented with 10% fetal calf serum and 1% antibiotic-antimycotic solution. The cultures were incubated was changed three to four times weekly and the cells were subcultured.

For measurement of the rate of BGP secretion from the cells, each cell type was grown to confluence in 60-mm culture plates and 5 ml of fresh media was added. After 24 h the media was again changed and the subsequent appearance of BGP in media was determined by triplicate radioimmunoassay of aliquots removed at appropriate times. The rate of BGP synthesis relative to other secreted protein was assessed after 24 h culture in media containing 10 µCi/ml of [3H]leucine. For measurement of BGP stability in media, the media were removed from the cell layer after 24 and 48 h and placed in a clean culture dish. Aliquots were again removed at appropriate times and assayed for BGP. Intracellular BGP levels were measured in trypsin dissociated cells after one wash with culture media and two washes with phosphate-buffered saline. The final cell pellet was lysed by freeze-thawing after addition of 0.7 ml of 6 M guanidine HCl in 0.1 M Tris buffer at pH 8/10 cells and assayed for BGP.

Gel Filtration—The molecular weight of rat BGP in media from osteosarcoma cells was determined by gel filtration over Sephacryl G-100 as previously described (2). The molecular weight of media BGP and of intracellular BGP was also measured under denaturing conditions using 6 M guanidine HCl in 0.1 M Tris buffer at pH 8 in a column (0.9 x 150 cm) of Sephacryl S-300. For media analysis, 10 ml of media were freeze-dried, dissolved in 2 ml of eluent buffer, and applied to the Sepharclamp column. For analysis of intracellular BGP, the lysed cell pellet from ROS 17/2 cells was applied to the Sephacryl S-200 column directly. In several experiments, 3000 ng of calf BGP was added as an internal molecular weight marker. Control experiments demonstrated that calf and rat BGP co-elute on Sephacryl S-200 columns.

Acrylamide Gel Electrophoresis—The procedures used in 15% polyacrylamide gel electrophoresis, gel slicing, and radioactivity counting have been described (8). [3H]leucine labeled BGP was added as an internal marker of electrophoretic mobility since control experiments demonstrated that calf BGP co-migrates with rat BGP. Experiments with trypsin and chymotryptic digests of BGP indicated that proteolytic cleavage always generates components with higher mobility than BGP.

RESULTS

Synthesis of Bone Gla Protein by Osteosarcoma Cells in Culture—The ability of clonal rat osteosarcoma cell lines to synthesize BGP is compared with other properties of these cells in Table I. As can be seen, there is a strong positive correlation between BGP synthesis, parathyroid hormone responsiveness, and alkaline phosphatase activity. The two cell lines which synthesize BGP, 17/2 and 2/3, are also the only two lines which form an ossified sarcoma when implanted into a suitable rat host (9). The 17/2 cell line has an intracellular BGP level equivalent to the amount of BGP secreted in 2/3 h, while the 25/1 and 25/4 cells have no detectable intracellular BGP. Thus, the absence of BGP in the medium of 25/1 and 25/4 cells must be due to an inability to synthesize the protein rather than failure to secrete it.

The rates of BGP accumulation in the media of different clonal cell lines are compared in Fig. 1. For both cell lines which synthesize BGP, 17/2 and 2/3, the concentration of BGP in the media levels off after 30 h. It is unlikely that this reflects a dynamic steady state between further BGP synthesis and destruction of BGP in the media by proteases, since less than 10% of the BGP in media removed from cells was lost in 48 h at 37°C. Furthermore, the plateau in BGP concentration cannot be explained by decreased synthesis due to exhaustion of a serum factor, since the same BGP level is reached with 2% serum with 10% serum.

Characterization of the Bone Gla Protein Secreted by Osteosarcoma Cells—Media BGP from 17/2 cells and pure BGP from rat bone have the same apparent molecular weight as shown by their identical elution volumes from Sephacryl G-100 (Fig. 2). Media BGP and BGP from bone also eluted in the same position on Sephacryl S-200 as calf BGP which was added as an internal molecular weight marker (data not shown). The use of calf BGP as an internal marker is possible because the antibodies for rat BGP and calf BGP do not cross-react and so the level of the two proteins can be determined independently by radioimmunoassay (8, 11). As seen in Fig. 2, BGP is less than 0.2% of the total [3H]leucine labeled protein.
secreted by the osteosarcoma cells. The small fraction of total protein secretion devoted to BGP is consistent with our earlier observation that BGP accounts for less than 0.2% of the protein synthesized in calf bone culture (6).

Media BGP and BGP purified from rat bone also have identical electrophoretic mobilities in 20% acrylamide gels (Fig. 3). Electrophoresis experiments with calf BGP used as an internal marker showed that media BGP and BGP from bone both have the same electrophoretic mobility as calf BGP, $R_F = 0.57$. No immunoreactive BGP with higher or lower electrophoretic mobility was detected in the culture media.

**Discovery of a Putative Intracellular Precursor to the Bone Gla Protein**—The molecular weight of immunoreactive protein in rat osteosarcoma cells was investigated by gel filtration. In the experiment shown in Fig. 4, calf BGP was added as an internal molecular weight marker to facilitate comparison of intracellular and media BGP. As can be seen in Fig. 4A, most of the immunoreactive intracellular rat protein emerges at an earlier elution position than BGP consistent with the presence of a higher molecular weight component. Since 6 M guanidine HCl is present from the moment of cell lysis, proteolytic processing cannot occur and the higher molecular weight immunoreactive protein cannot result from noncovalent association of rat BGP with intracellular proteins.

**Fig. 2.** Gel filtration of $^3$H-labeled, secreted proteins on Sephadex G-100. A confluent 10-cm culture plate was cultured for 24 h with 10 μCi/ml $[^3$H]leucine. The medium was desalted by gel filtration on Sephadex G-25 and then void volume fractions were pooled, lyophilized, and applied to the column. The arrow indicates the elution position of the peak fraction of pure rat BGP on the same column. Rat BGP data points represent the average of triplicate radioimmunoassays. Column, 2 x 150 cm; buffer, 5 mM NH$_4$HCO$_3$; flow rate, 11 ml/h; 5°C. ○ − ○, cpm/ml; ● − ●, rat BGP, ng/ml.

**Fig. 3.** Gel electrophoresis of secreted bone Gla protein. Fractions 46 to 52 in Fig. 2 were pooled, lyophilized, and electrophoresed in 20% polyacrylamide gel at pH 8.9. Gels were sliced for counting and Gla protein assay as described under “Experimental Procedures.” Rat BGP data points represent the average of triplicate radioimmunoassays. ● − ●, cpm/mm gel slice; ○ − ○, rat BGP, ng/mm gel slice.

**Fig. 4.** Gel filtration of secreted and intracellular proteins on Sephacryl S-200. A, intracellular proteins from 2.3 x 10$^7$ ROS 17/2 cells with 3000 ng of calf BGP added as a molecular weight marker. B, secreted protein from 5 ml of ROS 17/2 culture media with 3000 ng of calf BGP added as a marker. The intracellular proteins were prepared as described under "Experimental Procedures." Column, 0.9 x 150 cm; buffer 6 M guanidine HCl, 0.1 M Tris, pH 8.3, 3.7 ml/h; 25°C. ● − ●, rat BGP, ng/ml; ○ − ○, calf BGP, ng/ml. Both calf and rat BGP data points represent the average of triplicate radioimmunoassay.
**Biosynthesis of γ-Carboxyglutamate-containing Bone Protein**

The results of the present investigation support the provisional identification of osteoblasts as the cells which synthesize the bone Gla protein. The clonal osteosarcoma cell lines which synthesize BGP are also the cell lines with high PTH responsiveness and alkaline phosphatase activity (9). Both properties are characteristic features of osteoblasts in cell culture. Further, only the two cell lines which synthesize BGP in culture form mineralized osteosarcomas when implanted into a rat (9). The synthesis of BGP by bone cells is also supported by previous studies which prove that BGP is made in fetal rat mineral (4, 8). We have also shown that rabbit bones which have only 5% of the normal BGP level have normal mineral content and normal strength (4).

The identification of BGP as the protein secreted by osteosarcoma cells focuses attention on BGP itself as the unit of biological function. Some possible functions for this protein can accordingly be excluded. No protein of only 49 residues has yet been identified as an enzyme nor has a protein of this size been assigned a structural function. However, most hormones and chemotactic factors do have a low molecular weight. The presence of BGP in plasma and the variation of the plasma BGP level with bone metabolic diseases (4, 8, 11) and the identification of BGP as a potential inhibitor of hydroxyapatite crystalization (2, 22) have led to the hypothesis that the BGP may have a role in skeletal homeostasis (4, 11). The major hypothesis which has been suggested for the function of BGP is a role in biological mineralization (1, 2, 22). The abundance of BGP, approximately 1 molecule/hydroxyapatite crystal in bone (2), supports this view as does its only documented in vitro activity as a potent inhibitor of hydroxyapatite crystalization (2, 22). We have recently discovered that BGP is not present in fetal rat mineral (4, 8). We have also shown that rabbit bones which have only 5% of the normal BGP level have normal mineral content and normal strength (4).

![Diagram](image)

**FIG. 5.** Gel electrophoresis of intracellular Gla protein fractions. A, Fraction 28, Fig. 4(A). B, Fraction 31, Fig. 4(A). Twenty percent polyacrylamide gel at pH 8.9. Gels were sliced and assayed for BGP as described in "Experimental Procedures." - - rat BGP, ng/l mm gel slice; ○○ calf BGP, ng/l mm gel slice. Both calf and rat BGP data points represent the average of triplicate radioimmunassays.

(12). Experiments carried out with bovine serum albumin, chymotrypsin, RNase, and calf BGP as molecular weight markers on this column show that the major immunoreactive intracellular component has a molecular mass of approximately 9,000 daltons compared to the 58,000-dalton BGP from bone.

Due to the asymmetric elution profile of the intracellular immunoreactive protein (Fig. 4), fractions from the right and left sides of the peak were analyzed independently by acrylamide gel electrophoresis. As can be seen in Fig. 5B, the lower molecular weight component from the right side of the peak has an electrophoretic mobility identical with BGP from rat bone and with the added calf BGP internal marker (RP = 0.57). In contrast, the higher molecular weight component from the left side of the peak has a lower mobility (RP = 0.29, Fig. 5A). This reduced electrophoretic mobility is consistent with the larger size of the intracellular BGP. Thus, the skewed appearance of the intracellular BGP from Sephacryl S-200 can be explained by the presence of the two components, a major component of higher molecular weight and lower electrophoretic mobility than BGP from rat bone and a minor component identical with BGP in molecular weight and in mobility.

**DISCUSSION**

The results of the present investigation support the provisional identification of osteoblasts as the cells which synthesize the bone Gla protein. The clonal osteosarcoma cell lines which synthesize BGP are also the cell lines with high PTH responsiveness and alkaline phosphatase activity (9). Both properties are characteristic features of osteoblasts in cell culture. Further, only the two cell lines which synthesize BGP in culture form mineralized osteosarcomas when implanted into a rat (9). The synthesis of BGP by bone cells is also supported by previous studies which prove that BGP is made and is fully γ-carboxylated in bone culture. In addition, independent studies demonstrate that a chicken bone microsomal system incorporates labeled CO₂ into a γ-carboxyglutamic acid (13) and that one of the labeled proteins is identical in size with the 6500-dalton vitamin K-dependent protein of chicken bone (14). Finally, presence of 4-hydroxyproline at position 9 in the calf BGP sequence (8) shows that the protein has been modified by prolyl hydroxylase, an enzymatic marker used widely to distinguish osteoblasts from osteoclasts in mixed cell populations (8).

The protein secreted by osteosarcoma cells is identical with BGP from rat bone in electrophoretic mobility and in molecular weight. We therefore conclude that BGP is not derived from a larger precursor by an extracellular proteolytic cleavage. This inference is supported by the preliminary experimental result that addition of 100 μg/ml of Warfarin to the osteosarcoma cells does not change the molecular weight of the secreted product. Warfarin is a potent inhibitor of γ-carboxylation and subsequent extracellular proteolytic cleavage in the only other well characterized vitamin K-dependent system, the blood coagulation factors (10).

Our current studies indicate that there is an intracellular precursor to BGP. Using a specific radioimmunassay for rat BGP, we have shown that there are two immunoreactive components among intracellular proteins. One of these components is identical with BGP from bone. The other component has a higher molecular weight in 6 M guanidine HCl and a lower electrophoretic mobility in 20% acrylamide gels than BGP. The presence of both proteins in osteosarcoma cells suggests that the putative precursor is processed to BGP prior to secretion. The existence of a precursor to BGP would raise the intriguing prospect that the precursor contains a recognition signal which targets BGP as a protein to be post-translationally modified by the γ-carboxylase system. The presence of intracellular precursors to prothrombin (15-17) indicate that a similar recognition signal may also target prothrombin for γ-carboxylation. A precursor might have other biological functions in the synthesis of BGP, as suggested by the existence of precursors to other low molecular weight, secreted proteins such as insulin (18), glucagon (19), and calcitonin (20). Studies are in progress to explore the relationship between precursor processing to BGP and the formation of γ-carboxyglutamic acid.

The identification of BGP as the protein secreted by osteosarcoma cells focuses attention on BGP itself as the unit of biological function. Some possible functions for this protein can accordingly be excluded. No protein of only 49 residues has yet been identified as an enzyme nor has a protein of this size been assigned a structural function. However, most hormones and chemotactic factors do have a low molecular weight. The presence of BGP in plasma (4, 8, 11) and the identification of BGP as a potent inhibitor of hydroxyapatite crystalization (2, 22) have led to the hypothesis that the protein may have a role in skeletal homeostasis (4, 11). The major hypothesis which has been suggested for the function of BGP is a role in biological mineralization (1, 2, 22). The abundance of BGP, approximately 1 molecule/hydroxyapatite crystal in bone (2), supports this view as does its only documented in vitro activity as a potent inhibitor of hydroxyapatite crystalization (2, 22). We have recently discovered that BGP is not present in fetal rat mineral (4, 8). We have also shown that rabbit bones which have only 5% of the normal BGP level have normal mineral content and normal strength (4). While...
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these results appear to preclude a role for BGP in mineralization itself, the coincidence of BGP appearance and the transition from fetal amorphous calcium phosphate to adult hydroxyapatite suggests that the protein may function in mineral maturation (8). The results of the present study are of pivotal importance to further investigations on BGP function since we can now safely focus on the properties of the 49-residue protein found in bone.

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REFERENCES