The Vitamin K-dependent Bone Protein Is Accumulated within Cultured Osteosarcoma Cells in the Presence of the Vitamin K Antagonist Warfarin*

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Osteosarcoma cells grown in normal culture medium secrete bone γ-carboxyglutamic acid protein (BGP, osteocalcin) which is identical with BGP purified from the bone matrix. Two tests indicate that the secreted medium protein contains the full complement of three γ-carboxyglutamate residues present on BGP purified from the bone matrix. First, the secreted protein from ROS 17/2 and bone matrix BGP have identical isoelectric points (pI = 4.6). Second, they have identical hydroxypapite binding behavior. If warfarin is added to the culture medium, the secreted protein has a higher isoelectric point (pI = 4.6) and a lower affinity for hydroxypapite characteristic of thermally decarboxylated or non-γ-carboxylated BGP. The observed shift in isoelectric point of secreted BGP after warfarin treatment from pH 4.0 to 4.6 is also reflected in the presence of pI = 4.1 and pI = 4.6 species intracellularly. These isoelectric species correspond to fully carboxylated BGP and nonecarboxylated BGP, which are in the process of secretion. Addition of 10 μg/ml of warfarin causes a specific 47% reduction in secretion rate of BGP, while at the same time, the intracellular BGP concentration increases 3-fold. These phenomena appear related to the interruption by warfarin of the normal sequence of processing of precursor BGP proteins, as a new, immunoreactive species with a higher isoelectric pH not present in control cells appears to be responsible for the increased intracellular antigen within warfarin-treated cells. Our results show that vitamin K-dependent processing is important for normal secretion of BGP from the cell.

Bone contains a protein of unknown function which has 3 residues of the vitamin K-dependent amino acid, γ-carboxyglutamic acid (1, 2). This protein has been named the bone Gla1 protein, also referred to as osteocalcin. Bone culture experiments show that bone is a site of BGP synthesis and that the newly synthesized BGP is fully γ-carboxylated (3). Comparison of the amino acid sequence of BGP's from several species has shown that the protein structure is highly conserved during evolution (4–8). While the appearance of Gla in bone appears to be closely related to the appearance of the bone mineral phase in all species studied, the appearance of immunoreactive BGP appears delayed (9–12). Although a definite role for BGP in mineralization has yet to be established, long-term vitamin K deficiency in rats results in an overmineralization of bone. On this basis, a role in preventing excess mineralization has been proposed (13).

A clonal rat osteosarcoma cell line which has many of the biochemical markers associated with osteoblasts can synthesize BGP (14, 15). The steroid hormone 1,25-dihydroxyvitamin D3 stimulates BGP synthesis by these osteosarcoma cells (16). BGP is initially synthesized by these cells as a 9,000-dalton intracellular precursor which is processed to the 5,800-dalton BGP prior to secretion from osteosarcoma cells (15).

Intracellular precursor forms have been described for a number of different secreted proteins, including prothrombin, a well-characterized vitamin K-dependent blood coagulation protein (17). The precursor to prothrombin has been reported to be processed through several intracellular stages prior to secretion from the cell (18–22). The radioimmunoassay for rat BGP has been used to study further the effect of the vitamin K antagonist, warfarin, on BGP synthesized by osteosarcoma cells and the relationship of γ-carboxylation to BGP synthesis and secretion. Warfarin has been used previously to deplete bone of γ-carboxyglutamic acid and BGP (5, 13, 23), and to inhibit the accumulation of newly synthesized BGP in cultured bone (3). We report here that during warfarin treatment an immunoreactive non-γ-carboxylated BGP can be secreted but at a lower rate. The lower rate of secretion of BGP from cells is accompanied by an intracellular accumulation of immunoreactive BGP. Evidence is presented for specific warfarin inhibition of an intracellular processing step from BGP precursor to BGP.

EXPERIMENTAL PROCEDURES

Materials—Urea, acrylamide, bisacrylamide, TEMED, and amphotelytes were obtained from Bio-Rad. Sephadex G-100 was purchased from Pharmacia, and 14C-[4,5-3H]leucine were purchased from Amersham. Trizma base and Nonidet P-40 were bought from Sigma. Fetal calf serum, Coons F12 medium, and antibiotic-antimycotic solution were bought from Irvine Scientific. Sodium warfarin was obtained from Endo Laboratories. Goat anti-rabbit-γ-globulin was from Calbiochem.

Osteosarcoma Cell Culture—Clonal rat osteosarcoma cells (ROS 17/2 cells, 14) were grown in Coons F12 medium supplemented with 10% fetal calf serum and 1% antibiotic-antimycotic solution. The cultures were incubated at 37°C in a humidified atmosphere of 90% air and 10% CO2. Medium was changed three to four times weekly and the cells were subcultured two to three times a week. Osteosarcoma cells grown in this medium had a doubling time of 1 to 2 days.
For measurement of the rate of BGP secretion from cells, each culture was grown to confluence in 60-mm culture plates and 5 ml of fresh media (Coons F12 with 10% fetal calf serum) were added. After 24 h, the media was again changed to Coons F12 with 2% fetal calf serum with or without warfarin, and the subsequent appearance of BGP in media was determined by triplicate radioimmunoassay of aliquots removed at appropriate times. Methods for purifying rat bone Gla protein, obtaining specific antiserum, and performing radioimmunoassays were described previously (11, 12, 15, 16). Cells were released by trypsin and washed sequentially in culture medium and phosphate-buffered saline, intracellular BGP was determined by radioimmunoassay after cell lysis in 6 M guanidine, 0.1 M Tris, pH 8.

Isoelectric Focusing—Isoelectric focusing of Sephadex G-100 purified media proteins was performed in 10% polyacrylamide gels with a pH range from 3 to 8. The ampholine mixture consisted of 50% 3:5, 32% 3:10, and 18% 4:6 with 0.1% Tween 20 (3). Proteins were focused at a constant 300 V for 14 to 18 h at 4°C. Parallel blank gels were run for pH determination. Isoelectric focusing of intracellular proteins was performed in 10% polyacrylamide gels with an ampholine mixture of 80% 3:10, 20% 2.5:4, with 9.5 M urea, 2% Nonidet P-40. Cells were prepared for focusing by trypsin-release from culture plates and sequential washing in culture medium and phosphate-buffered saline, then lysed in 9.5 M urea and 2% Nonidet P-40. For BGP measurement, 1- or 2-mm gel slices were placed in 0.5 ml of radioimmunoassay diluent (11, 15) and extracted for 18 to 24 h. In some experiments, calf BGP was used as an internal marker for isoelectric focusing because calf and rat BGP's have nearly identical isoelectric points. 20% polyacrylamide gel electrophoresis was performed as previously described (3).

Hydroxyapatite Binding—Binding curves consisted of 50, 5, 0.5, 0.05, 0.006, and 0 mg of hydroxyapatite added to 500 ml of culture medium from control, untreated rat osteosarcoma cells. The gels were adsorbed to hydroxyapatite at 4°C for 10 min. Hydroxyapatite binding was determined by triplicate RIA of 25 ml of supernatant medium to measure the amount bound to hydroxyapatite. Hydroxyapatite binding of purified rat BGP was determined in the identical uncultured medium by addition of a known amount of 125I-labeled rat BGP (5). The results in Fig. 2 are the results of three separate experiments.

RESULTS

Characterization of BGP Secreted by Warfarin-treated Osteosarcoma Cells—Medium BGP secreted by ROS 17/2 cells has been shown to be identical with BGP from rat bone in molecular weight and in electrophoretic mobility (15). We have used the specific radioimmunoassay for rat BGP together with isoelectric focusing gels and gel filtration chromatography to characterize the BGP secreted by normal and warfarin-treated ROS 17/2 cells. Fig. 1A shows that the isoelectric point of Sephadex G-100 purified BGP from the medium of ROS 17/2 cells is identical with the isoelectric point of bone matrix BGP (pI = 4.0). Additional evidence that the medium BGP is fully y-carboxylated comes from the identical high affinity hydroxyapatite binding behavior of bone matrix BGP and medium BGP secreted from control untreated ROS 17/2 cells (Fig. 2).

Medium BGP secreted by control ROS 17/2 cells or secreted by ROS 17/2 cells with added warfarin (10 lg/ml) elutes as a single low molecular weight peak of very similar size on Sephadex G-100 gel filtration (Fig. 3). Thus, the protein secreted by warfarin-treated cells has nearly the same molecular weight as the 5,800-dalton rat BGP. The small difference in the Sephadex G-100 elution position is consistent with the small change in elution position observed when the BGP has been thermally decarboxylated (24).

The isoelectric point and hydroxyapatite binding properties of medium BGP secreted by warfarin-treated cells are consistent with a non-y-carboxylated BGP. Cultures with 100 µg/ml of added warfarin secreted BGP with altered isoelectric points, with the major band having an isoelectric point of 4.6 (Fig. 1B). In addition, BGP from warfarin-treated culture medium exhibits a lower affinity for hydroxyapatite than that from control medium or purified rat BGP (Fig. 2). The conclusion that the BGP from warfarin medium is non-y-carboxylated is reinforced by the observation that thermally decarboxylated and non-y-carboxylated BGP exhibit the same decreased affinity for hydroxyapatite and the higher isoelectric point of 4.5 (5, 24, 25).

Effect of Warfarin on Secretion Rate and Intracellular Levels of BGP—Addition of 10 µg/ml of warfarin to culture media inhibits the secretion of BGP by ROS 17/2 cells (Fig. 4). Radioimmunoassay of culture medium after warfarin addition shows that cultures with added warfarin have only 53% of the control levels of medium BGP after 24 h in culture. The
Warfarin Inhibits Secretion of Bone Gla Protein

Fig. 3. Sephadex G-100 gel filtration of media proteins from warfarin-treated ROS 17/2 cells. Column (2 × 150 cm) buffer, 5 mM NH₄HCO₃, load, 10 ml of media, 5 °C. ••• at BGP; O—O, calf BGP added as an internal molecular weight marker. A, control media; B, media with 100 µg/ml of added warfarin.

Fig. 4. Secretion of bone Gla protein by ROS 17/2 osteosarcoma cells. BGP in medium was determined as described under "Experimental Procedures." Each data point is the average of triplicate culture plates assayed by RIA. O—O, control; ••••, 10 µg/ml of added warfarin. Arrow indicates the time of addition of warfarin.

Calculated secretion rate of BGP is 9.7 ng of BGP/h/10⁶ cells in control cultures, while the secretion rate is decreased to 4.0 ng/h/10⁶ cells after addition of warfarin. (The secretion rates were calculated as the difference in the amount of BGP between 4 and 24 h (Fig. 4).) A repeat experiment with five replicate plates for each time point gave nearly identical results, with warfarin-treated cells secreting 56% of controls. The degradation rate of BGP is negligible in control and warfarin-treated medium, since BGP added at 100 ng/ml at the start of the experiment results in secretion curves which are offset by approximately 100 ng at each time point.

A second effect of warfarin on ROS cells is the observed increase in intracellular BGP concentration (Fig. 5). After 13 h there was a nearly 3-fold increase in the intracellular BGP concentration when compared to control cells. Because cells are harvested by trypsin dissolution, any contribution from cell surface-associated BGP may be eliminated as trypsin rapidly destroys immunoreactivity of BGP.

In comparison, addition of up to 100 µg/ml of warfarin had only a small effect on the secretion or synthesis of total protein (Table I). Secreted [³H]leucine-labeled protein was inhibited by only 14%, while intracellular [³H]leucine-labeled protein increased by 15%. When warfarin was added at 333 µg/ml, significant differences in total protein synthesis and secretion were observed (Table I).

Effect of Warfarin on the Composition of Intracellular Immunoreactive Proteins—Previous studies have shown that a larger BGP precursor is present which is processed to BGP prior to secretion from the cell. Sephacryl S-200 gel filtration of intracellular BGP had revealed the presence of a higher molecular weight immunoreactive BGP (15). 20% polyacrylamide gel electrophoresis of the Sephacryl S-200 peak fraction showed that the higher molecular weight protein has a reduced electrophoretic mobility compared with secreted BGP or purified bone matrix BGP (15).

To show that intracellular proteins are not artifacts due to aggregation, or degradation of BGP during purification, the intracellular BGP precursors have been further characterized with an isoelectric focusing gel system to resolve total intracellular BGP species in a single step. Because denaturants

Table I
Effect of warfarin on protein synthesis

| Sample    | Media (ng/ml) | Cells (ng/ml) |
|-----------|---------------|---------------|
| Control   | 1.00          | 1.00          |
| Warfarin  |               |               |
| 3.3 µg/ml | 1.08          | 1.08          |
| 10 µg/ml  | 0.86          | 1.15          |
| 33 µg/ml  | 0.85          | 1.09          |
| 100 µg/ml | 0.86          | 0.93          |
| 333 µg/ml | 0.26          | 0.32          |
FIG. 6. Isoelectric focusing of total intracellular BGP proteins. Cells from confluent monolayers were lysed in 9.5 M urea, 2% Nonidet P-40 and the lysate focused on a 3.5-8.0 pH gradient in 9.5 M urea, 2% Nonidet P-40. A, control cells; B, cells treated with 100 μg/ml of warfarin. The numbers in the figure indicate the pH at that position in the gel.

(9.5 M urea) and detergents (2% Nonidet P-40) are present from the moment of cell lysis, neither aggregation nor proteolytic processing is likely to occur.

As shown in Fig. 6A, three major peaks of immunoreactive proteins are present with untreated control cell lysates. The three major peaks have isoelectric pH of 4.1, 5.4, and 5.6 on this gel system. The pI = 4.1 species is probably the fully γ-carboxylated BGP we reported within ROS cells (15), as the pI of 4.1 is very close to the pI of 4.0 expected for the 49-residue bone Gla protein. The pI = 5.4 and 5.6 species appear to be different isoelectric pH forms of the intracellular BGP precursor, as the 9,000-dalton precursor which migrated as a single band on 20% polyacrylamide gels resolved into 2 bands on nondenaturing isoelectric focusing gels (data not shown).

The addition of warfarin (100 μg/ml) causes a dramatic change in the pattern of isoelectrically focused intracellular proteins. BGP antigens in warfarin-treated cells band with isoelectric pH values of 4.6, 5.6, and 6.0, with the majority of the intracellular immunoreactive material comprised by a new peak with an isoelectric point of 6.0 (Fig. 6B). As expected, the putative γ-carboxylated pI = 4.1 species present in control cells is no longer present in warfarin-treated cells.

Because the experiment shown in Fig. 6 represents the total intracellular BGP from equal numbers of warfarin-treated and untreated cells, the relative concentrations of intracellular components can be compared. Control cells have 23.3 ng/10^7 cells of γ-carboxylated BGP (pI = 4.1), while warfarin-treated cells have no detectable peak at pH 4.0. Warfarin-treated cells do contain 1.6 ng/10^7 cells of a component at pH 4.6, an isoelectric pI which matches the isoelectric pH of the γ-carboxylated BGP (Figs. 1B and 6B). The other major effect of warfarin on osteosarcoma cells is on the putative BGP precursor protein with an isoelectric point of 6.0 (Fig. 6). All of the increased intracellular BGP in warfarin-treated cells seems to be due to the appearance of large amounts of this new component, because the concentrations of the other immunoreactive BGP species (pI = 4.6, 5.6) are not significantly elevated. The disappearance of the pI = 5.4 species and the appearance of elevated amounts of a pI = 6.0 intracellular precursor after warfarin treatment has been consistently observed in repeat experiments.

**DISCUSSION**

Comparison of our results with BGP and the reported biosynthetic processing of the vitamin K-dependent blood coagulation factor prothrombin reveals several similarities. Esmon et al. and Grant and Suttie have isolated intracellular precursors of rat prothrombin from rat livers (18, 19). More recently, Graves et al. have employed purified anti-prothrombin antibody to further characterize the precursor prothrombin in rat hepatoma cells (20, 22). These studies reported the sequence of processing events and the vitamin K dependence of intracellular precursor processing to prothrombin. They have observed analogous inhibition of secretion and intracellular accumulation of prothrombin precursors in vitamin K-deficient hepatoma cells in vitro (20, 22). Their results were also similar to results in our study because they found intracellular prothrombin precursors migrate as a single homogeneous band indistinguishable from prothrombin on sodium dodecyl sulfate-polyacrylamide gels, but these intracellular proteins can be resolved into 5 distinct precursor prothrombins by isoelectric focusing gels (22).

Evidence for a larger 9,000-dalton intracellular BGP precursor for the 5,800-dalton BGP have been previously described (15). This larger component migrated as a single homogenous band on 20% polyacrylamide gels. Precursor processing to BGP appears to occur intracellularly, because only the 5,800-dalton BGP was detected in the culture medium, whereas BGP as well as precursor could be detected inside the cell (15). This result is a departure from the observation on the prothrombin system where some of the intracellular precursors are found in the medium of rat hepatoma cell culture (22). Another difference in the osteosarcoma cell-BGP system is that proteolysis of prothrombin in media appears to be a major problem in the hepatoma cell culture, but this is not a problem in osteosarcoma cell culture, possibly due to the presence of fetal calf serum (15, 22).
In the present investigation, the characterization of BGP precursors has been continued using a procedure which enables visualization of the entire spectrum of immunoreactive intracellular proteins by a single step separation on a urea-Nondet P-40 isoelectric focusing gel followed by RIA. With this method two major intracellular BGP precursors with pI = 5.4 and 5.6 are found in urea-Nondet P-40 isoelectric focusing gels (Fig. 6A). It should be noted here that the purified 9,000-dalton putative precursor we reported previously (15) can be resolved into two major bands with similar isolectric points on non-denaturing isoelectric focusing gels. We believe that the two bands visualized by urea-Nondet P-40 isoelectric focusing are probably both precursor proteins of 9,000 daltons. In the absence of γ-carboxylation, precursors which are normally γ-carboxylated might be expected to shift to a more basic isoelectric point consistent with the loss of the 3 negatively charged carboxyl groups. Indeed, we have found that the majority of intracellular proteins in warfarin-treated cells is due to a new species with pI = 6.0 and that the pI = 5.4 band has disappeared (Fig. 6, A and B).

An obvious advantage of the ROS 17/2 osteosarcoma cell-thrombin system for studying the vitamin K-dependent γ-carboxylation and intracellular precursor processing is the easily detectable difference in size of the precursor BGP and BGP (20). If a short polypeptide sequence of 3,000 daltons or less carries the recognition signal for the vitamin K-dependent γ-carboxylase enzyme system, such a small polypeptide may be difficult to detect within the much larger 75,000-dalton BGP system in comparison to the H35 hepatoma cell-prothrombin system for studying the vitamin K-dependent γ-carboxylation and intracellular precursor processing is the easily detectable difference in size of the precursor BGP and BGP (20). If a short polypeptide sequence of 3,000 daltons or less carries the recognition signal for the vitamin K-dependent γ-carboxylase enzyme system, such a small polypeptide may be difficult to detect within the much larger 75,000-dalton BGP system. Indeed, all 5 of the intracellular precursor prothrombins migrate together on sodium dodecyl sulfate gels and it has been reported that the non-γ-carboxylated precursor prothrombin is the same size as the γ-carboxylated precursor based on identical sodium dodecyl sulfate gel mobility (20, 22). In the case of BGP, the precursor is a protein approximately 3,000 daltons larger than BGP, based upon its gel filtration mobility (15). One possible sequence of intracellular processing events is that the conversion of a larger 9,000-dalton species to the smaller 5,800-dalton BGP occurs after γ-carboxylation. For this model, a postulated signal peptide contained within a precursor may work to direct the γ-carboxylation event. We speculate that the pI = 6.0 material which accumulates and is present only in warfarin-treated cells may be the substrate for the γ-carboxylase enzyme.

Higher molecular weight Gla-containing antigenic proteins have been reported in extracts of chicken bone (26). The component which has an apparent molecular mass of 10,000 daltons may correspond to the intracellular peptides we report here.

At present, the characteristic of the precursor which confers upon it the increased apparent molecular weight is unknown. An amino acid signal sequence which targets proteins for γ-carboxylation has long been sought. Further studies on characterization of the intracellular events leading to secretion of BGP from cells will be necessary to answer these questions.

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