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CELL-FREE TRANSLATION OF THE VITAMIN K-DEPENDENT BONE PROTEIN OSTEOCALCIN

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The primary gene product of the vitamin K-dependent bone matrix protein, osteocalcin, has been identified by immunoprecipitation of cell-free translated proteins from 4 week rat calvariae mRNA preparations. Peptides of 9.8kd and 12kd, precipitated with a polyclonal affinity selected species specific antibody raised to purified rat osteocalcin, accounted for 1-2% of labelled proteins and were displaced by rat osteocalcin. These studies demonstrate that the 5800 molecular weight osteocalcin is synthesized as a precursor of approximately twice its size. The size of the propeptide, with a molecular weight of 4.3kd, is consistent with other known secreted vitamin K-dependent blood proteins.

Osteocalcin is extracted from bone as a small (M_r 5800 dalton) peptide (1,2). It is characterized by the presence of three γ-carboxylated glutamic acid (Gla) residues introduced by a vitamin K-dependent post-translational modification of glutamic acid residues. Although the precise function of osteocalcin is unknown, its calcium-binding properties (3), and stimulated synthesis by $1,25(OH)_2D_3$ (4,5) suggest an important regulatory role in bone.

In isolated osteoblast cultures (6) and tissue extracts (7) a higher molecular weight protein (~10,000), which is immunologically related to osteocalcin peptide, has been reported. We demonstrate here the identification of an osteocalcin precursor isolated from cell-free translation products which were produced using mRNA extracted from neonatal rat calvariae.

MATERIALS AND METHODS

RNA extraction. RNA was extracted by a modification of the procedure of Rowe et al. (8). Calvariae were removed from 4-week-old Sprague Dawley rats (Charles River, Wilmington, MA) and immediately frozen in liquid N_2. The collected bones were ground under liquid N_2 and the resulting powder suspended in 50mM EDTA, 10mM TRIS 1% sodium dodecyl sulfate pH 7.6, homogenized for 30 seconds with a Tekmar polytron (Cincinnati, OH) and digested with 300 ug/ml Proteinase K (Sigma Chemical Co., St. Louis, MO) at 50°C for 1 hr. Subsequent phenol-chloroform extraction was carried out and total nucleic acids were ethanol precipitated. Total nucleic acids were fractionated into RNA and DNA

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(9) by dissolution in 6M guanidine HCl to a final concentration of 400 ug/ml, followed by precipitation with 0.5 volumes of ethanol (-20°C). RNA was dissolved in 0.01M sodium acetate, 1mM EDTA, 10mM TRIS-HCl, 0.1% sodium dodecyl sulfate (pH 7.5), heated to 70°C for 1 minute, then adjusted to 0.5M NaCl. Oligo dT cellulose chromatography (Pharmacia P-L Biochemicals, Inc. Piscataway, NJ) was carried out as described by Bantle et al. (10). Yields of polyadenylated RNA, determined by optical density at 260nM, ranged from 20-30 ug/gram of bone and represented 2-3% of the total RNA.

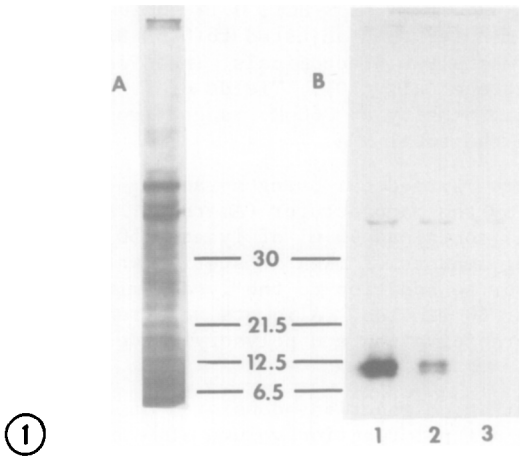
Cell free translation. Studies were carried out using a rabbit reticulocyte lysate kit as recommended by the manufacturer (Amersham, Arlington Heights, IL). Reaction mixtures (50 ul) contained 40 ul of lysate, 50 uCi L-[4,5-^{3}H] leucine 130 Ci/mmol and varying amounts of mRNA in sterile water. mRNA was heated to 70° for 1 minute prior to addition to the lysate mixture. Incubations were carried out at 30°C for 60 minutes. Aliquots were removed at 60 min for trichloroacetic acid (TCA) precipitation and polyacrylamide gel electrophoresis (11).

Immunoprecipitation. Immunoprecipitation reactions consisted of cell free translation products (30 ul) brought to a final reaction volume of 0.5ml in 50mM TRIS, 150mM NaCl, pH 7.6. Non-immune goat serum (Pel-Freez Biologicals, Rogers, AK) diluted 1:50 in the same buffer (25 ul) was added as carrier. Affinity purified goat anti-rat osteocalcin antisera, prepared by a previously reported procedure (12), was generously provided by Drs. Peter Hauschka and Thanos Mavrakos (Dept. of Human Biochemistry, Children's Hospital, Boston, MA). Reaction mixtures were incubated for 36 hours at 27°, with constant shaking. A second two hour incubation at 27° followed the addition of rabbit antigoat antisera (Cappel Laboratories, Cochranville, PA). Precipitations were collected through a 0.7M sucrose cushion and washed (13).

Polyacrylamide gel electrophoresis. Immunoprecipitated samples and labelled molecular weight markers were electrophoresed on 7-18% linear gradient polyacrylamide-SDS gels with a 4% stacking region (11). The gels were prepared for fluorography with Enhance (New England Nuclear) vacuum dried and autoradiographed for 24-48 hrs using Kodak X-Omat AR film.

RESULTS

Cell free translation of mRNA isolated from rat calvariae yielded a wide range of labelled proteins (Figure 1A). Incubation of these cell free translation products with the affinity purified anti-osteocalcin antisera typically resulted in precipitation of 1-2% of the total labelled proteins as determined by TCA precipitation. The results of immunoprecipitation of the translation products are shown in Figure 1B. Two major bands are present at 9.8kd and 12kd and one minor band is seen at ~50,000 kd. Addition of 100 ng of unlabelled osteocalcin to the immunoprecipitation mixture resulted in displacement of labelled protein from the major bands at 9.8kd and 12kd (Figures 1 and 2). Nonspecific antisera failed to precipitate labelled protein. The minor band seen at approximately 50kd co-migrated with the immunoglobulin fraction and results from nonspecific binding of labelled proteins to immunoglobulin (9).



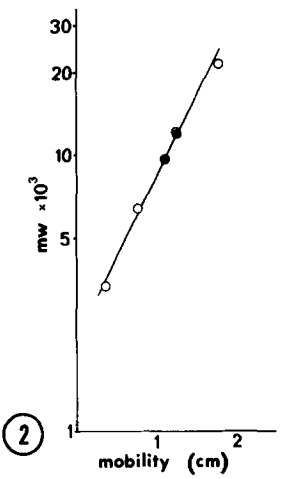


Figure 1: SDS polyacrylamide gel electrophoresis of in vitro translation products of mRNA isolated from rat calvariae. A is total in vitro translation products; B represents immunoprecipitation of in vitro translation products: Lane 1, with affinity purifed anti-rat osteocalcin antisera, Lane 2, with affinity purified antisera and 100ng unlabelled osteocalcin, Lane 3 with nonspecific antisera.

Figure 2: Molecular weights of immunoprecipitated peptides determined by mobility on SDS-polyacrylamide gel. [^{14}C]methylated molecular weight standards (Amersham, Arlington Heights, IL), soybean trypsin inhibitor (21.5kd), cytochrome C (12.5kd), aprotinin (6.5kd), and insulin chain B (3.4 kd) are shown as open circles. Immunoprecipitated osteocalcin bands, shown as solid circles, migrate to 9.8kd and 12kd.

DISCUSSION

These data show that in the rat the primary gene product of osteocalcin is approximately twice the size of the mature protein (5.8kd) which is extracted from bone, and to which our antisera was raised. This is in general agreement with a past report of a 9kd osteocalcin precursor (6) and more recent reports of 13.5kd (14) and 99 amino acid (15) precursors. That we have demonstrated peptides of both 9.8kd and 12kd which show strong immunocrossreactivity to osteocalcin is surprising and there are several possible explanations for these results: 1) translation of partially degraded mRNA, 2) slow elongation of the growing polypeptide or premature chain termination, 3) proteolysis of a longer peptide or 4) precipitation of immunologically similar but structurally distinct proteins.

Slow elongation or premature chain termination cannot be ruled out. However, the synthesis of a wide range of higher molecular weight proteins,

including procollagen, indicates that the mRNA extracted was of good quality and capable of translation. Likewise proteolysis cannot be excluded; however, the reproducibility of these results (n = 4) suggests that cleavage must occur consistently at a single site. Precipitation of structurally different but immunologically similar bone proteins remains a possibility. It is unlikely, however, that either of the immunoprecipitated bands represents the precursor of matrix Gla protein (16), a 9650d protein that has been reported to show some sequence homology to osteocalcin. If it is produced in a preproform as is osteocalcin, and other vitamin K-dependent proteins (17), its precursor size would be predicted to be greater than 13kd.

It is of interest that the leader sequence of osteocalcin precursor has been reported to show a degree of homology to prothrombin, clotting factors IX and X and protein C (17), other vitamin K-dependent proteins. This finding strengthens the hypothesis that the pro sequence in some way regulates γ-glutamyl carboxylation of these proteins. It is very unlikely, however, that our findings result from cross reactivity with these clotting proteins as our antisera was raised to osteocalcin, 5.8kd, which does not contain the leader sequence.

Although purely speculative, another possible explanation for the finding of two precursor forms is that post-transcriptional processing of osteocalcin mRNA results in two distinct mRNA forms. In the calcium field, RNA polymorphism has been described for calcitonin (18), and vitamin D-dependent calcium binding protein (19). Further studies to establish the relationship between these two immunoprecipitable proteins are underway.

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