Vol. 173, No. 1, 1990 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1990 Pages 471-479

# COMPARISON OF TWO PHOSPHOPROTEINS IN CHICKEN BONE AND THEIR SIMILARITIES TO THE MAMMALIAN BONE PROTEINS, OSTEOPONTIN AND BONE SIALOPROTEIN II

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Received October 23, 1990

Summary. Two phosphorylated proteins of ~66 kDa and ~60 kDa mass with different DEAE-Sephacel elution patterns were isolated from chicken bone and were shown to be genetically distinct by both biochemical and immunological analysis. A tryptic peptide from the 60 kDa protein was identified that was similar to a sequence of the rat bone sialoprotein II. Both proteins showed RGD inhibited cell-attachment with the MG-63 osteosarcoma cell, and the ~66 kDa phosphoprotein appeared to promote cell adhesion better than human vitronectin. The two phosphoproteins appear to share functional and biochemical characteristics and to be homologous to the mammalian bone phosphoproteins, osteopontin and bone sialoprotein II.


Phosphorylated noncollagenous proteins are found in all vertebrate mineralized tissues and have been hypothesized to play a crucial role in initiating or controlling the spatial distribution of mineral in the extracellular matrix (1,2,3). For chicken bone, numerous phosphorylated polypeptides, ranging in molecular weight from 6 kDa to 150 kDa, containing both O-phosphothreonine (Thr-P) and O-phosphoserine (Ser-P) residues have been extracted from bone and purified to homogeneity (4,5,6,7). Partial $NH_2$-terminal amino acid sequence and immunological analysis using both monoclonal and polyclonal antibodies developed against several of these polypeptides, demonstrated that many of the smaller molecular mass species were derived from common precursors. These data further suggested that at least two genetically distinct phosphoproteins were present in chicken bone (7,8,9). Subsequent work demonstrated that the primary

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0006-291X/90 $1.50

gene product of one of these species was a glycosylated phosphoprotein with an apparent molecular mass of ~66 kDa (9). Partial protein sequence analysis and the complete primary structure of this ~66kDa BPP deduced from the cDNA sequence demonstrated that this protein has a ~37% protein similarity with the mammalian bone phosphoprotein, osteopontin (10,11,12,13).

A second phosphorylated protein of ~60 kDa has now been isolated. The DEAE-Sephacel elution characteristics, amino acid composition, sequence analysis, and immunological characteristics of this protein demonstrated that it was genetically distinct from the ~66 kDa phosphoprotein. It appeared to be be similar to the rat and human bone phosphoprotein, sialoprotein II (BSP II) (14, 15, 16). Functional cell binding studies showed that both proteins promoted integrin mediated cellular adhesion.

## Methods and Materials

Phosphoprotein Extraction and Purification from Tissue.
Non-collagenous proteins were extracted from 14 wk old chicken long bones by mild 0.3 N HCl extraction as previously described or from cultures of embryonic osteoblasts. (9,17). Preliminary chromatography on DEAE sephacel and molecular sieving on Sephacryl S-300 (7,9) was used to isolate both of the major phosphoproteins. Protein purity was determined by amino terminal sequence analysis in either of two ways: directly on samples obtained from filtration through Sephacryl S-300 for the ~66 kDa phosphoprotein; or after subsequent preparative, SDS (18) polyacrylamide electrophoresis of ~1.2 mg using a 5-10% continuous-gradient gel of the ~60 kDa phosphoprotein (18). Phosphoproteins were specifically detected on SDS-polyacrylamide gels by staining with Rhodamine B (7,19). Complete tryptic digestion was performed after reduction and alkylation, then resolved by HPLC on a C18 column, as described previously (9). All sequence analyses were carried out on a protein sequencer (ABI 470A Applied Biosystems, Inc. CA) equipped with an on line ABI 120A HPLC at the Microchemistry Laboratory, Harvard University, Cambridge, MA. Thrombin cleavage of 20-40 $\mu$g of purified 66 kDa and 60 kDa phosphoproteins was carried out with 0.9 units of human plasma thrombin (Sigma, Biochemicals St. Louis, MO) in 50 $\mu$l of 50 mM Tris Acetate pH 70 at 37$^{o}$ C for 45 minutes. Total amino acid analysis and Ser-P and Thr-P contents were determined as previously described (5). Total protein extracts from cultured embryonic chicken osteoblasts were prepared as previously described (8).

Antibody Production and Characterization. Polyclonal antibody to the ~66 kDa phosphoprotein, was prepared as described (20). The 60 kDa antibody was prepared from protein purified by DEAE-Sephacel chromatography followed by preparative SDS - polyacrylamide electrophoresis. The protein bands were excised from the gel, ground to fine particles, and mixed with 1 ml of Freunds complete adjuvant and 1 ml of PVP. Rabbits were subsequently inoculated using the schedule developed by Gundberg et al. (21) for the generation of antibodies to osteocalcin. Western blot analysis and antigen detection have been described (20).

Cell Adhesion Assay The direct attachment assays were carried out as described (22) using freshly trypsinized human osteosarcoma fibroblasts (MG-63).

Briefly, 100 $\mu$L of a single-cell suspension containing $10^4$ cells was placed in a flat-bottom microtiter well that had been previously coated with serial dilutions of either human vitronectin, the 60 kDa, or the 66 kDa chicken phosphoproteins. After one hour at 37°C, unattached cells were washed away and attached cells were fixed and stained. In the inhibition of attachment assays, soluble peptides were added to the medium as previously described (23) and the wells were coated with 3 $\mu$g/ml of the three proteins being tested. The exact peptide content of the stock solutions used for the inhibition of adhesion assays were determined by quantitative amino acid analysis. At the end of each assay, adherent cells were stained with 0.5% o-Toluidine Blue dissolved in 3% paraformaldehyde, the excess stain was washed away with water, and the cells were then destained with 1% SDS and the color quantitated with a vertical pathway spectrophotometer at 600nm.

## Results and Discussion

In order to determine if phosphorylated proteins were present in the bone protein extracts, both the A280 and A830 profiles of the DEAE-Sephacel chromatography were monitored as a preliminary means of detecting both the total protein and phosphate containing protein peaks (Figure 1A). Two peaks were identified eluting at 0.25- 0.30 M and >0.3 M of the NaCl concentration gradient. A representative SDS-polyacrylamide-gel electrophoresis analysis of fractions across the DEAE-Sephacel chromatograph showed that peaks D and E contained prominent protein species of 60-70 kDa. The high relative abundance of the two phosphoprotein containing peaks relative to peak C in which osteocalcin elutes, suggest on a per mass ratio that these proteins are present in bone (Figure 1B) in at least equal quantities to osteocalcin. The DEAE-

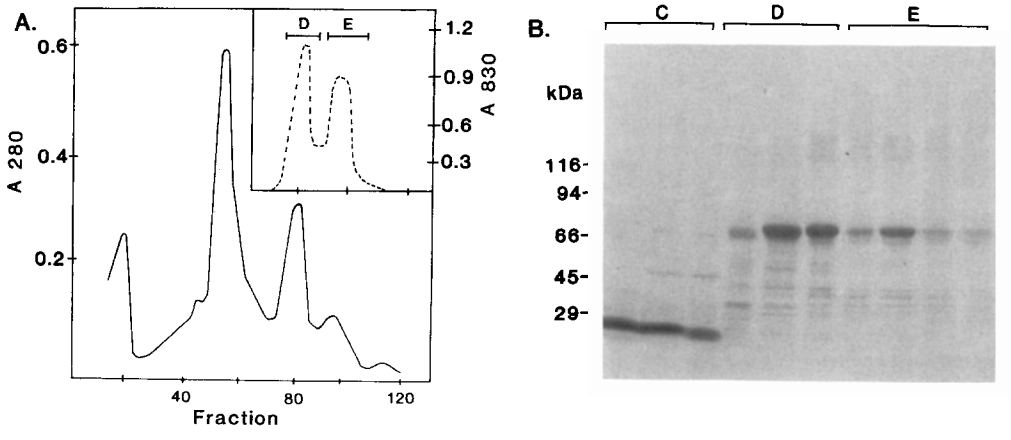



Figure 1. DEAE-chromatographic profile of the acid extractable non-collagenous proteins of bone. A) Optical density profile of total protein A280. Inset shows optical density profile of phosphate containing proteins at A830. B) SDS-polyacrylamide gel analysis of selected fractions from peaks C, D and E.

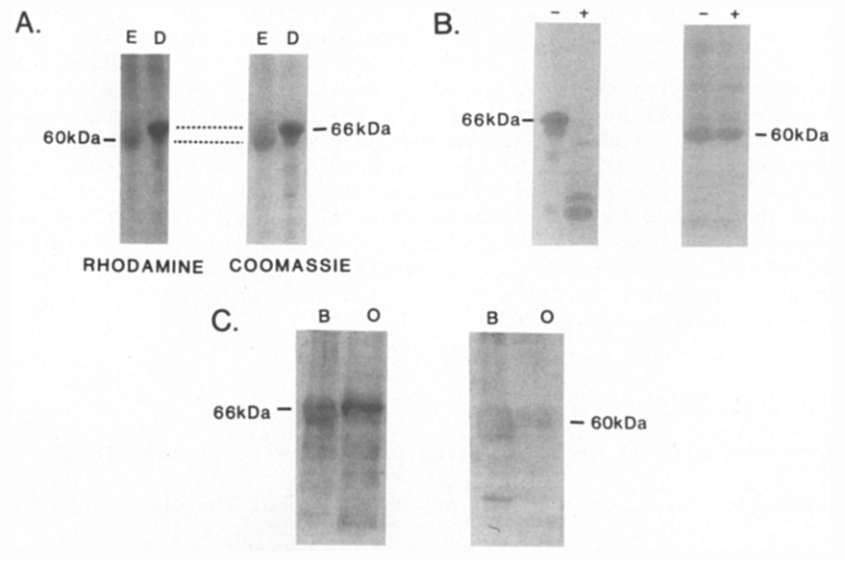



Figure 2. Biochemical and Immunological characterization of the DEAE-chromatographic peaks D and E. A) Demonstration of protein phosphorylation of the pooled fractions of peaks D and E by specific staining with Rhodamine B. B) Demonstration of thrombin proteolysis of the 66 kDa phosphoprotein but resistance of the 60 kDa phosphoprotein to cleavage under identical enzymatic treatment; (-) denotes no enzyme; (+) addition of enzyme. C) Antibody specificity of polyclonal antibody developed against ~66 kDa or ~60 kDa to either total acid extracted protein in bone (B) or total protein extracted from 30 day embryonic osteoblast cell cultures (O). Note lower levels of ~60 kDa protein accumulated in osteoblast cell cultures.

Sephacel fractions corresponding to peaks D and E were pooled as denoted in Figure 1B. Subsequent purification of the major 60-70 kDa protein species of peaks D and E was carried out by one round of Sephacel S-300 chromatography. The resultant protein species were examined by SDS - polyacrylamide gel electrophoresis and stained with either Coomasie Blue or Rhodamine B which is specific for phosphorylated protein (7,19). Two proteins of ~66 kDa and ~60 kDa were detected and their phosphorylated nature was confirmed by Rhodamine B staining (Figure 2A.) Table I shows the amino acid composition of the two proteins. While both proteins have approximately the same Ser-P and Thr-P contents, each appears to be compositionally unique. The higher Asx and Glx contents of the ~60 kDa protein and its later DEAE-Sephacel elution would suggest that it has a greater acidic amino acid content. It is interesting to note the similiarity of the respective amino acid contents of the ~66 kDa and

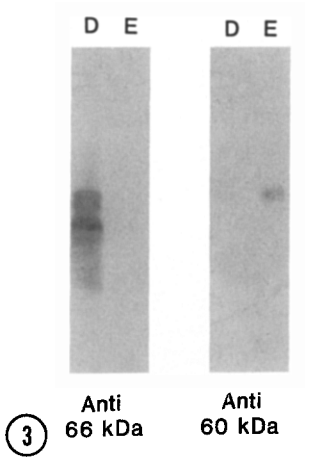
Table I. Comparison of Amino Acid Compositions of the ~66 kDa and ~60 kDa Chicken Phosphoproteins with Rat-Bone Phosphoproteins

| Residues/1000 | 66 BPP kDa | 60 BPP kDa | Osteopontin[a] | BSP II |
|---|---|---|---|---|
| Asn + Asp | 175 | 132 | 166 | 113 |
| Thr | 36 | 36 | 37 | 76 |
| Ser | 92 | 92 | 148 | 60 |
| Gln + Glu | 165 | 265 | 163 | 235 |
| Pro | 51 | 53 | 48 | 36 |
| Gly | 51 | 102 | 20 | 103 |
| Ala | 88 | 91 | 68 | 86 |
| Val | 83 | 40 | 44 | 40 |
| Met | 0 | 0 | 7 | 7 |
| Ile | 14 | 16 | 24 | 10 |
| Leu | 42 | 31 | 54 | 33 |
| Tyr | 15 | 15 | 17 | 79 |
| Phe | 20 | 7 | 20 | 23 |
| His | 41 | 8 | 51 | 17 |
| Lys | 58 | 31 | 58 | 50 |
| Arg | 54 | 53 | 31 | 33 |
| P-Ser | 15 | 25 | 38 | N.D. |
| P-Thr | 3 | 3 | 4 | N.D. |
| P(%) | 2.6 | N.D. | 4.4 | 0.5 |
| Molecular Mass[c] | 66 kDa | 60 kDa | 60-70 kDa | 60-70 kDa |
| Residues[d] | 265 | N.D. | 317 | 302 |

[a]Data for osteopontin is from Oldberg et al. (24).
[b]Data for bone sialoprotein II is from Oldberg et al. (16).
[c]Molecular masses are estimated based on migration on a polyacrylamide gel in comparison to globular molecular weight standards.
[d]Residues per molecule are based on cDNA sequence analysis.
N.D. = not determined.

~60 kDa phosphoproteins to the mammalian bone phosphoproteins osteopontin and bone sialoprotein II (13,14,15,16,24).

A series of experiments were performed to further determine if the ~66 kDa and the ~60 kDa phosphoproteins were genetically distinct proteins. In previous experiments both rat and human osteopontin had been shown to be susceptible to proteoloysis by thrombin (25,26). Both the ~66 kDa and ~60 kDa phosphoproteins were subjected to thrombin treatment under identical conditions; however, only the ~66 kDa protein showed cleavage (Figure 2B). Polyclonal antibodies were developed in rabbits against both the ~66 kDa and the ~60 kDa phosphoproteins. Initial experiments compared the immunoreactivity of these polyclonal antibodies against total proteins extracted from either 14 wk chicken bone or the proteins synthesized by embryonic osteoblast culture (Figure 2C). While the ~66 kDa antibody shows a strong reaction against proteins from both the post embryonic bone and embryonic osteoblast cultures, the ~60 kDa antibody reacts strongly only with the proteins extracted from the



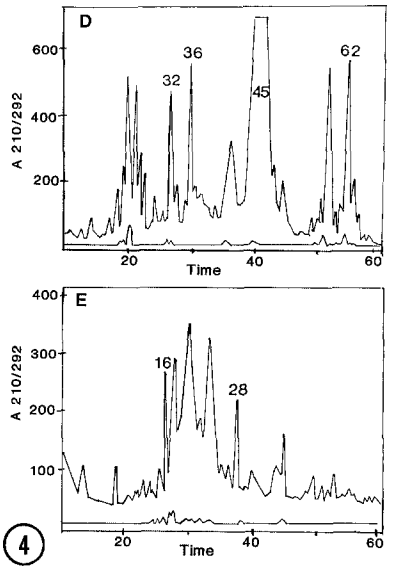




Figure 3. Immunological cross reactivity of ~66 kDa and ~60 kDa phosphoproteins. Equal quantities of DEAE-peaks D and E were resolved by SDS-polyacrylamide gel electrophoresis and subjected to Western blot analysis with either the polyclonal antibody to the ~66 kDa phosphoprotein or the ~60 kDa phosphoprotein. The protein fraction and antibody used in each reaction is denoted in the figure.

Figure 4. Tryptic peptide analysis of DEAE-peaks D and E. Each DEAE peak was subjected to further purification to homogeneity as described in the materials and methods. The purified ~66 kDa and ~60 kDa phosphoproteins were then subjected to complete tryptic digestion and the resultant fragments resolved by HPLC on a C18 column. The numbered peaks were those selected for sequence analysis and are summarized in table II.

bone of mature chickens. However it is present at low levels in embryonic osteoblasts. Similarly, the ~66 kDa antibody shows reaction with several discrete smaller molecular mass proteins shown to be proteolytic fragments (9), while the ~60 kDa reacts predominantly with one protein species of ~60 kDa. The cross reactivity of each antibody to the separate phosphoprotein species was also tested (Figure 3). Neither antibody cross reacted with the other's antigen, demonstrating that each protein appeared to be immunologically unique.

To confirm that each of the phosphoproteins was genetically separate, both amino terminal sequence analysis and internal sequence analysis of tryptic fragments were performed. The tryptic digestions of the two phosphoproteins produced singular HPLC profiles (Figure 4). A summary of the sequence analysis is presented in table II. Comparison of these data for both the amino terminal sequence and those tryptic peptides which had similar elution times,

Table II. Comparison of Sequence Analysis of the Two Major Chicken Bone Phosphoproteins to Rat Bone Phosphoproteins

| Derivation of Sequence | | Cycle .....5.....10.....15.....20.....25 |
|---|---|---|
| N-terminal (chicken) tryptic peptides | 66 kDa | W-P-V-S-K-S-R-Q-H-A-I-S-A- |
| \| | 32 | -G-D-S-V-A-Y-G-F-R- |
| \| | 33 | -E-P-L-A-X-X-S-X-V-D-T-S-N-Q-X-L-E-S- |
| \| | 45 | -Y-H-Q-D-H-V-D-S-Q-S-Q-E-H-L-Q-X-T-X-N-D- |
| \| | 62 | -K-L-I-E-D-D-A-T-A-E-V-G-D-S-Q-L-A-G-L-W-L-P-K- |
| N-terminal (chicken) tryptic peptides | 60 kDa | D-D-P-S-V-F-D-S-L-G-G-R-H-R-R-S-E-G-T-S-G-X-P- |
| \| | 16 | -Y-A-Y-P-P-L-H-R- |
| \| | 28 | -D-D-P-S-V-F-D-S-L-G-G-R- |
| N-terminal (rat) osteopontin internal sequence osteopontin 143-153 | | L-P-V-K-V-A-D-F-G-S-S-D-D-K-A-H- |
| | | -G-R-G-D-S-L-A-Y-G-L-R- |
| N-terminal rat Bone Sialoprotein II internal sequence BSP II 51-61 | | F-S-M-K-N-F-H-R-R-I-K-A-D-E-S- |
| | | -Y-F-Y-P-P-L-K-R-F-P |

Undetermined amino acids are denoted by X. Rat sequences are from Oldberg, et al. (16,24), while chicken sequences for the ~66 kDa phosphoproteins are from Gotoh et al (9).

failed to detect any common sequences between the two proteins. An internal sequence of the ~60 kDa phosphoprotein was identified (tryptic peptide 16) that was identical to the sequence found in the rat bone phosphoprotein, sialoprotein II (16).

A recognized biological characteristic of both the mammalian bone proteins osteopontin and bone sialoprotein II, is their ability to promote integrin mediated cell adhesion, due to the presence of an RGD sequence (16,24,27,28,29). In order to test the ability of the two chicken phosphoproteins to promote RGD mediated cellular adherence, their ability to promote cellular attachment was tested. These results are summarized in Figures 5A and B. The attachment of cells to the 66 kDa and 60 kDa phosphoproteins exhibited patterns similar to human osteopontin and bone sialoprotein II when compared to the attachment of human vitronectin (B). Similarly, the RGD containing peptide inhibited cell attachment to all three proteins while the non-RGD peptide had no effect on attachment, suggesting an integrin mediated mechanism of cell adhesion (28).

In summary, the data presented here confirm previous reports that at least two genetically distinct phosphoproteins are present in chicken bone. Preliminary sequence data and comparison of the two proteins' biological

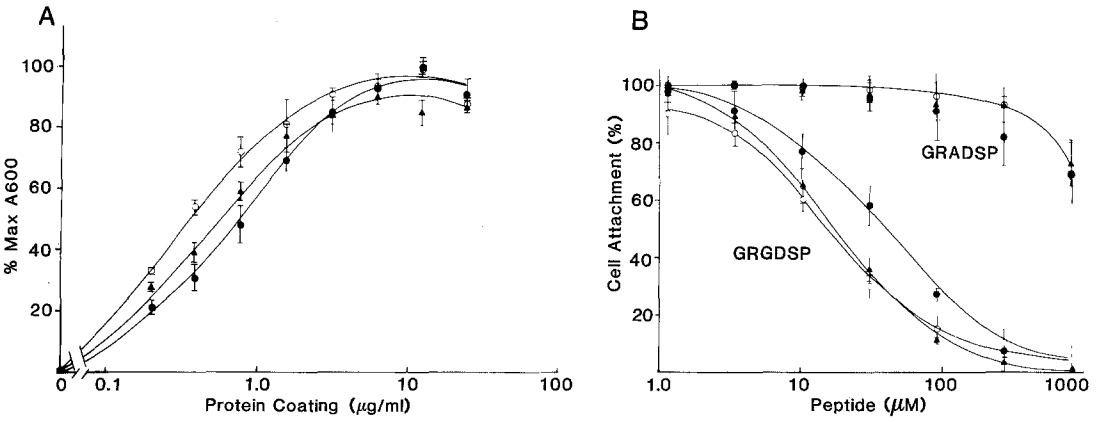



Figure 5A. Cell Attachment Assays. MG-63 cell attachment to vitronectin (▲), 66 kDa (o) or 60 kDa (●) chicken phosphoprotein coated microtiter wells at the indicated coating concentrations was determined. The assays were performed with 1.2 x $10^4$ cells/well.

Figure 5B. Inhibition of cell attachment to immobilized 66 kDa phosphoprotein (o), 60 kDa phosphoprotein (●) or vitronectin (▲) by the synthetic peptide GRGDSP. Microtiter wells were coated at 3 μg/mL with the test proteins and MG-63 cell attachment in the presence of the indicated concentrations of soluble peptides was determined. The assay was performed with $10^4$ cells/well and the $A_{600}$ of the ligand coated wells with no peptide added were set at 100%. The data represent the mean of four experiments with standard deviations indicated.

activities suggest that these proteins are the avian homologues to the mammalian proteins, osteopontin and bone sialoprotein II.

## Acknowledgments

The authors wish to acknowledge Dr. William Lane for assistance with the protein sequencing. This research was funded in part from the National Institutes of Health (HD 22400 and AM 34078), the Peabody Foundation, Inc. and an Institutional Grant from the Orthopaedic Research and Education Foundation, funded by the Bristol-Myers/Zimmer Corporation.

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