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IDENTIFICATION OF TWO PHOSPHORYLATION MOTIFS IN BOVINE OSTEOPONTIN*

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SUMMARY Fourteen phosphoserines and one phosphothreonine have been localized in a partial amino acid sequence of bovine milk osteopontin. Twelve of the phosphoserines are located in a Ser-X-Glu/Ser(P) sequence motif, suggesting that the phosphorylations are catalyzed by the mammary gland casein kinase. Two phosphoserines were found not to be located in a mammary gland casein kinase recognition sequence. Instead these two phosphoserines were located in the motif Ser-X-X-Glu which is a recognition sequence for casein kinase II. These data indicate that there might be more than one kinase active in the phosphorylation of osteopontin isolated from bovine milk. Furthermore, the serine in the cell-binding sequence Arg-Gly-Asp-Ser was shown not to be phosphorylated.

Osteopontin is an acidic glycoprotein rich in aspartic acid, glutamic acid and serine, where many of the serines carry an O-linked phosphate group. Osteopontin was first isolated from the mineralized matrix of bovine bone (1). Since then it has been characterized in tissues and cells from several species, including man (2), rat (3), mouse (4), pig (5) and chicken (6).

In bone, osteopontin is synthesized by preosteoblasts, osteoblasts and osteocytes, and secreted into the osteoid and subsequently incorporated in the bone (7,8). The expression at an early developmental stage indicates that osteopontin might be involved in the initiation of the bone calcification process (9). In addition to bone cells, osteopontin is synthesized by neurons in the inner ear (9), as well as osteopontin mRNA has been detected in kidney distal tubular cells, uterus, lung, brain and epidermis (10,11,12).

Osteopontin is also present in physiologic fluids such as urine (13,14,15) and milk (16). In urine it is the major inhibitor of precipitation of calcium salts especially calcium oxalate

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Abbreviations used: MGCK: mammary gland casein kinase; PTH: phenylthiohydantoin; HPLC: high-performance liquid chromatography; RGDS: Arg-Gly-Asp-Ser.

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(13,14,15). The major phosphorylated proteins in milk are the caseins where all phosphate groups are attached to serines or threonines found in the sequence motif Ser/Thr-X-Glu/Ser(P) (17). The same applies to the five phosphate groups in the milk protein PP3 (18). This suggests that the mammary gland casein kinase (MGCK) has a high degree of specificity in the selection of phosphorylation sites.

The phosphorylation of osteopontin is thought to be important in the interaction with calcium and calcium phosphate. Osteopontin isolated from rat bone is reported to contain 12 phosphoserines and one phosphothreonine (19), but the phosphoamino acids of osteopontin have not yet been localized in the amino acid sequence of any species. Recently, we have isolated osteopontin from bovine milk (20). In the present paper we describe the localization of one phosphothreonine and 14 phosphoserines in peptides covering 47% of the osteopontin sequence. Two of the phosphoserines are not located in the sequence recognition motif of the mammary gland casein kinase.

MATERIALS AND METHODS

Materials Endoproteinase Lys-C was obtained from Boehringer Mannheim (6800 Mannheim, Germany). Vydac C_{18} (10 μm) was from The Separations Group (Hesperia, CA 92345). Reagents used for sequencing were purchased from Applied Biosystems (Foster City, CA 94404). All other standard chemicals used were of analytical grade.

Peptide generation Osteopontin, isolated from bovine milk (20) was digested with endoproteinase Lys-C (EC 3.4.21.40) using an enzyme:substrate ratio of 1:250 (w/w) in 0.1 M ammonium bicarbonate, pH 8.0, at 37 °C for 18 h. A trypsin (EC 3.4.21.4) digest of osteopontin was performed using an enzyme:substrate ratio of 1:100 (w/w) in 0.1 M ammonium bicarbonate, pH 8.0, at 37 °C for 6 h. A peptide generated by the endoproteinase Lys-C digestion (peptide 5, Fig. 2) was further digested with thermolysin (EC 3.4.24.2) at a concentration of 10 μg/ml in 0.1 M pyridine-acetate, pH 6.5, 5 mM $CaCl_2$ at 55 °C for 3 h.

Isolation of peptides Peptides from the digests were separated by reverse-phase HPLC on a Vydac C_{18} column. The separation was carried out on a Pharmacia LKB system (Pharmacia, S-75128 Uppsala, Sweden), consisting of a 2248 LC gradient pump connected to a 2252 LC controller and a 2510 Uvicord SD detector equipped with a 226 nm filter and a flow cell with a 2.5 mm path length. The peptides were separated in 0.1% trifluoroacetic acid and eluted with a gradient of acetonitrile developed over 50 min (0-5 min: 0% B; 5-40 min: 0-50% B; 40-50 min: 50-95% B) at a flowrate of 0.85 ml/min. Peptides were detected in the effluent by measuring the absorbance at 226 nm. The peptides were repurified by reverse-phase HPLC under the same conditions as described above, except that this time 0.05% heptafluorobutyric acid was used as the ion-pairing agent.

Amino acid composition analysis Phosphoserine/-threonine containing peptides were detected by short time hydrolysis and subsequent amino acid analysis. Peptides were hydrolysed under vacuum at 110 °C for 2 h in the presence of 6 M HCl, 0.05% phenol, 1% thioglycolic acid. The amino acids were analyzed essentially as described by Barkholt & Jensen (21).

Phosphoserine conversion Peptides containing O-linked phosphate were treated with ethanethiol to convert phosphoserine into S-ethylcysteine. The conversion was performed as a β-elimination followed by the addition of ethanethiol, essentially as described by Meyer et al. (22).

Amino acid sequence analysis Amino acid sequence analysis was performed on an Applied Biosystems model 477A sequencer with on-line identification of the phenylthiohydantion (PTH)

derivatives using an Applied Biosystems 120A HPLC. PTH-S-ethylcysteine was identified by amino acid sequencing, as a peak eluting at 21.40 minutes, just before the diphenylthiourea peak in the system used.

RESULTS AND DISCUSSION

Osteopontin was purified from bovine milk as described (20). Peptides were obtained by endoproteinase Lys-C digestion and separated by reverse-phase HPLC (Fig. 1). Phosphopeptides were detected by amino acid analysis and further purified. To localize the phosphoserines directly by amino acid sequencing, these residues were converted into S-ethylcysteine by ethanethiol treatment (22). None of the peptides examined contained carbohydrate.

At present we have localized one phosphothreonine and 14 phosphoserines in osteopontin (Fig. 2). Twelve of the identified phosphoserines are located in a Ser/Thr-X-Glu/Ser(P) sequence motif, suggesting that the phosphorylations are catalyzed by the mammary gland casein kinase (MGCK) (17). Ser(P)7 and Ser(P)168 are not located in the MGCK recognition sequence, but these phosphoserines are both situated in Ser-X-X-Glu/Ser(P) sequences. This sequence may form a recognition site for another kinase, casein kinase II (23).

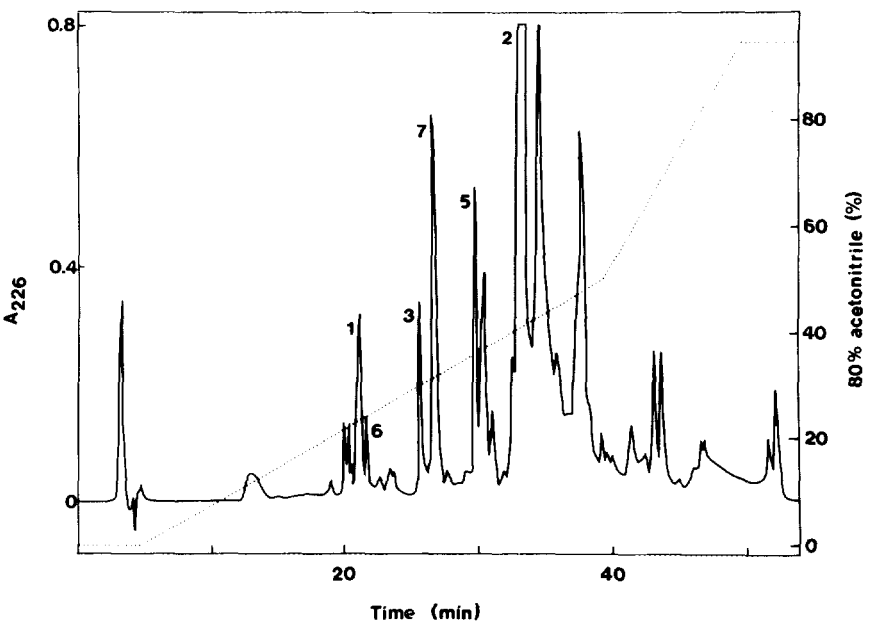

Fig. 1. *Reverse-phase HPLC separation of peptides obtained from osteopontin by digestion with endoproteinase Lys-C.* Peptides were eluted with a gradient of 80% acetonitrile in 0.1% trifluoroacetic acid (dotted line) on a Vydac C_{18} (10 μm) column (4 x 250 mm). The column was operated at 40 °C and the flow rate was 0.85 ml/min. Peptides were detected in the effluent by recording the absorbance at 226 nm (solid line). Phosphate containing fractions were identified by amino acid analysis as described in materials and methods. The peaks are numbered according to Fig. 2.

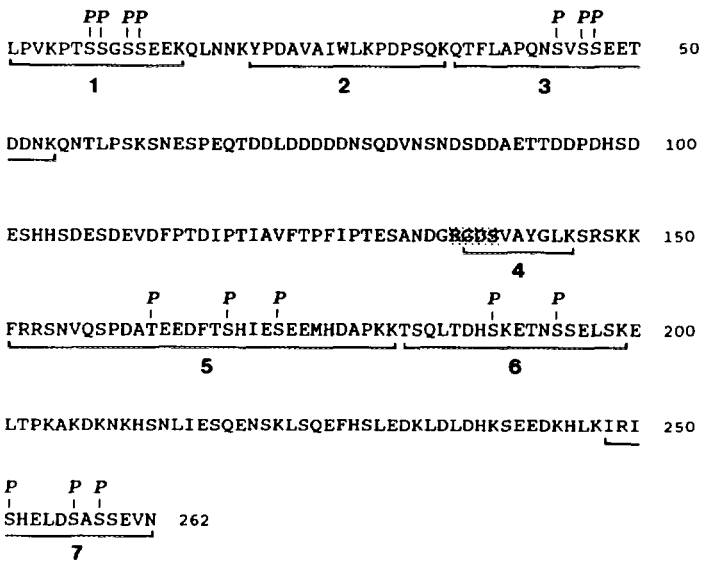

Fig. 2. *Localization of phosphoserines in bovine milk osteopontin.* The amino acid sequence was deduced from the cDNA sequence of bovine osteopontin according to Kerr et al. (24), with the exception that Thr40 has been substituted with an alanine residue. Solid lines indicate isolated and characterized peptides. Peptides are numbered as indicated. P denotes identified phosphorylation. The RGDS-peptide involved in cell-attachment is marked (hatched box).

In the amino acid sequence of the bovine osteopontin (24) a total of 23 serine residues are located in a MGCK recognition sequence. The present work covers thirteen of these serine residues. Twelve are found to be phosphorylated as expected, but ethanethiol treatment of Ser198 gave no ethylcysteine indicating that this serine residue, though located in the MGCK recognition sequence, is not phosphorylated.

Special attention has been paid to Ser139 which is part of the RGDS cell-binding sequence. The presence of this serine could imply a site that, when phosphorylated, alters the function of the protein, perhaps precluding or enhancing cell-binding (25). In an earlier study on osteopontin structure we have generated tryptic peptides. Sequencing of one of these peptides (peptide 4, Fig. 2) representing residues 137-145 gave a normal yield of Ser139 showing that this serine residue is not phosphorylated in milk osteopontin.

Phosphothreonine residues are not converted by the ethanethiol treatment and have to be localized by other methods. Bovine osteopontin contains 18 threonines, of which three (Thr6, Thr162 and Thr192) are located in a MGCK recognition sequence. Amino acid sequencing of peptides 1 and 6 (Fig. 2) gave expected yields of PTH-threonines in cycles corresponding to Thr6 and Thr192, respectively, showing that these threonines are not phosphorylated. Sequencing of peptide 5 (Fig. 2) gave no PTH-threonine in the cycle 12, suggesting that Thr162 is phosphorylated. To verify this assumption, peptide 5 (Fig. 2) was subjected to thermolysin

digestion and the resulting peptides were characterized. A peptide corresponding to residues 157-165 was identified by amino acid sequencing. Subsequent mass spectrometric analysis of the peptide verified the presence of a phosphothreonine at position 162 in bovine milk osteopontin (data not shown). The phosphorylations of all the characterized peptides were confirmed by mass spectrometric analysis.

A total of 12 phosphoserines and one phosphothreonine has been estimated present in rat bone osteopontin (19). Approximately one half of the serine residues in the amino acid sequence of bovine milk osteopontin have been examined resulting in the identification of 14 phosphoserines. This result indicates that milk osteopontin has a higher content of phosphorylated amino acids than bone osteopontin. To elucidate this difference in phosphorylation between milk osteopontin and the bone counterpart further studies are needed.

It has been shown that recombinant mouse osteopontin is able to autophosphorylate tyrosine residue(s) (26) and protein-bound sulphate has been reported present in rat bone marrow osteopontin (27). This sulphate was not removed by deglycosylation indicating the presence of a sulphated tyrosine in mineralized osteopontin. In bovine osteopontin two tyrosine residues are present at positions 20 and 142 (peptides 2 and 4, Fig. 2). Sequencing of peptides 2 and 4 gave expected yields of PTH-tyrosine in cycles corresponding to Tyr20 and Tyr142, respectively, indicating that the tyrosines of bovine milk osteopontin are not modified.

Bovine osteopontin contains three aspargine residues (Asn63, Asn85 and Asn93) located in the putative glycosylation sequence Asn-X-Ser/Thr. Amino acid sequencing of peptide 6 (Fig.2) gave a normal yield of PTH-aspargine in cycle 12, showing that Asn93 is not glycosylated in bovine milk osteopontin.

Uropontin, a protein identical to osteopontin has recently been isolated from human urine (13,14,15). Experiments have shown that a uropontin concentration of approximately 0.1 μM is sufficient for inhibition of crystal growth. This concentration is too low for inhibition to be on the basis of chelation of calcium. It therefore seems likely that crystal growth is inhibited by an interaction of uropontin with the crystals. From one litre of bovine milk we have isolated approximately 22 mg osteopontin, corresponding to a concentration of 0.4 μM. The function of osteopontin in milk is not clear but a function as an inhibitor of crystal growth, similar to uropontin in urine, is a possibility.

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