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IN VITRO PHOSPHORYLATION OF MOUSE OSTEOPONTIN EXPRESSED IN *E. COLI*

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To understand the role of post-translational modifications on the structure and function of osteopontin, a secreted glycosylated phosphoprotein, we expressed mouse osteopontin in *E. coli* as a fusion protein with glutathione-S-transferase (GST). The purified fusion protein was cleaved by factor Xa generating GST (26 kDa) and recombinant osteopontin (60 kDa). The fusion protein was phosphorylated *in vitro* by cytosolic, microsomal, and casein kinase II fractions from mouse kidney homogenates. The fusion protein and recombinant osteopontin were also phosphorylated by the catalytic subunit of cAMP-dependent protein kinase. The suitability of the fusion and recombinant proteins as model substrates for the study of the function(s) and post-translational modifications of osteopontin is discussed.

Osteopontin (2ar, BSPI, SPPI, PP69, Eta-1, 2B7) is a secreted glycosylated phosphoprotein expressed by bone (1, 2, 3), kidney (1, 4), deciduum and placenta (4), activated T-lymphocytes (5), macrophages (6), smooth muscle cells of the vascular system (7), and carcinomas and sarcomas (8). Osteopontin is also present in plasma and milk (8).

Analysis of the predicted amino acid sequences of osteopontin from various species reveals several conserved motifs. These motifs include phosphorylation sites for protein kinases, a polyaspartate sequence, an N-glycosylation site, several O-glycosylation sites, a Gly-Arg-Gly-Asp-Ser (GRGDS) cell-surface receptor binding motif, and two heparin binding domains (9-17).

It has been proposed that osteopontin may be involved in the initiation or regulation of mineralization of the extracellular matrix (18), cell attachment and spreading (9), bone resorption (19), metastasis and cell migration (8), early immune response to bacterial infection and genetic resistance to infection by *Rickettsia tsutsugamushi* (5), mobility and state of activation of macrophages, and enhancement of IgM and IgG secretion by polyclonal B-lymphocytes (20).

Tissue-specific phosphorylation of osteopontin may be essential to modulate its biological function(s). Osteoblasts secrete a number of phosphorylated forms of osteopontin (21), whereas normal rat kidney cells secrete both phosphorylated and non-phosphorylated forms of this protein (22).

Abbreviations: BSPI, bone sialoprotein I; SPPI, secreted phosphoprotein I; Eta-1, Early T-lymphocyte activation 1.

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Few data are available on the identity of the protein kinases responsible for the phosphorylation of osteopontin (23). Identification of these kinases can be facilitated by the availability of non-phosphorylated form(s) of osteopontin in sufficient amounts to be used as a substrate *in vitro*. To approach this problem, we have cloned and expressed in *E. coli*, for the first time, mouse osteopontin as a fusion protein with glutathione-S-transferase (GST) and shown it to be a substrate for protein kinases.

MATERIALS AND METHODS

Materials: Glutathione-agarose beads (sulfur linkage), thrombin, glutathione, and the catalytic subunit of bovine heart cAMP-dependent protein kinase were purchased from Sigma (St. Louis, MO). [γ-^{32}P]ATP (>3000 Ci/mmol) was purchased from Amersham (Arlington Heights, Il). BamHI, EcoRI and factor Xa were purchased from Boehringer Mannheim (Indianapolis, IN), Casein kinase II was purified from mouse kidneys as described by Meggio et al (24), 2ar cDNA was a gift from D. Denhardt (Rutgers's University, N.J.), and the pGEX vector was a gift from R. Boise (Children's Hospital, Boston).

Bacterial strains and plasmids: All plasmids were transformed into *E. coli* strain DH5α (F$^-$, *end*A1, *hsd*R17, *sup*E44, *thi*A, *rec*A1, *gyr*A96, *rel*A1, Δ(*Arg*F, *lac*ZYA), *U*169, ϕ80 Δ *lac*Z, Δ M15) (Gibco BRL, Gaithersberg, MD). pGEX-3x vector is described by Smith and Johnson (25).

Polymerase chain reaction amplification of osteopontin DNA sequences and cloning into pGEX-3x expression vector: Nucleotides 119 to 1018 of mouse osteopontin cDNA (11) were amplified by PCR using the primers PA (5'-TGGATCCTCCCGGTGAAATGT-3') and PB (5'-AGAATTCCTGCTTAATCC-3'). The amplified DNA (1 Kb) was purified and ligated into the BamHI/EcoRI sites of pGEX-3x, resulting in a plasmid named pNNK10.

Purification of GST-osteopontin: Overnight cultures of *E. coli* transformed with either parental or recombinant pGEX vectors were diluted 1:20 with LB broth supplemented with 100 μg/ml ampicillin and grown at 37°C with shaking for 1 h prior to the addition of IPTG to a final concentration of 0.5 mM. After 30 min of additional growth, the bacteria were harvested by centrifugation. The pellet was resuspended in PBS containing 1% Triton X100 and 5 mM EDTA, and the bacteria lysed by mild sonication. GST-osteopontin was purified from lysed bacteria by affinity chromatography on glutathione-agarose beads as described by Smith and Johnson (25).

Phosphorylation assay: Phosphorylation of purified GST-osteopontin by either the cytosolic, microsomal, or purified casein kinase II preparations was carried out in 50 μl of Tris-HCl buffer pH 8.0, containing 150 mM NaCl, 12 mM $MgCl_2$, 30 μM [γ-^{32}P]ATP (specific activity of 1 μCi/mmol), and 5 μg of GST-osteopontin. The reactions were started by the addition of 2-3 μg of cytosolic or microsomal kinase preparations or 100 ng of purified casein kinase II preparation. The reactions were incubated at 37°C for 20 min, then stopped by boiling in Laemmli buffer (26). Phosphorylation of purified GST-osteopontin or recombinant osteopontin by the catalytic subunit of cAMP dependent protein kinase was carried out in 50 μl of Tris-HCl buffer pH 7.4, containing 100 mM NaCl, 10 mM $MgCl_2$, 20 μM [γ-^{32}P]ATP (specific activity of 1 μCi/mmol), and 5 μg of GST-osteopontin or recombinant osteopontin. The reactions were started by the addition of 0.1 units of the catalytic subunit of cAMP dependent protein kinase, incubated at 30°C for 15 min, and terminated by boiling in Laemmli buffer (26).

RESULTS AND DISCUSSION

Expression of GST-osteopontin in E. coli: The coding region of mouse osteopontin, excluding the signal peptide (amino acids 1-16), was amplified from a mouse cDNA clone by PCR using the primers PA and PB (see Materials and Methods) which contained BamHI and EcoRI endonuclease restriction sites, respectively (Figure 1). The amplified mature osteopontin DNA was cloned into the BamHI-

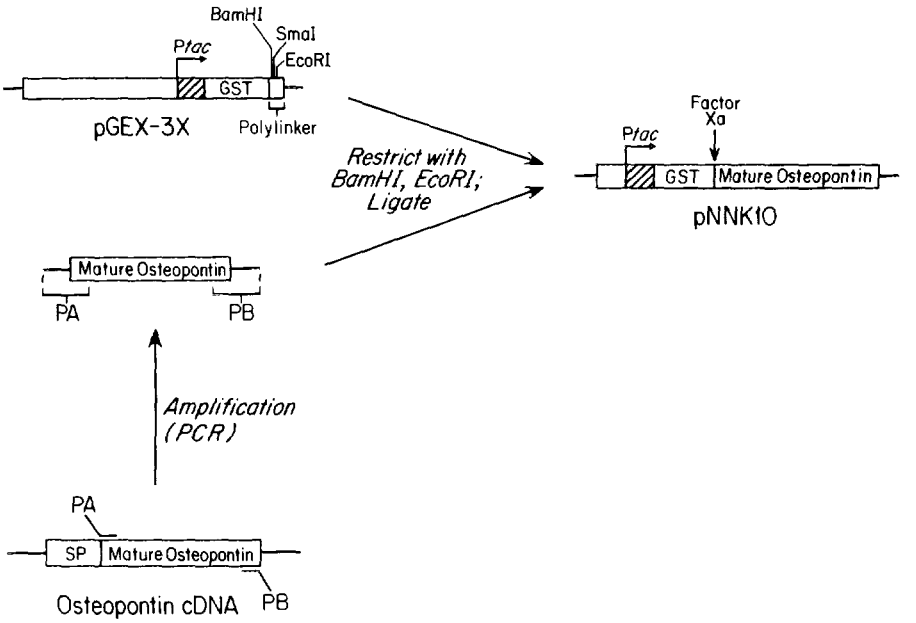

Figure 1. Schematic for construction of pNNK10: a GST-osteopontin expression vector. pNNK10 was constructed as described in text. sp, signal peptide (residues 1-16); mature osteopontin, residues 17-294; ptac, *tac* promoter; GST, glutathione-S-transferase; factor Xa, the cleavage site for human factor Xa.

EcoRI sites of the prokaryotic expression vector pGEX-3x (25). The resulting plasmid (pNNK10) contains the region coding for the 278 amino acid mature osteopontin fused, in frame, to the 3' end of the open reading frame coding for glutathione-S-transferase (Figure 1). The expression of fusion protein is regulated by the IPTG-inducible *tac* promoter (see ref. 27).

Induction of DH5α(pNNK10) cells with IPTG for 3 h resulted in the abundant synthesis of a soluble fusion protein of 80 kDa (Figure 2A, lanes 5 and 6) which was absent from induced DH5α(pGEX-3x) (lanes 1 and 2) or DH5α control cells (lanes 3 and 4). This protein was purified from bacterial lysates by affinity chromatography on glutathione-agarose beads (lane 6). Induction of DH5α(pNNK10) cells with IPTG for 3 h also yielded minor proteins that copurify with the 80 kDa protein. These minor proteins could be minimized by inducing DH5α(pNNK10) cells in early log phase for only 30 min and purifying the 80 kDa protein in the presence of 5 mM EDTA. These modifications resulted in a yield of approximately 5 μg of the 80 kDa protein per ml of culture (Figure 2B).

Cleavage of GST-osteopontin by thrombin and factor Xa: It has been previously reported that osteopontin contains three putative thrombin cleavage sites at the carboxyl-side of residues Arg_{144}, Arg_{153}, and Arg_{157} (11), and that thrombin cleavage of osteopontin from rat and human results in the generation of a 32 kDa polypeptide (8). Thrombin cleavage of the 80 kDa protein generated a 33 kDa polypeptide, whose amino-terminal sequence (SKSRSFQVSDEQYPDAT) was nearly identical to the amino-terminal sequence of the 32 kDa polypeptide of rat osteopontin (8) and identical to the

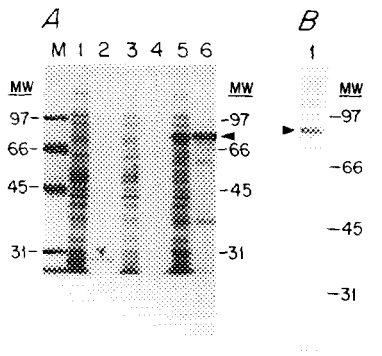

Figure 2. Expression of GST-osteopontin fusion protein in *E. coli* cells. A. Samples (equivalent to 100 ml of cells) were separated by electrophoresis on a 10% SDS-polyacrylamide gel (26). The gel was stained with Coommassie blue. (MW) molecular weight markers, (1) 10,000 x g supernatant (10S) from lysed DH5α(pGEX-3x) cells, (2) GST eluted from glutathione-agarose beads, (3) 10S from control cells (lacking any fusion protein), (4) proteins from control cells eluted from glutathione-agarose beads, (5) 10S from DH5α(pNNK10) cells, (6) GST-osteopontin fusion protein eluted from glutathione-agarose beads. Figure 2A is a composite from different regions of the same gel. B. GST-osteopontin (2 μg) isolated from DH5α(pNNK10) cells induced in early log phase for 30 min and purified in the presence of 5 mM EDTA. Solid arrow heads mark the position of GST-osteopontin; open arrow head marks the position of GST.

sequences located at the carboxyl-side of Arg_{153} of mouse osteopontin (11). These results demonstrated that the 80 kDa fusion protein contains osteopontin sequences, and that the major thrombin cleavage site of the 80 kDa protein is identical to the major thrombin cleavage site of native osteopontin from rat and human (8). In addition to the major thrombin cleavage site, a minor cleavage site was also detected four amino acids from the amino-terminus of the 33 kDa polypeptide.

Cleavage of the fusion protein with factor Xa resulted in the generation of two polypeptides of 26 kDa and 60 kDa, which corresponded to GST and mature mouse osteopontin (recombinant osteopontin), respectively (Figure 3B). The apparent molecular weight of recombinant osteopontin was approximately twice the calculated molecular weight of 32,350 (11). This anomalous electrophoretic migration of recombinant osteopontin is consistent with the anomalous migration exhibited by mouse (56 to 67 kDa; 11) and rat (65 kDa; 22) osteopontin translated *in vitro*. This anomalous migration of osteopontin has been explained, at least in part, by its relative high content of acidic amino acids (11, 22).

Phosphorylation of GST-osteopontin and recombinant osteopontin in vitro: *E. coli* does not phosphorylate proteins in the same manner as do eukaryotic cells (28). In fact, amino acid analysis of GST-osteopontin purified from *E. coli* revealed no phosphorylated residues (the level of detection is less than one phosphorylated residue per molecule, assuming an average recovery of 50% of the phosphorylated residues). Moreover, mouse osteopontin contains consensus sequences for various protein kinases, including several consensus sequences for casein kinase II and cAMP-dependent protein kinases. It was expected, therefore, that osteopontin expressed in bacteria would be an appropriate substrate for eukaryotic protein kinases. To determine whether GST-osteopontin could be

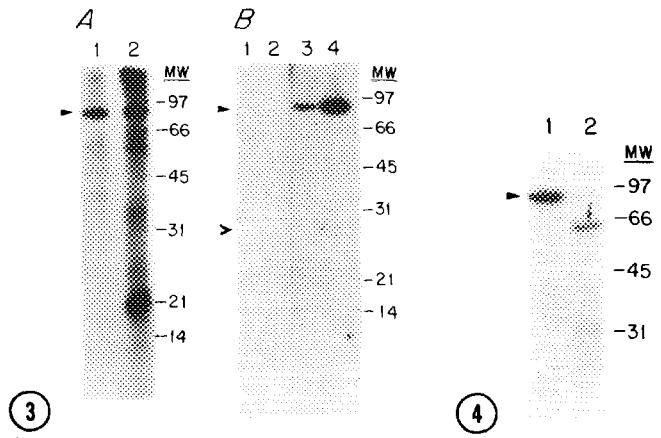

Figure 3. Phosphorylation of GST-osteopontin *in vitro*. A. Phosphorylation of GST-osteopontin by 3 μg of cytosolic fraction (1), or 2 μg of microsomal fraction (2). B. Phosphorylation of GST osteopontin by casein kinase II. (1) control (no enzyme added), (2) GST with 100 ng purified casein kinase II, (3) GST-osteopontin with 100 ng casein kinase II and 50 mg/ml heparin, (4) GST-osteopontin with 100 ng casein kinase II. The reactions were stopped by boiling in Laemmli buffer (26), then applied onto a 10% SDS-polyacrylamide gel and electrophoresed for 3 h at 150 V. The gels were stained with Coommassie blue for 15 min, destained in several changes of 500 ml of 20% methanol:10% acetic acid, dried, and then exposed to Kodak XAR film overnight with intensifing screens at -70^{0}C. This figure is a composite of different regions of the same gel. Solid arrow heads mark the position of GST-osteopontin; open arrow head marks the position of GST.

Figure 4. Phosphorylation of GST-osteopontin and recombinant osteopontin by the catalytic subunit of cAMP-dependent protein kinase. (1) GST-osteopontin, (2) recombinant osteopontin. The reactions were stopped by boiling in Laemmli buffer (26). The samples were electrophoresed and the gels processed as described in the legend to Figure 3. The minor band observed at 31 kDa is a degradation product of recombinant osteopontin that is probably due to contamination of commercial preparations of factor Xa by another protease. Solid arrow head marks the position of GST-osteopontin; solid arrow marks the position of recombinant osteopontin.

phosphorylated *in vitro*, the fusion protein was incubated in the presence of [γ-^{32}P]ATP with either a cytosolic or a microsomal fraction of mouse kidney homogenate. Figure 3A shows that GST-osteopontin could be phosphorylated by the cytosolic (lane 1) or the microsomal (lane 2) fractions in the absence of exogenous activators of protein kinases. In addition to phosphorylating GST-osteopontin, the microsomal fraction phosphorylated several endogenous substrates (62, 55, 50, 35 and 20 kDa). Phosphorylation of these endogenous substrates also occurred in the absence of added GST-osteopontin.

Phosphorylation of GST-osteopontin by the cytosolic fraction was significantly inhibited by 50 mg/ml heparin (data not shown). This result and the observation that a casein kinase II-type enzyme extracted from embryonic chicken tibia phosphorylated chicken osteopontin (23), led us to test whether the phosphorylation of GST-osteopontin by the cytosolic fraction was also, in part, due to a casein kinase II-type enzyme. Casein kinase II was purified from the cytosolic fraction by the procedure of Meggio et al. (24). This preparation of casein kinase II phosphorylated GST-osteopontin (Figure 3B, lane 4) and this phosphorylating activity was significantly inhibited by heparin (lane 3). Neither the cytosolic, microsomal (data not shown), nor purified casein kinase II fractions (Figure 3B, lane 2) phosphorylated GST. Therefore, the observed phosphorylation of the fusion protein occurred within

the osteopontin domain. In addition to being phosphorylated by the above kinases, GST-osteopontin was phosphorylated by cAMP-dependent protein kinase (Figure 4, lane 1). Purified recombinant osteopontin, released from the fusion protein by factor Xa, also served as a substrate for cAMP-dependent protein kinase (Figure 4, lane 2).

Mouse osteopontin appears to be a complex substrate with at least 58 consensus phosphorylation sites for different types of kinases. These putative phosphorylation sites are not randomly distributed throughout the protein but they appear as if they were organized in eight clusters. For example, between residues 100 and 126 there are 9 potential phosphorylation sites for either casein kinase I, casein kinase II or mammary gland casein kinase. In addition to potential phosphorylation sites for these independent casein kinase family of enzymes, osteopontin also contains potential phosphorylation sites for cAMP- and cGMP-dependent protein kinases, calmodulin-dependent protein kinase, and protein kinase C. Prince et al. (29) reported that osteopontin from rat bone contains an average of 12 phosphoserine and 1 phosphothreonine. Thus, in osteopontin there are several fold more potential phosphorylation sites than those found phosphorylated in osteopontin from bone. Possibly not all of the potential sites will be phosphorylated at any given time, since some sites may not be accessible to protein kinases or some tissues may not contain all of the kinase activities required for the phosphorylation of osteopontin. Furthermore, the clustering of sites suggests that certain phosphorylated residues can serve as specificity determinants (30). Phosphorylation of a Ser/Thr residue by any kinase can generate a site for phosphorylation of an adjacent residue by either casein kinase I or mammary gland casein kinase (30, 31). Conversely, phosphorylation at one site by a particular kinase may suppress the phosphorylation of a nearby residue, such as the mutually exclusive phosphorylation of hormone-sensitive lipase by cAMP-dependent protein kinase and calmodulin-dependent protein kinase (31).

The presence of phosphorylation sites for dependent protein kinases in osteopontin suggests that the generation of distinct phosphorylated forms of this protein depends on the cell type and/or the metabolic state of the cell. Consistent with this notion, osteopontin from osteosarcoma cells and RCA1 osteoblastic cells showed an increased [$^{32}PO_4$]-labeling following treatment with 1,25-dihydroxyvitamin D_3 (21). The variety and number of potential phosphorylation sites found in osteopontin, therefore, may serve as the structural basis for the differential phosphorylation observed in this protein (21).

Expressing mouse osteopontin in bacteria provides large amounts of a non-phosphorylated form of the protein that can be used as a substrate for specific phosphorylation *in vitro*. Our results demonstrate that mouse osteopontin can be readily phosphorylated *in vitro* by protein kinases present in cytosolic and microsomal fractions from mouse kidney homogenates, by purified casein kinase II, and by the catalytic subunit of cAMP-dependent protein kinase. Our results on the phosphorylation of mouse osteopontin by casein kinase II are consistent with previous results indicating that a casein kinase II-type activity extracted from chicken bone can phosphorylate chicken osteopontin (23). No results have been reported, previous to our work, on the phosphorylation of osteopontin by cAMP-dependent protein kinase. The fact that both, casein kinase II and cAMP-dependent protein kinase can phosphorylate osteopontin *in vitro* is provocative in light of reports describing heparin-sensitive ectokinase (32) and cAMP-dependent protein ectokinase (33) activities on the surface of cells. It is

possible that these enzymatic activities can be responsible, at least in part, for the phosphorylation of osteopontin *in vivo*.

Additional studies on recombinant osteopontin, now in progress, will allow us to identify kinases other than casein kinase II and cAMP-dependent protein kinase that phosphorylate osteopontin, as well as other enzymes involved in its post-translational modifications. These studies will allow us to generate various forms of osteopontin *in vitro* that can be used subsequently to determine their possible role in calcification, in the stimulation of B-lymphocytes, and in cell attachment and migration.

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