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MOUSE OSTEOPONTIN EXPRESSED IN *E. COLI* EXHIBITS AUTOPHOSPHORYLATING ACTIVITY OF TYROSINE RESIDUES

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Osteopontin is a secreted glycosylated phosphoprotein found in various normal and transformed tissues. Mouse osteopontin expressed in bacteria has been found to autophosphorylate *in vitro* using ATP or GTP as phosphoryl donors. The reaction does not occur using inorganic orthophosphate as the donor. Only tyrosine residues are phosphorylated. Neither serine nor threonine residues, both of which are found phosphorylated in osteopontin extracted from bone, is autophosphorylated *in vitro*. The autophosphorylation of tyrosine residues by a secreted protein such as osteopontin may provide additional insight into its biological functions.

The secreted phosphoprotein osteopontin (BSPI, bone sialoprotein I; SPPI, secreted phosphoprotein I; Eta-1, early T-lymphocyte activation-1) is expressed by various cell types including osteoblasts (1, 2, 3), kidney cells (1), activated T-lymphocytes (4), and smooth muscle cells of the vascular system (5). Osteopontin is also expressed by carcinomas and sarcomas (6, 7, 8), and its concentration is found to be increased in the plasma of patients with cancer or sepsis (6). The primary structure of osteopontin, deduced from cDNA sequences (4, 9, 10, 11, 12, 13), exhibits several distinct motifs including several phosphorylation sites for various protein kinases, one N- and several O-glycosylation sites, one Gly-Arg-Gly-Asp-Ser (GRGDS) cell surface receptor-binding sequence, three thrombin cleavage sites, and two heparin binding sites.

Based on the primary sequence of osteopontin, experimental evidence and theoretical considerations, several functions have been proposed for this protein in bone, including a role in the initiation and ultrastructural localization of apatite crystals during mineralization (14). This hypothesis has been strengthened by recent biochemical studies (15), and by immunohistochemical (16, 17) and high-resolution electron microscopic immunocytochemical localization of the protein at the sites of mineralization in bone (3, 18) and calcified cartilage (17) as well as in mineralizing cultures of osteoblasts (19). Osteopontin is also thought to interact with an integrin receptor on the cell surface through its GRGDS sequence (9, 20, 21, 22); this interaction may be modulated by phosphophorylation of serine residues (23). Phosphorylation of osteopontin may also play a role in bone resorption. The protein has been localized by immunocytochemistry to the area of bone facing osteoclasts (18, 21, 24), and it has been found to modify osteoclast activity *in vitro* (25). Since

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phosphorylation of osteopontin appears to play an important role in several of its proposed functions in bone, we have expressed a non-phosphorylated form of mouse osteopontin in *E. coli*, and found that it can be phosphorylated by protein kinases *in vitro* (26). During the course of these studies, we observed that the recombinant protein exhibits autophosphorylating activity of tyrosine residue(s) *in vitro*. We report here on the characterization of the autophosphorylating activity of recombinant osteopontin.

MATERIALS AND METHODS

Purification of GST-osteopontin: The fusion protein, GST-osteopontin, was purified from *E. coli* lysates by affinity chromatography on glutathione-agarose (sulfur linkage; Sigma Co., St. Louis, MO) (26).

Autophosphorylation of GST-osteopontin: One microgram of GST-osteopontin was incubated with 0.06 μM of [γ-^{32}P]ATP (3000 Ci/mmol; Amersham, Arlington Heights, IL), or 0.11 μM of [γ-^{32}P]GTP (>4500 Ci/mmol; ICN, Irvine, CA) in 40 μl of 20 mM Tris-HCl buffer, pH 7.4, containing 100 mM NaCl, 10 mM $MgCl_2$, 10 mM $MnCl_2$ at 30°C for 30 min. The Tris-HCl buffer, pH 7.4, can be substituted by HEPES buffer, pH 7.4 (see below). The reactions were stopped by boiling in Laemmli buffer (27) containing 50 mM EDTA and 10 mM ATP to avoid phosphorylation catalyzed by free divalent cations. The reactions were then applied onto a 10% SDS-polyacrylamide gel and electrophoresed for 3 h at 150 V. The gels were stained in 0.5% Coommassie blue for 15 min, destained in several changes of 500 ml of 20:10% methanol:acetic acid, dried and autoradiographed using Kodak X-AR film (Eastman Kodak Co., Rochester, NY).

Phosphoamino acid analysis: One hundred micrograms of phosphorylated or nonphosphorylated GST-osteopontin were hydrolyzed in 4 M HCl at 110°C for 4 h (28, 29). The samples were analyzed for phosphoamino acids on a Beckman (model 121 M) amino acid analyzer using a Beckman WII column, pre-equilibrated with 0.16 M sodium citrate, pH 1.3 (30, 31).

Autophosphorylation of recombinant osteopontin: One hundred micrograms of GST-osteopontin were digested with 2 μg of factor Xa (Boehringer-Mannheim, Indianapolis, IN) and recombinant osteopontin was purified as described (26). The recombinant protein was dialyzed against two changes of 2 liters of 20 mM HEPES, pH 7.4, containing 150 mM NaCl. Approximately 0.5 μg of recombinant osteopontin was incubated with [γ-^{32}P]ATP and processed as described above.

RESULTS

Autophosphorylation of GST-osteopontin using ATP or GTP: Fig. 1 shows that GST-osteopontin was labeled after incubation with [γ-^{32}P]ATP (Fig. 1A, lane 2) or [γ-^{32}P]GTP (Fig. 1B, lane 2). When the fusion protein was inactivated by heating to 80°C for 10 min before incubation with [γ-^{32}P]ATP (Fig. 1A, lane 1) or [γ-^{32}P]GTP (Fig. 1B, lane 1), no labeling was detected. These results suggested that the labeling of GST-osteopontin after incubation with [γ-^{32}P]ATP or [γ-^{32}P]GTP was due to an autophosphorylation reaction requiring structural integrity of the protein. The fact that SDS, urea, or cold ATP did not displace the radioactive label from the protein (data not shown) was further evidence that the labeling of GST-osteopontin using [γ-^{32}P]ATP or [γ-^{32}P]GTP as a phosphoryl donor was the result of autophosphorylation of the protein. The reaction proceeded in the presence of Mg^{2+} (Fig. 1A, lane 4; Fig. 1B, lane 4) but was significantly stimulated by Mn^{2+} (Fig. 1A, lane 2; Fig. 1B,

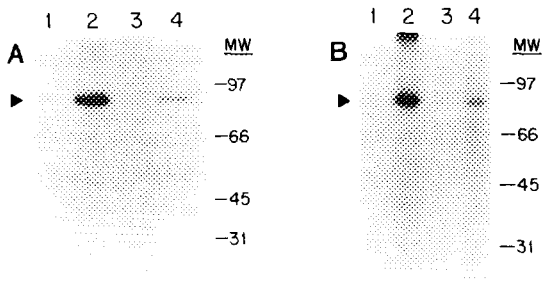

Fig. 1. Autophosphorylation of GST-osteopontin. A. Lane 1, reaction containing heat inactivated (80°C for 10 min) GST-osteopontin; lane 2, reaction containing native GST-osteopontin; lane 3, reaction as in lane 2, plus 5 mM spermine (Sigma Co.), lane 4, reaction as in lane 2, but without $MnCl_2$, MW; molecular weight markers. B. Lanes 1, 2, 3 and 4 same as in A. Closed arrowheads indicate the position of GST-osteopontin. The band seen near the origin of electrophoresis on lane 2 of panel B is probably due to aggregated GST-osteopontin. The gels were exposed to Kodax X-AR film for 48 h at room temperature (A) or for 16 h at -75°C with intensifying screens (B).

lane 2). This effect of Mn^{2+} is not unique to GST-osteopontin, since it also has been shown to stimulate the autophosphorylation and kinase activity of both insulin (32) and epidermal growth factor (33) receptors. Autophosphorylation of GST-osteopontin was inhibited by spermine (Fig. 1A, lane 3; Fig. 1B, lane 3) in a fashion similar to that observed with the tyrosine-specific kinases I and II from spleen (34). [^{32}P]-orthophosphate was not a substrate for the autophosphorylation of GST-osteopontin (data not shown). These observations indicate that the autophosphorylation reaction requires a source of high energy phosphate such as ATP or GTP which function as phosphoryl donors.

Osteopontin is autophosphorylated on tyrosine residues: To determine the nature of the autophosphorylated residues, GST-osteopontin and GST-osteopontin incubated with ATP were hydrolyzed under conditions designed to cleave peptide bonds but preserve a significant number of phosphoamino acids (4 M HCl at 110°C for 4 h; 28, 29). The results of the phosphoamino acid analysis are shown in Fig. 2. GST-osteopontin (Fig. 2, trace 1) showed no peak above background. GST-osteopontin incubated with ATP (Fig. 2, trace 2) exhibited a peak that co-eluted with the O-phosphotyrosine standard (Fig. 2, trace 3). There were no peaks detected which coeluted with either O-phosphoserine or O-phosphothreonine (Fig. 2, trace 4). The material from trace 2 was collected, further hydrolyzed with 4M HCl at 110°C for 12 h (29), and reanalyzed for its amino acid composition. The result was a single peak that co-eluted with the tyrosine standard (data not shown), confirming that the peak from trace 2 was O-phosphotyrosine. Autophosphorylation of tyrosine residues was further established by the finding that only the autophosphorylated protein was recognized by an O-phosphotyrosine antibody (Oncogene Science, Inc., Uniondale, NY) in a Western blot assay (data not shown). That the O-phosphotyrosine residues reside in the osteopontin domain of the fusion protein, was demonstrated by the fact that GST alone was not autophosphorylated, nor could it be phosphorylated by any of the various protein kinase preparations obtained from mouse kidney (26). GST was also not phosphorylated when incubated with GST-osteopontin (data not shown). Furthermore, purified recombinant osteopontin (M_r 60,000) was autophosphorylated when incubated

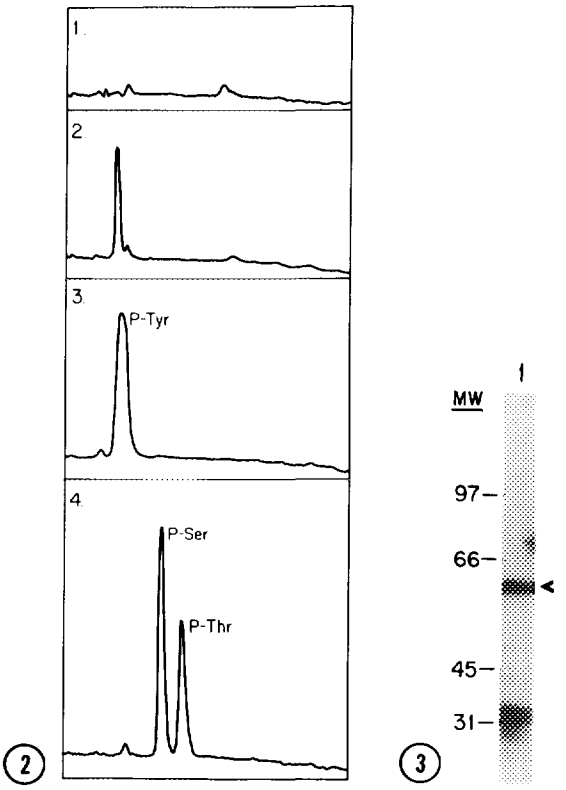

Fig. 2. GST-osteopontin is autophosphorylated on tyrosine residues. Trace 1, GST-osteopontin incubated in the absence of ATP; trace 2, GST-osteopontin incubated in the presence of ATP; trace 3, O-phosphotyrosine standard; trace 4, O-phosphoserine/O-phosphothreonine standard.

Fig. 3. Autophosphorylation of recombinant osteopontin. Lane 1, recombinant osteopontin; MW, molecular weight markers. The bands observed at about 31 kDa are degradation products of recombinant osteopontin, probably due to contamination of commercial factor Xa by another protease. Open arrow indicates the position of recombinant osteopontin.

with [γ-^{32}P]ATP (Fig. 3, lane 1). This observation demonstrates that the autophosphorylating activity resides within osteopontin.

DISCUSSION

Our results demonstrate that mouse osteopontin expressed in bacteria is able to autophosphorylate tyrosine residues *in vitro* when incubated with ATP or GTP. Although generally only O-phosphoserine and O-phosphothreonine have been detected in phosphoproteins present in bone of a large number of vertebrates (30, 31, 35) including osteopontin (36, 37), the primary sequence of this protein does contain consensus phosphorylation sites for tyrosine kinases that could be potential targets for autophosphorylation (see below). It is unlikely that the autophosphorylating activity of recombinant osteopontin is due to a bacterial tyrosine kinase contaminating our preparations, since *E. coli* cells contain little or no tyrosine kinase activity (38).

The actual O-phosphotyrosine residues of osteopontin which are autophosphorylated *in vitro* remain to be identified. $Tyrosine_{150}$ is a possible candidate, since this residue is located within a conserved sequence (RGDSLAY, residues 144 to 150) that conforms to a consensus site for phosphorylation by tyrosine kinases (39). In mouse osteopontin $tyrosine_{277}$, located within the sequence KEDDRY (residues 272 to 277), is another candidate.

It has been reported that osteopontin is sulfated on tyrosine residues (40). Sulfation of this protein was observed only during bone tissue mineralization (41). It is presently not known whether the same tyrosine residues of osteopontin become sulfated or phosphorylated, or how any of these modifications may affect one another. Similarly, there are no *in vivo* data as to whether tyrosine residues are phosphorylated *in vivo* either by autophosphorylation or by protein kinases, although Senger et al (42) reported that osteopontin from neoplastic cells (7, 43) was phosphorylated either through autophosphorylation or by an unidentified kinase on undetermined residues. Likewise, the description by Krane and Glimcher (44) of an extracellular activity that catalyzed the transfer of phosphoryl groups from ATP to an acceptor protein within the organic matrix of bone may also represent an autophosphorylation of residual osteopontin, tightly complexed with the collagen in the preparations used. In any event, the possibility that autophosphorylation of tyrosine residues may occur *in vivo*, and may, as in other systems (45, 46), initiate a cascade of intracellular events including the stimulation of protein kinases is an intriguing suggestion, which may link the autophosphorylation of tyrosine to some of the distinct biological functions of osteopontin.

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