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Phosphorylation of Osteopontin Is Required for Inhibition of Vascular Smooth Muscle Cell Calcification*

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Osteopontin (OPN) is a non-collagenous, glycosylated phosphoprotein associated with biomineralization in osseous tissues, as well as ectopic calcification. We previously reported that osteopontin was co-localized with calcified deposits in atherosclerotic lesions, and that osteopontin potently inhibits calcium deposition in a human smooth muscle cell (HSMC) culture model of vascular calcification. In this report, the role of phosphorylation in osteopontin's mineralization inhibitory function was examined. The ability of OPN to inhibit calcification completely depended on post-translational modifications, since bacteria-derived recombinant OPN did not inhibit HSMC mineralization. Following casein kinase II treatment, phosphorylated OPN (P-OPN) dose-dependently inhibited calcification of HSMC cultured *in vitro* about as effectively as native OPN. The inhibitory effect of osteopontin depended on the extent of phosphorylation. To determine the specific structural domains of OPN important for inhibition of calcification, we compared OPN fragments (N-terminal, C-terminal, and full-length), and compared the inhibitory effect of both phosphorylated and non-phosphorylated fragments. While none of the non-phosphorylated OPN fragments effected calcification, P-OPN caused dose dependent inhibition of HSMC calcification. P-OPN was treated with alkaline phosphatase to create dephosphorylated OPN. Dephosphorylated OPN did not have an inhibitory effect on calcification. The expression of OPN mRNA and P-OPN secretion by HSMC were decreased in a time-dependent manner during culture calcification. These results indicate that phosphorylation is required for the inhibitory effect of OPN on HSMC calcification, and that regulation of OPN phosphorylation represents one way in which mineralization may be controlled by cells.

Vascular calcification is often encountered in the development of atherosclerotic intimal lesions and is a common consequence of aging (1). In diabetic patients and individuals with renal failure, vascular calcification contributes to both the morbidity and mortality associated with these diseases (2). For example, vascular calcification is positively correlated with increased risk of myocardial infarction and increased risk of dissection following angioplasty (3). Moreover, calcification is a major cause of failure for both native and tissue prosthetic heart valves, affecting 1–2% of the aging population (4). Until recently, vascular calcification was considered to be a passive, degenerative, and end-stage process of vascular disease. However, bone morphogenetic proteins including bone morphogenetic proteins-2, and noncollagenous bone matrix proteins such as osteopontin, osteonectin, osteocalcin, and matrix Gla protein have been demonstrated in calcified vascular tissues (5–8). In addition, vascular cell calcification *in vitro* was regulated by calcitropic hormones such as parathyroid hormone-related peptide (9) and vitamin D (10), as well as lipid oxidation products (11). These findings suggest that the process of vascular calcification may share some mechanisms with mineralization seen in bone, cartilage, and teeth, and that vascular calcification is in fact an actively regulated process.

OPN[1] is a secreted, glycosylated phosphoprotein found normally in mineralized tissues such as bones and teeth, in addition to kidney, urine, and epithelial lining cells of numerous organs. OPN is associated with calcified deposits in soft tissues, such as Monckeberg's sclerosis, aortic stenosis, prosthetic valves, renal stones, and tumor-associated calcifications. We and others have reported that OPN is abundant at sites of calcification in atherosclerotic plaques and in calcified aortic valves (7, 12). OPN is a multifunctional protein that promotes cell adhesion and migration (13), inhibits hydroxyapatite formation (14), and binds Ca^{2+} (15). OPN can exist in multiple forms depending on the extent of post-translational modification. In addition to sulfation (16), glycosylation (17), and transglutamination (18), osteopontin can undergo extensive phosphorylation. A highly phosphorylated form of OPN can be isolated from the mineralized extracellular matrix of bone tissue (19) and is synthesized by osteoblasts (20, 21). Breast milk has also been shown to contain highly phosphorylated OPN (22). In some cells, OPN phosphorylation is highly regulated. For example, normal rat kidney cells as well as smooth muscle cells secrete both phosphorylated and non-phosphorylated OPN (23, 24). Likewise, JB6 epidermal cells treated with phorbol esters secrete phosphorylated OPN while JB6 cells treated with vitamin D_3 secrete non-phosphorylated OPN (25). While an extensive tissue survey has yet to be performed, it is likely that tissue-specific expression of OPN differs not only in pro-

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[1] The abbreviations used are: OPN, osteopontin; DMEM, Dulbecco's modified Eagle's medium; HSMC, human smooth muscle cell; P-OPN, phosphorylated OPN; reOPN, recombinant OPN; CKII, casein kinase II; FBS, fetal bovine serum; PAGE, polyacrylamide gel electrophoresis; GST, glutathione *S*-transferase; HA, hydroxyapatite; PPACK, thrombin-[D-phenylalanyl-*N*-[4-(aminoiminomethyl)amino]-1-[(chloroacetyl)-butyl]-L-prolinamide.

tein levels but phosphorylation state. Such differences in the extent of phosphorylation of OPN may be important in OPN's physiological function, in particular, in the formation of mineralized tissues.

Previously we reported that native smooth muscle derived-OPN inhibited calcium deposition in a bovine smooth muscle cell calcification system, and that OPN was localized to the surface of calcified deposits (26). In this study, we investigated the role of phosphorylation in OPN's ability to inhibit calcification *in vitro*. We found that bacterial-derived recombinant OPN (reOPN) containing no post-translational modifications did not inhibit HSMC mineralization, while native OPN derived from rat neonatal smooth muscle cells inhibited HFSMC culture calcification. The ability of OPN to inhibit mineralization could be restored to reOPN using casein kinase II (CKII) to generate phosphorylated OPN (P-OPN). P-OPN dose dependently inhibited calcification and was about as effective as native OPN. The inhibitory effect of osteopontin on HSMC culture calcification was strictly dependent on the number of phosphorylated sites. Moreover, phosphorylated OPN treated with alkaline phosphatase to generate dephosphorylated OPN did not inhibit HSMC culture calcification. Finally, both the expression of endogenous OPN mRNA and phosphorylated OPN secretion decreased in a time-dependent manner during HSMC culture calcification. These results indicated that phosphorylation of OPN is required for its inhibitory effects on HSMC biomineralization, and that this is an actively regulated process in HSMC probably contributing to the propensity of the cultures to calcify.

MATERIALS AND METHODS

Reagents—Dulbecco's modified Eagle's medium (high glucose, 4.5 g/liter of glucose) (DMEM) and fetal bovine serum (FBS) were purchased from Life Technologies, Inc. (Grand Island, NY). Casein kinase II was purchased from Calbiochem (LA Jolla, CA). $H_3[^{32}P]O_4$, [γ-^{32}P]ATP, and [α-^{32}P]dCTP were obtained from NEN Life Science Products Inc. (Boston, MA). Unless otherwise mentioned, all other reagents were obtained from Sigma.

Native Osteopontin and Neutralizing Antibody—Native OPN was purified from the conditioned medium of rat neonatal smooth muscle cell cultures as described previously (29). This preparation was judged to be >95% pure, on the basis of Coomassie staining and N-terminal sequence analysis. Goat anti-rat osteopontin antibody OP-199 and nonimmune goat serum were prepared, and IgG fractions were purified as described previously (27).

Generation of ReOPN Fragments—Full-length human reOPN was generated as described previously (27). An expression plasmid containing histidine-tagged protein was generated by cloning a polymerase chain reaction fragment containing the full-length splice variant of human OPN (OPN10), amino acid residues 1–317, into the *Bam*HI site of vector pQE30 (Qiagen, Chatsworth, CA). *Escherichia coli* transformed with the His-OPN plasmid was grown in LB with 100 μg/ml ampicillin and induced with isopropyl-1-thio-β-D-galactopyranoside at 37 °C to express the histidine-tagged protein. The reOPN was purified from bacterial cells according to the manufacturer's instructions (QIA expression kit, Qiagen), chromatographed on Ni^{2+}-nitrilotriaacetic acid resin, and eluted with 0.2 M imidazole. The purified reOPN was analyzed by SDS-PAGE.

Osteopontin N- and C-terminal proteins were generated by thrombin cleavage of bacterially expressed GST-OPN fusion proteins. Expression plasmids containing GST-OPN were generated by cloning polymerase chain reaction-amplified N-terminal (amino acid residues 17–169) and C-terminal (amino acid residues 170–317) osteopontin fragments into *Bam*HI/*Eco*RI sites of pGEX-2T (Amersham Pharmacia Biotech). The N-terminal 10N and 30N fragments were amplified from cDNAs encoding two different splice forms of OPN, OP10, and OP30, respectively. The 30N fragment is identical to the 10N fragment except that it includes the alternate splice exon 5 (amino acid residues 59–72). The C-terminal 10C fragment was amplified from OP10. The plasmid OP10 was provided by Dr. Larry Fisher (28). OP30 was obtained from ATCC (29). The GST-OPN fusion constructs were DNA sequence verified. *E. coli* JM109 cells transformed with these GST-OPN plasmids were grown in LB with 150 μg/ml ampicillin and then induced with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside for 2 h at 37 °C to express the fusion proteins. The GST-OPN fusion proteins were purified basically according to the manufacturer's instructions (GST gene fusion system, Amersham Pharmacia Biotech) with glutathione-Sepharose beads. The OPN N- or C-terminal fragments were separated from GST-bound beads by treating with 0.1 unit of biotinylated thrombin/μg of GST-OPN (Novagen, Madison, WI) for 2 h. The cleavage reaction was stopped with biotinylated-PPACK (400 ng/unit of biotinylated thrombin). Supernatants were collected and biotinylated thrombin and PPACK were removed by incubation with streptavidin-agarose beads (Pierce) and separation of beads from supernatant.

Cell Culture—HSMC were obtained by enzymatic digestion as described previously (30). Briefly, medial tissues were separated from segments of human fetal aorta obtained at autopsy. Small pieces of tissue (1 to 2 mm^3) were digested overnight in DMEM supplemented with 165 units/ml collagenase type I, 15 units/ml elastase type III, and 0.375 mg/ml soybean trypsin inhibitor at 37 °C. Single cell suspensions were placed in 6-well plates and cultured for several weeks in DMEM supplemented with 20% FCS at 37 °C in a humidified atmosphere containing 5% CO_2. Cultures which formed colonies were collected at confluence and maintained in growth medium (DMEM containing 15% FBS and 1 mM sodium pyruvate supplemented with 100 units/ml penicillin and 100 mg/ml streptomycin; final inorganic phosphate concentration = 1.4 mM). Purity of cultures was assessed by positive immunostaining for α-SM actin and calponin, and absence of von Willebrand factor staining as described previously (30). HSMC up to passage 8 were used for these experiments.

Induction of Calcification—HSMCs were routinely subcultured in growth medium. At confluence, the cells were switched to calcification medium (DMEM containing 15% FBS in the presence of 2 mM inorganic phosphate (unless otherwise stated) supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin) for up to 14 days. The medium was replaced with fresh medium every 2 days. For time course experiments, the first day of culture in calcification medium was defined as day 0.

Quantification of Calcium Deposition—Cells were decalcified with 0.6 N HCl for 24 h. The calcium content of HCl supernatants was determined colorimetrically by the *o*-cresolphthalein complexone method (Calcium Kit; Sigma) as described previously (9). After decalcification, the cells were washed three times with phosphate-buffered saline and solubilized with 0.1 N NaOH, 0.1% sodium dodecyl sulfate (SDS). The protein content was measured with a BCA protein assay kit (Pierce, Rockford, IL). The calcium content of the cell layer was normalized to protein content.

Preparation of P-OPN—The reOPNs (10 μg) were phosphorylated in the presence of 0.3 mM ATP with or without [γ-^{32}P]ATP (specific activity 1 μCi/mmol) and 100 ng of CKII in 100 μl of assay buffer (20 mM HEPES, pH 7.5, 15 mM NaCl, 12 mM $MgCl_2$). At various times during the reaction, incorporation of [γ-^{32}P]ATP into proteins was monitored by spotting 1 μg of proteins on glasswool, followed by washing with 5% trichloroacetic acid to remove unincorporated [γ-^{32}P]ATP and counting incorporated ^{32}P in 5 ml of liquid scintillant. Incorporation of ^{32}P into proteins was evaluated by SDS-PAGE on 10% gels, and radiolabeled proteins were detected by autoradiography. Western blot confirmed that the isolated protein was OPN.

RNA Isolation and Northern Blot Analysis—Total RNA was isolated from HSMCs by extraction with Trizol as suggested by the manufacturer (Life Technologies, Inc.). Twenty micrograms of total RNA were electrophoresed on 1% agarose gels containing formaldehyde, and transferred to a nylon filter (Zeta-Probe GT, Bio-Rad). Blots were prehybridized at 42 °C for 1 h in a buffer containing 50% formamide, 0.75 M NaCl, 50 mM Tris-HCl (pH 7.5), 1% SDS, 10% dextran sulfate, 20 μg/ml denatured salmon sperm DNA, and 1× Denhardt's solution and then hybridized at 42 °C for 24 h with cDNA probe for human OPN which was labeled with [α-^{32}P]dCTP (3000 Ci/ml; NEN Life Science Products Inc., Boston, MA) by use of a random priming method (Megaprime cDNA labeling system, Amersham Pharmacia Biotech). Blots were washed and autoradiographed with x-ray film at −70 °C. The amounts of RNA were quantified by densitometric scanning and normalized by comparison with GAPDH.

Preparation of Dephosphorylated OPN—5 μg of P-OPN was dephosphorylated in the presence of 2 units of alkaline phosphatase in 50 mM HEPES (pH 10), 1 mM $MgCl_2$ for up to 24 h at 37 °C. The samples were analyzed by SDS-PAGE on 10% gels, and radiolabeled proteins were detected by autoradiography.

Metabolic Labeling of HSMCs and Immunoprecipitation of OPN—HSMCs were cultured in DMEM supplemented with 15% FBS until confluent and then switched to calcification medium containing 2 mM

phosphate. HSMC were grown in the calcification medium for 10 days to promote mineralization. To detect phosphorylated OPN, HSMC were incubated in phosphate-free DMEM for 30 min, followed by incubation in phosphate-free DMEM (1 ml/dish) containing [^{32}P]orthophosphate (1 mCi of ^{32}P/dish) for 6 h. After 6 h incubation, the medium was carefully collected. The supernatants were immunoprecipitated with anti-OPN antibody (OP-199) or a goat IgG as a negative control at 4 °C. Immune complexes were recovered by binding to protein A-Sepharose and washing five times with IP wash buffer (50 mM Hepes, pH 7.4, 50 mM NaCl, 50 mM sodium fluoride, 10 mM sodium pyrophosphate, 5 mM EDTA, 1% Nonidet P-40, 2 μg/ml aprotinin, 0.5 μg/ml leupeptin, and 200 μM phenylmethylsulfonyl fluoride). The immunoprecipitated proteins were suspended in 20 μl of sample buffer (0.07 mM Tris-HCl, pH 6.8, 3% SDS, 10% glycerol, and 0.01% bromphenol blue). The samples were analyzed by SDS-PAGE on 10% gels, and radiolabeled proteins were detected by autoradiography. Western blot confirmed that the isolated protein was OPN.

Statistics—Data were analyzed for statistical significance by ANOVA with post-hoc Scheffe's F analysis, unless otherwise stated. These analyses were performed with the assistance of a computer program (StatView version 4.11, Abacus Concepts, Berkeley, CA).

RESULTS

We have developed an *in vitro* model for human vascular calcification. In this system elevating inorganic phosphate to the hyperphosphatemic range (2 mM) induced matrix calcification. We first examined the effect of native smooth muscle cell-derived OPN (native OPN) on HSMC calcification. Native OPN was previously shown to be both phosphorylated and glycosylated (24). Native OPN inhibited HSMC calcification in a dose-dependent manner (calcified control (vehicle-treated cells) *versus* 15 nM native OPN-treated cells: 153.4 ± 27.1 *versus* 62.7 ± 4.8 (μg/mg protein), mean ± S.D. (n = 3)) (Fig. 1*A*). This finding was consistent with previous studies showing that native OPN inhibited bovine smooth muscle cell calcification (26). We next examined the effect of bacterial-derived rat and human reOPNs on HSMC calcification. In contrast to native OPN, both rat and human reOPNs dose dependently promoted calcification (calcified control (vehicle-treated cells) *versus* 15 nM rat reOPN-treated cells: 153.4 ± 27.1 *versus* 244.6 ± 31.5 (μg/mg of protein), mean ± S.D. (n = 3)) (calcified control (vehicle-treated cells) *versus* 15 nM human reOPN-treated cells: 153.4 ± 27.1 *versus* 254.4 ± 14.9 (μg/mg protein), mean ± S.D. (n = 3)) (Fig. 1, *B* and *C*). Since the bacterial products contain neither phosphorylation nor glycosylation, these data suggested that the ability of OPN to inhibit calcification was dependent on post-translational modification.

In order to compare the bioactivity of phosphorylated and non-phosphorylated OPN, human reOPN was phosphorylated with CKII. CKII phosphorylated OPN in a time-dependent manner for up to 90 min (Fig. 2). A mean molar ratio of phosphate:OPN of approximately 20 was achieved (Table I). This is in good agreement with the number of putative CKII phosphorylation sites found in the human OPN sequence (23).

We next examined the effect of P-OPN on HSMC calcification. P-OPN inhibited calcification in a dose-dependent manner, and at 75 nM P-OPN, calcium deposition decreased to 31% of control cultures (calcified control (vehicle-treated cells) *versus* 75 nM phosphorylated OPN-treated cells: 142.2 ± 2.5 *versus* 42.8 ± 5.0 μg/mg of protein, mean ± S.D. (n = 3)) (Fig. 3*A*). To determine whether the extent of OPN phosphorylation effected its inhibitory potential, we prepared differentially phosphorylated OPNs by incubating reOPN with CKII for limiting periods of times. OPN was phosphorylated with CKII for times ranging from 5 to 90 min. As shown in Fig. 3*B*, P-OPN inhibited calcification in proportion to the extent of phosphorylation (calcified control vehicle-treated cells *versus* 15 nM OPN phosphorylated with CK II for 90 min-treated cells: 169.6 ± 1.5 *versus* 73.8 ± 7.9 μg/mg of protein, mean ± S.D. (n = 3)) (Fig. 3*B*).

To identify the specific phosphorylated domain of OPN important for inhibition of calcification, we made use of reOPN N- and C-terminal fragments. These fragments represent the fragments that result from thrombin cleavage of osteopontin at serine 169. The N-terminal fragment contains amino acids 1–169 and the C-terminal fragment contains amino acids 170–317 of human OPN. In addition, two different N-terminal fragments were prepared, 10N and 30N, representing two alternative splice variants described for human OPN (28, 29). The 10N fragment differs from the 30N fragment by deletion of exon 5. This exon encodes 14 amino acids including potential sites for phosphorylation (28, 29). Each fragment was phosphorylated by incubating with CKII for 90 min. As shown in Table I, mean molar ratios of phosphate:30N-terminal, 10N-terminal, 10C-terminal OPN fragments of 12.1, 9.7, and 8.6, respectively, were achieved. The slightly lower level of phosphorylation of the 10N fragment compared with the 30N fragment is likely due to the extra phosphorylation sites present in the 30N fragment that contains exon 5 (29). The effect of N-terminal and C-terminal P-OPN fragments on HSMC calcification was then investigated. Whereas nonphosphorylated OPN fragments did not significantly decrease calcification, all phosphorylated OPN fragments potently inhibited calcification (calcified control (vehicle-treated cells) *versus* 15 nM phosphorylated OPN (full-length) *versus* 15 nM phosphorylated 30N-OPN *ver-*

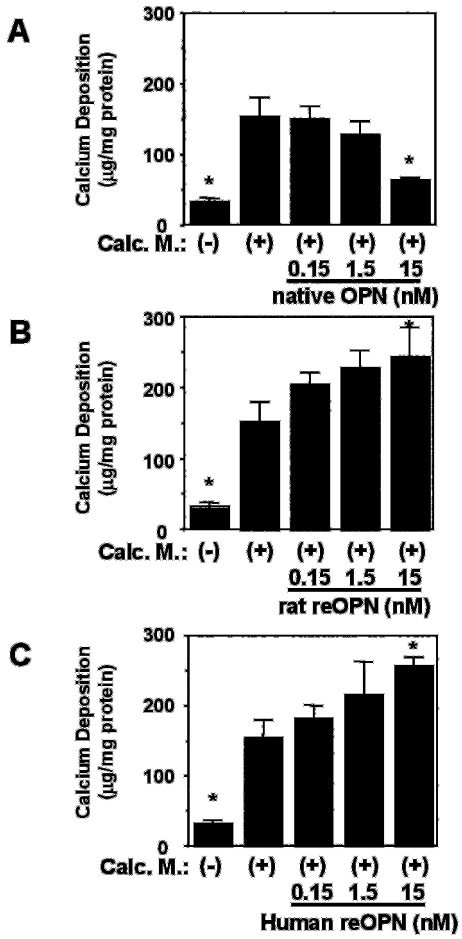

FIG. 1. **Effect of native OPN and reOPN on HSMC calcification.** HSMCs were cultured in growth medium or calcification medium for 4 days in the presence of indicated concentration of: *A,* native OPN or its vehicle alone; *B,* rat reOPN or its vehicle alone; or *C,* human reOPN or its vehicle alone. The calcium contents were measured by the *o*-cresolphthalein complexone method, normalized by cellular protein content, and are presented as mean ± S.D. (n = 3). The differences compared with calcified control were statistically significant (*, $p < 0.01$, Scheffe's test). The + and − indicate the presence and absence of calcification medium, respectively.

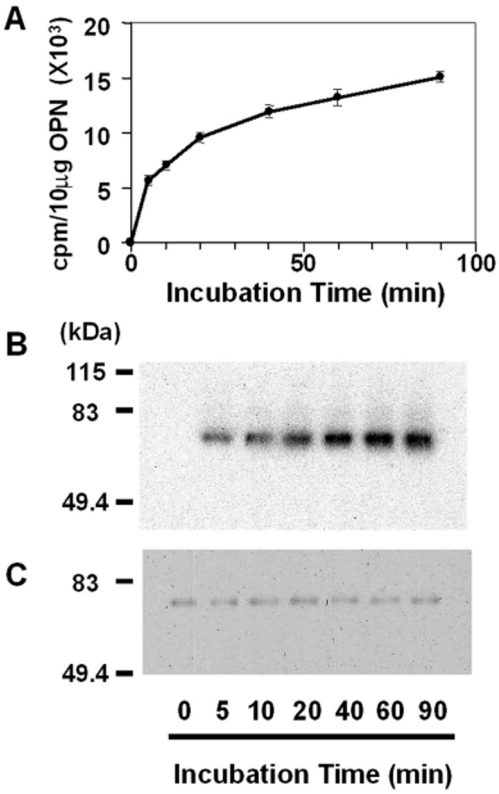

FIG. 2. **Time course of OPN phosphorylation by CKII.** *A,* phosphorylation was performed by incubation of 15 nM OPN with CKII for the indicated times as described under "Materials and Methods." Incorporation of ^{32}P into proteins was evaluated by trichloroacetic acid precipitation and SDS-PAGE on 10% gels. *A,* time course of trichloroacetic acid precipitable counts/min in OPN following incubation with CKII. Data represent mean ± S.D. ($n = 3$). *B,* radiolabeled P-OPN was electrophoresed, transferred to membrane, and detected by autoradiography (*C*). Immunoblot was performed with anti-rat OPN polyclonal antibody. The position of migration of molecular weight markers (kDa) is indicated on the *left side*.

TABLE I
Incorporated phosphate per mole of OPN

Phosphorylation was performed by incubation of 10 μg of human recombinant OPN with CKII for 90 min as described under "Materials and Methods." Incorporation of ^{32}P into proteins was evaluated by spotting 1 μg of proteins on glasswool, followed by washing with 5% trichloroacetic acid to remove unincorporated [γ-^{32}P]ATP and counting incorporated ^{32}P in 5 ml of liquid scintillant. The data indicate mean molar ratio of phosphate:OPN and are presented as mean ± S.D., $n = 3$.

OPN	Mol of phosphate/mol of OPN
Full-length	20.0 ± 0.4
30N	12.1 ± 0.4
10N	9.7 ± 0.3
10C	8.6 ± 0.4

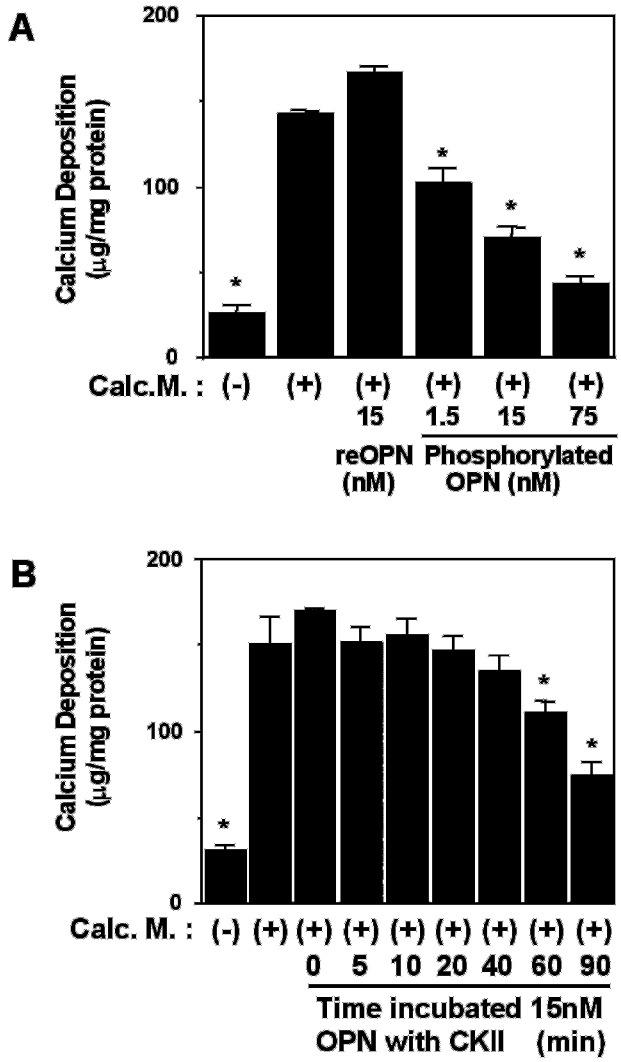

FIG. 3. **Effect of OPN phosphorylated with CKII on HSMC calcification.** *A,* human reOPN was phosphorylated with CKII for 90 min. HSMCs were cultured in growth medium or calcification medium for 4 days in the presence of the indicated concentrations of phosphorylated OPN or its vehicle. The calcium contents were measured by the *o*-cresolphthalein complexone method, normalized by cellular protein content and are presented as mean ± S.D. *, differences compared with calcified control were statistically significant ($p < 0.01$, Scheffe's test). The + and − indicate the presence and absence of calcification medium, respectively. *B,* human reOPN was phosphorylated with CKII for the indicated times to generate differentially phosphorylated P-OPNs. HSMCs were cultured in growth medium or calcification medium for 4 days in the presence of 15 nM of each P-OPN or vehicle. The calcium contents were measured by *o*-cresolphthalein complexone method, normalized by cellular protein content, and are presented as mean ± S.D. ($n = 3$). *, differences compared with uncalcified control were statistically significant ($p < 0.01$, Scheffe's test). The + and − indicate the presence and absence of calcification medium, respectively.

sus 15 nM phosphorylated 10N-OPN *versus* 15 nM phosphorylated 10C-OPN-treated cells: 145.0 ± 10.2 *versus* 27.3 ± 3.1 *versus* 25.8 ± 0.6 *versus* 27.6 ± 3.5 *versus* 20.6 ± 0.6 μg/mg protein, mean ± S.D. ($n = 3$)) (Fig. 4*A*). These data suggested that the organization of phosphate groups guided by OPN primary structure in both the N- and C-terminal fragments were most critical for anticalcification properties of OPN. Furthermore, these data indicate that anticalcification properties of OPN are RGD-independent in this *in vitro* model system. This is consistent with previous observations that OPN's ability to bind and block hydroxyapatite crystal growth most likely explains its ability to inhibit biomineralization *in vitro* (26, 31).

We next examined the effect of alkaline phosphatase on OPN's ability to inhibit HSMC calcification. P-OPN was dephosphorylated by alkaline phosphatase treatment as confirmed by 10% SDS-PAGE (Fig. 5*A*) with no loss of osteopontin protein (Fig. 5*B*). Although P-OPN inhibited calcification, after treatment with alkaline phosphatase, calcium deposition was restored (calcified control (vehicle-treated cells) *versus* recombinant OPN *versus* phosphorylated OPN *versus* dephosphorylated OPN-treated cells: 147.3 ± 9.6 *versus* 172.5 ± 5.8 *versus* 44.6 ± 5.2 *versus* 162.4 ± 10.4 μg/mg of protein, mean ± S.D. ($n = 3$)) (Fig. 5*C*). These data suggested that alkaline phosphatase could be a physiological regulator of OPN's anticalcification activity.

Finally, we examined the expression and phosphorylation

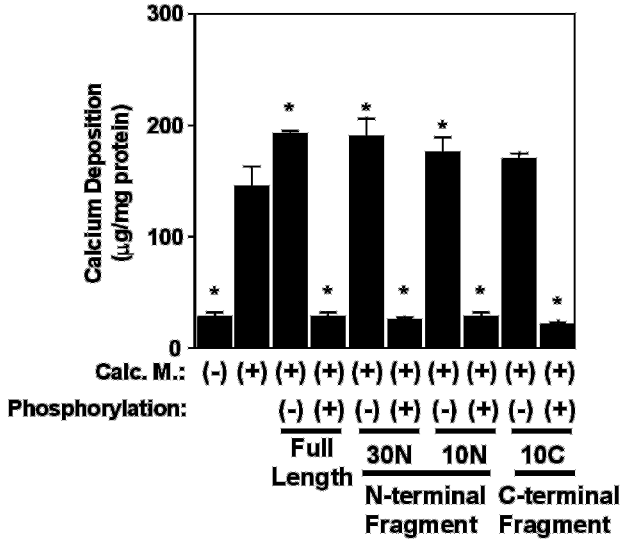

FIG. 4. **Effect of OPN fragments on HSMC calcification.** Human recombinant OPN (full-length), 30N-OPN(N-terminal fragment), 10N-OPN(N-terminal fragment), and 10C-OPN(C-terminal fragment) were phosphorylated with CKII for 90 min. HSMCs were cultured in growth medium or calcification medium for 4 days in the presence of 15 nM phosphorylated (+) or nonphosphorylated (−) full-length OPN, N-terminal fragment (30N and 10N), or C-terminal fragment (10C). The calcium contents were measured by *o*-cresolphthalein complexone method, normalized by cellular protein content, and are presented as mean ± S.D. ($n = 3$). *, differences compared with uncalcified control were statistically significant ($p < 0.01$, Scheffe's test).

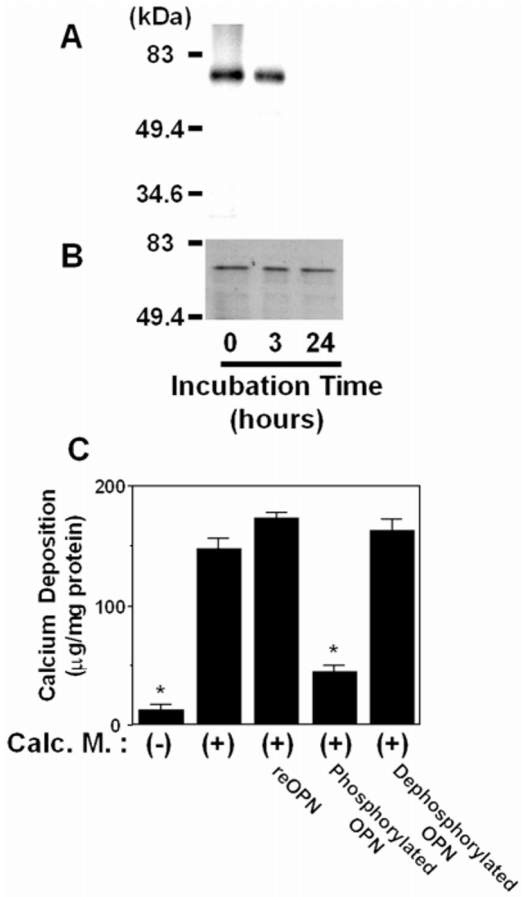

FIG. 5. **Effect of OPN dephosphorylated with alkaline phosphatase on HSMC calcification.** Human recombinant OPN was phosphorylated with CKII for 90 min and then P-OPN was treated with 2 units of alkaline phosphatase for 0, 3, or 24 h. Dephosphorylated OPN was evaluated by SDS-PAGE on 10% gels, transferred to membrane, and detected by (*A*) autoradiography and (*B*) specific immunoblot for OPN. The position of migration of molecular weight markers (kDa) is indicated on the *left side*. *C,* HSMCs were cultured in calcification medium or growth medium in the presence of 15 nM recombinant OPN, 15 nM OPN phosphorylated with CKII, 15 nM OPN dephosphorylated with alkaline phosphatase for 24 h, or its vehicle. The calcium contents were measured by *o*-cresolphthalein complexone method, normalized by cellular protein content, and are presented as mean ± S.D. ($n = 3$). *, differences compared with uncalcified control were statistically significant ($p < 0.01$, Scheffe's test). The + and − indicate the presence and absence of calcification medium, respectively.

state of endogenous OPN during *in vitro* calcification of HSMC cultures by Northern blot analysis. A 1.6-kilobase OPN mRNA was detected in both calcified and non-calcified HSMC. The expression of OPN mRNA was clearly decreased during the calcification process (Fig. 6). To determine the phosphorylation state of OPN during culture calcification, HSMC were metabolically labeled by the addition of [^{32}P]orthophosphate to the culture medium. The labeled osteopontin in the medium was immunoprecipitated and separated by 10% SDS-PAGE and visualized by autoradiography. While a strong band corresponding to phosphorylated OPN was visualized in non-calcifying HSMC, no phosphorylated OPN was detected calcifying HSMC (Fig. 7*A*). This was not due to the inability of the antibody to detect non-phosphorylated OPN since Western blot analysis indicated that OPN protein was present in both non-calcified and calcified HSMC culture supernatants (Fig. 7*B*). Consistent with the mRNA data, somewhat less OPN was detected in calcified *versus* noncalcified cultures. These data suggest that decreased synthesis and secretion of phosphorylated OPN may contribute to calcification of HSMC under the conditions used in this study.

DISCUSSION

In this study, we have demonstrated that the ability of OPN to inhibit calcification of HSMC cultures is dependent on post-translational modification. Although bacteria-derived reOPN did not inhibit HSMC culture mineralization, rat native OPN showed strong anticalcification activity. We found that reOPN phosphorylated by CKII dose dependently inhibited calcification. Inhibition of calcification was proportional to the number of phosphorylated sites in OPN. While nonphosphorylated N-terminal and C-terminal reOPN fragments did not effect HSMC culture calcification, phosphorylated versions of these fragments strongly inhibited HSMC calcification. Furthermore, OPN dephosphorylated with alkaline phosphatase did not have an inhibitory effect on HSMC culture calcification. Finally, the expression of OPN mRNA, secretion of protein, and fraction of phosphorylated osteopontin decreased during calcification. These results indicate that phosphorylation of OPN is required for its inhibitory effect on HSMC culture calcification.

We previously found that the major mineral deposited in bovine smooth muscle cell cultures was hydroxyapatite (HA) (26). OPN has a high affinity for HA (31), and was previously shown to inhibit *de novo* HA formation in both metastable calcium phosphate solutions, and steady state agarose (32) and gelatin (33) gels. In contrast, OPN showed no ability to nucleate HA (32). Consistent with these findings, native OPN inhibited calcification of both human (present study) and bovine smooth muscle cell cultures (26), and was shown by immunogold electron microscopy to bind to growing hydroxyapatite crystals within the extracellular matrix (26). Thus, it is likely that the ability of OPN to bind to HA and block crystal formation underlies its potent effect on vascular smooth muscle calcification *in vitro.*

OPN is highly anionic due to its elevated content of the acidic amino acid, aspartate, and its high degree of phosphorylation. The primary structure of OPN contains over 20 potential phos-

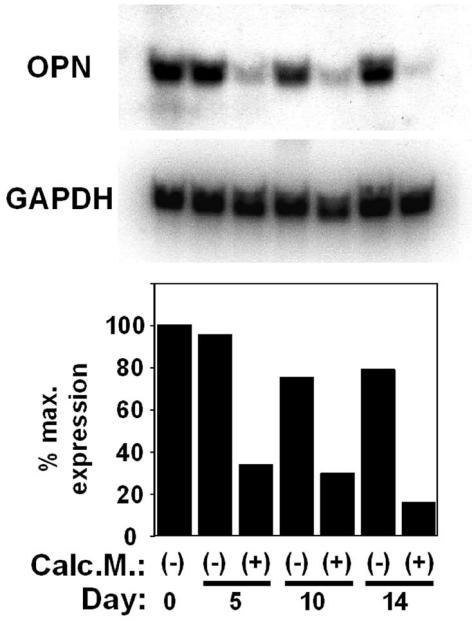

FIG. 6. **The expression of OPN in HSMC during calcification.** Confluent HFSMC were cultured in growth medium (−) or calcification medium (+) for the indicated times. Twenty micrograms of total RNA obtained from HFSMC at the indicated times were analyzed by Northern blot analysis with a cDNA for human OPN (*upper panel*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (*middle panel*). The densitometric data of scanned autoradiographs was normalized for GAPDH and plotted as the percentage of maximum expression (*lower panel*).

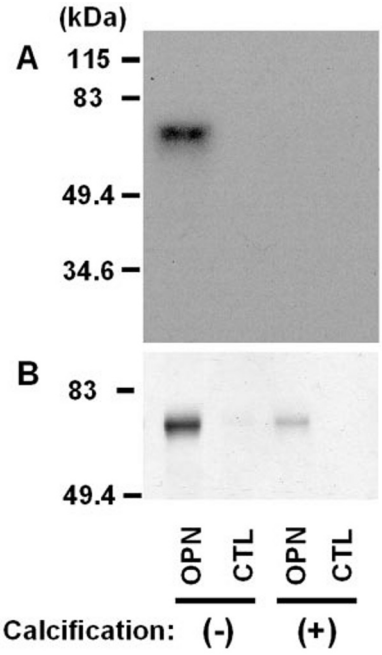

FIG. 7. **Immunoprecipitation of OPN during HSMC calcification.** Confluent HSMCs were cultured in calcification medium (+) or growth medium (−) for 10 days. HSMCs were incubated in phosphate-free DMEM (1 ml/dish) containing [^{32}P]orthophosphate (1 mCi of ^{32}P/dish) for 6 h. After 6 h incubation, the medium were carefully collected and immunoprecipitated with the anti-rat OPN antibody or a goat IgG as a negative control at 4 °C. Immune complexes were recovered by binding to protein A-Sepharose and washing five times with IP wash buffer. All samples were evaluated by SDS-PAGE on 10% gels, transferred the membrane, and detected by autoradiography (*upper panel*). Western blot analysis confirmed that the protein isolated by immunoprecipitation from HSMC was osteopontin (*lower panel*).

phorylation sites for various protein kinases (28, 29). Not all of these sites appear to be utilized, however, since it has been reported that rat bone OPN contains 12 phosphoserines and 1 phosphothreonine (19), bovine milk OPN contains 27 phosphoserines and 1 phosphothreonine (17), and chicken osteoblast OPN contained 7 phosphoserine and 1 phosphothreonine (33). The majority of the phosphorylations occur on serines within consensus phosphorylation motifs for casein kinases such as mammary gland Golgi casein kinase and CKII (34). Indeed, in purified systems, CKII was the predominant enzyme capable of phosphorylating chicken OPN (35), and Golgi kinase had strong activity toward rat recombinant OPN (36). Consistent with those findings, human recombinant OPN was phosphorylated with CKII and a mean molar ratio of phosphate:OPN of 20 was achieved in the present studies. Which of these enzymes phosphorylates OPN *in vivo*, however, is still controversial.

Our studies indicate that the presence of phosphorylated residues is particularly important for OPN's anticalcification effects in HSMC cultures. We found that bacterial-derived recombinant OPN, devoid of any post-translational modification, did not inhibit HSMC culture calcification, and on the contrary, showed a slight stimulatory effect. However, following phosphorylation with CKII, bacterial OPN was as potent as native OPN in inhibiting HSMC calcification. Furthermore, the anticalcific potency of OPN depended on the extent of phosphorylation, with minimal inhibition occurring unless >9 mol of phosphate were incorporated per mole of osteopontin. While the precise sites of phosphorylation in our CKII-treated bacterial OPN have not yet been identified, these data suggest that either specific phosphorylated sequences or arrangement of phosphorylated sequences is required for OPN function in anticalcification. In addition, dephosphorylation of P-reOPN with alkaline phosphatase completely inhibited calcification inhibitory activity. These studies are consistent with previous observations in cell-free systems, showing that treatment of OPN with alkaline phosphatase removed 84% of the covalently bound phosphate and reduced HA inhibiting activity by more than 40-fold (37). Phosphorylation has also been suggested to regulate the cell binding activity of OPN. In one study, partial dephosphorylation of bovine OPN by tartrate-resistant acid phosphatase resulted in decreased osteoclast binding (38). On the other hand, CKII treatment of recombinant rat OPN enhanced osteoclast adhesion, even though only low mean molar ratio of phosphate:OPN of approximately 1.5 was achieved (39).

The present studies are the first to use defined OPN peptide fragments to examine sequences important for OPN's calcification inhibitory activity. The data indicate that OPN's inhibitory activity on HSMC calcification is independent of the RGD sequence and polyaspartic acid domain since a fragment lacking both the RGD and polyaspartate sequences (10C) exhibited inhibitory potency equivalent to fragments which contained both domains (30N and 10N). This was somewhat unexpected, since previous studies in a cell-free system showed that poly-L-aspartic acid was nearly as potent as bone-derived OPN in inhibiting HA formation (37). One explanation of this discrepancy is that the calcium binding properties of OPN may be more important in inhibiting HA formation in cell-free systems than in our cell culture system, since the polyaspartic acid sequence and both phosphorylated and nonphosphorylated forms of OPN have been shown to bind calcium with specificity (40).

Finally, to determine whether regulation of OPN phosphorylation might occur during the development of HSMC culture mineralization, we examined endogenous OPN mRNA, OPN protein, and phosphorylated OPN levels with time in mineralizing cultures. Our data indicate that OPN mRNA levels and total as well as phosphorylated OPN protein levels decline as

HSMC cultures calcify. Thus OPN synthesis as well as phosphorylation are inversely correlated with tissue culture mineralization.

Our findings suggest that regulation of phosphorylation state may be a common mechanism controlling OPN's functional activities. Several recent studies support this notion. Normal rat kidney cells secrete both the phosphorylated (pp69) and non-phosphorylated (np69) form of OPN. pp69 is cell surface-associated, whereas np69 is not. On the other hand, np69 can form a heat-dissociable complex with fibronectin, while pp69 cannot (23). Furthermore, phorbol ester stimulation of P-OPN in JB6 epidermal cells was correlated with tumorigenic morphological changes and anchorage independent growth. On the other hand, calcitriol stimulated synthesis and secretion of nonphosphorylated OPN in JB6 cells, and these transformed cells lacked the tumorigenic properties observed in phorbol ester-treated cells (25). These observations, combined with our studies, suggest that phosphorylated and nonphosphorylated forms of OPN have different functional properties. Identification of mechanisms controlling OPN phosphorylation state is thus of paramount interest in future studies.

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