Inhibition of hydroxyapatite formation by osteopontin phosphopeptides

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Osteopontin (OPN) is an acidic phosphoglycoprotein that is believed to function in the prevention of soft tissue calcification. *In vitro* studies have shown that OPN can inhibit the formation of hydroxyapatite (HA) and other biologically relevant crystal phases, and that this inhibitory activity requires phosphorylation of the protein; however, it is not known which phosphorylated residues are involved. We have synthesized peptides corresponding to four phosphoserine-containing sequences in rat OPN: OPN7–17, containing phosphoserines 10 and 11; OPN41–52, containing phosphoserines 46 and 47; OPN248–264, containing phosphoserines 250, 257 and 262; and OPN290–301, containing phosphoserines 295–297. The abilities of these peptides to inhibit *de novo* HA formation were determined using a constant-composition autotitration assay. All four OPN phosphopeptides caused a dose-dependent increase in nucleation lag time, but did not significantly affect subsequent formation of the crystals. However, OPN41–52 (inhibitory constant 73.5 min/μM) and OPN290–301 (72.2 min/μM) were approx. 4 times more potent inhibitors than OPN7–17 (19.7 min/μM) and OPN247–264 (16.3 min/μM). 'Scrambling' the amino acid sequence of OPN290–301 resulted in decreased potency (45.6 min/μM), whereas omission of the phosphate groups from this peptide caused a greater decrease (5.20 min/μM). These findings have identified phosphorylated sequences that are important for the ability of rat bone OPN to inhibit HA crystal formation, and suggest that negative-charge density is an important factor in this activity.

Key words: calcification, hydroxyapatite, mineralization, osteopontin, phosphorylation, sialoprotein.

INTRODUCTION

Osteopontin (OPN) is an acidic glycoprotein of approx. 300 residues that is highly expressed in, but not restricted to, mineralized tissues [1]. Studies have suggested that OPN is a multi-functional protein, involved in a variety of pathological and physiological processes: cell migration, adhesion and signalling [2]; tumorigenesis and metastasis [3]; and regulation of osseous and ectopic calcification [4]. Several features of the protein are conserved between species, including a stretch of contiguous aspartic acid residues, which is thought to mediate the mineral-binding properties of the protein [5]. In addition, OPN contains an RGD (Arg-Gly-Asp) integrin-binding domain, a thrombin-cleavage site and numerous consensus sequences for glycosylation and kinase-mediated phosphorylation [1]. Post-translational modification of OPN can increase its mass by 10–15 kDa [6].

OPN is encoded by a single-copy gene, but a number of isoforms exist which differ mainly in the level of post-translational modification, particularly phosphorylation. OPN can be phosphorylated by casein kinase II, cAMP-dependent protein kinase, Golgi casein kinase and protein kinase C [7–9]. Bovine milk OPN contains 28 phosphorylated residues that have been mapped by amino acid analysis in conjunction with MS [10]. From a partial analysis of the rat bone isoform, Neame and Butler [11] reported the locations of ten phosphorylated residues (Ser^{10}, Ser^{11}, Ser^{46}, Ser^{47}, Ser^{250}, Ser^{257}, Ser^{262}, Ser^{295}, Ser^{297} and Ser^{298}), plus two other 'likely' sites (Thr^{154} and Ser^{160}). We have recently confirmed three of the phosphorylation sites of rat bone OPN (Thr^{154}, Ser^{262} and Ser^{295}) reported by these investigators, and found an additional ten sites (M. Keykhosravani, A. Doherty-Kirby, H. A. Goldberg, G. K. Hunter and G. Lajoie, unpublished work). It appears that the level of phosphorylation can be regulated within bone cells, as treatment of ROS 17/2.8 osteoblast-like cells with 1,25-dihydroxy vitamin D_3 resulted in the expression of a less-phosphorylated form of OPN [12]. The fact that the level of phosphorylation can be regulated, together with the existence of many OPN isoforms differing in their level of phosphorylation, implies an important role for post-translational modification in modulating OPN function.

OPN is abundant at sites of pathological calcification and in highly supersaturated physiological fluids. For example, OPN was found to be the major organic constituent of calcium-oxalate-containing kidney stones, where it occurs in layers alternating with those of mineral [13]. OPN is not normally expressed in blood vessels, but is found in atherosclerotic plaque [14,15]. It has been proposed that OPN is expressed by soft tissues to protect against dystrophic calcification [16].

Data from experimental animal models with a targeted disruption of the OPN gene support the notion that OPN functions in an inhibitory manner towards crystal formation *in vivo*. Implantation of glutaraldehyde-fixed aortic leaflets into OPN-null mice resulted in far higher levels of calcification compared with implantation in wild-type animals [17]. Crossing $opn^{-/-}$ mice with mice carrying a null allele for matrix Gla protein resulted in twice the level of arterial calcification observed in $mgp^{-/-}$ alone [18]. Similarly, OPN-null mice fed on a hyperoxaluria-inducing ethylene glycol diet developed significantly higher levels of intratubular deposition of calcium oxalate than wild-type animals, which also exhibited up-regulation of OPN expression in kidney [19].

OPN has also been shown to inhibit calcification *in vitro*. Studies performed in our laboratory and in others have shown that

Abbreviations used: Fmoc, fluoren-9-ylmethoxycarbonyl; HA, hydroxyapatite; OPN, osteopontin; RP-HPLC, reversed-phase HPLC; TFA, trifluoroacetic acid.

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OPN is a potent inhibitor of the *de novo* formation and seeded growth of hydroxyapatite (HA), the mineral found in bone [20–22]. It has also been reported that the calcification of vascular smooth muscle cells is potently inhibited by OPN [16]. Furthermore, OPN has been shown to inhibit the *in vitro* formation of other salts, such as calcium oxalate and calcite (calcium carbonate) [23,24].

Phosphorylation of OPN is important for its mineral-modulating activity. It was shown that enzymic dephosphorylation of native OPN decreases its ability to inhibit HA [20,21] and calcite [24]. Likewise, recombinant rat OPN expressed in bacteria increased mineralization of cultured vascular smooth muscle cells, whereas enzymically phosphorylated OPN was a potent inhibitor [25].

Although phosphorylation of OPN appears to be vital for its ability to inhibit mineral growth, it is not known whether or not phosphorylation at particular residues is required for this inhibitory mechanism. To address this question, we have synthesized peptides containing phosphorylated serine residues identified by Neame and Butler [11] in rat bone OPN, and tested these peptides for their effects on *de novo* HA formation using a constant-composition autotitration assay.

EXPERIMENTAL

Peptide synthesis

All amino acid derivatives and resins for the solid-phase peptide synthesis were purchased from NovaBiochem Limited (San Diego, CA, U.S.A.). Peptides were individually synthesized using Fmoc (fluoren-9-ylmethoxycarbonyl) chemistry and Wang resin on a Millipore 9050 Plus PepSynthesizer as previously described [26]. *N*-α-Fmoc-*O*-benzyl-L-phosphoserine was used as a building block for incorporation of phosphate groups into peptides. All protected amino acids were coupled with resin substitution/amino acid/HBTU [2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate]/HOBt (*N*-hydroxybenzotriazole · H_2O)/DIPEA (*N*,*N*′-di-isopropylethylamine) (1:3:2.85:3:3). The phosphorylated peptides were cleaved from the resin using TFA (trifluoroacetic acid)/water/TIS (tri-isopropylsilane) (18:1:1) for 2 h at room temperature (20 °C). These peptides were purified by preparative reversed-phase HPLC (RP-HPLC) using a Agilent Zorbax 300SB-C18 column. An initial purification was carried out for all peptides using a 5–45 % acetonitrile gradient containing 0.1 % TFA over 30 min at a flow rate of 4 ml/min. Fractions were collected, and those containing the expected peptides as determined by electrospray ionization-MS (Micromass Quattro II) were subjected to further RP-HPLC purifications. Quality control was performed via analytical HPLC, and $\geqslant$ 95 % purity was achieved for all peptides used in subsequent studies.

Constant-composition method

Stock solutions containing 5 mM $Ca(NO_3)_2$/150 mM NaCl ($2 \times Ca$) or 6 mM Na_2HPO_4/300 mM NaCl ($4 \times PO_4$) were filtered through 0.2-μm-pore-size membranes. An HA-forming solution was made by combining 3 ml of $2 \times Ca$, 1.5 ml of $4 \times PO_4$ and 1.5 ml of deionized water or peptide dissolved in deionized water. The final concentrations of phosphate, calcium and NaCl were therefore 1.5 mM, 2.5 mM and 150 mM respectively. The reaction solution was added to a double-walled glass vessel connected to a circulating water bath maintained at 37 °C, and the pH of the solution was adjusted to 7.4 using 50 mM NaOH. Water-saturated N_2 was bubbled through the solution to exclude atmospheric CO_2. A calomel pHC4006 electrode (Radiometer Analytical, Villeurbanne, France) connected to a TIM900 titration manager and ABU93 triburette (Radiometer Analytical) was immersed in the reaction solution. The triburette was customized so that two of its burettes operated in the 'master–slave' mode. The 'master' burette contained 150 mM sodium phosphate and 50 mM NaOH, the 'slave' contained 250 mM calcium nitrate and 1.5 M NaCl. The titration manager was programmed to maintain pH 7.4 until titrant volumes of 1 ml were added. All chemicals were purchased from Sigma (St. Louis, MO, U.S.A.) and were $\geqslant$ 99 % pure.

Table 1 Amino acid sequences and molecular masses of OPN phosphopeptides, scrambled OPN290–301 (OPN290–301S*) and non-phosphorylated OPN290–301 (OPN290–301NP)

Phosphoserine (pS) residues are in bold type.

Peptide name	Peptide sequence	Theoretical mass (Da)	Observed mass (Da)
OPN7–17	EFG**pSpS**EEKAHY	1443.27	1442.88
OPN41–52	PQNSV**pSpS**EETDD	1467.21	1466.88
OPN248–264	ID**pS**QASSKA**pS**LEHQ**pS**HE	2093.86	2093.55
OPN290–301	SHELE**pSpSpS**SEVN	1543.45	1542.88
OPN290–301S*	**pS**SHEL**pS**SEV**pS**NE	1543.45	1545.8
OPN290–301NP	SHELESSSSEVN	1303.55	1304.33

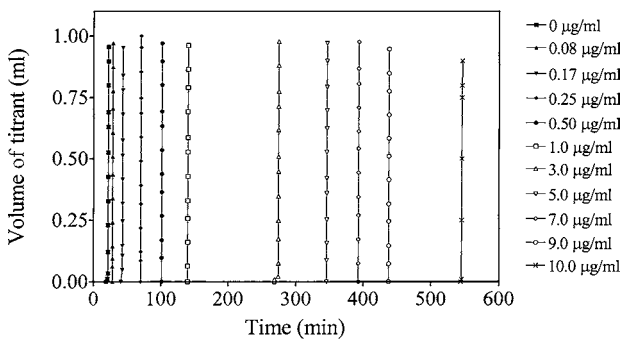

Figure 1 Constant-composition analysis of HA formation in the presence of OPN290–301

The volume of titrant was plotted against time for each peptide concentration tested. Nucleation lag time was defined as the time required for a 1 ml volume of titrant to be added to the reaction solution.

Quantification of the rates of inhibition

The point at which the titration curve intersected the time axis was defined as the nucleation lag time. These lag times were plotted against peptide concentration for each phosphopeptide tested. The slope of the regression line was used as the inhibition constant for that phosphopeptide.

RESULTS

The phosphopeptides synthesized were shown by MS to have the expected molecular masses (Table 1). A constant-composition method was employed to test the effects of these peptides on the formation of HA *in vitro*. Under the conditions used, nucleation of HA occurred at 20–30 min and subsequent HA formation was characterized by an almost-vertical titration curve. The titration curves obtained in the presence of OPN290–301 are shown in Figure 1. The peptide caused a dose-dependent increase in lag

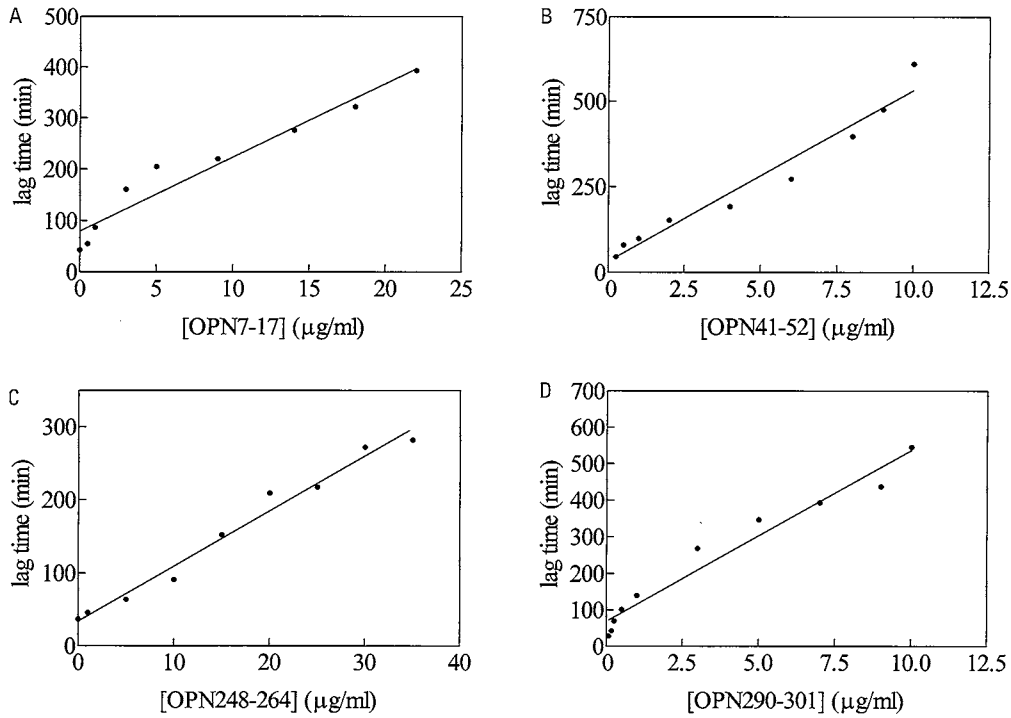

Figure 2 Determination of inhibition constants for OPN7–17 (A), OPN41–52 (B), OPN248–264 (C) and OPN290–301 (D)

The gradient of the regression line was used as an indicator of the inhibitory rate for each peptide analysed. OPN7–17, $y = 13.8x + 90.6$ ($r^2 = 0.943$); OPN41–52, $y = 50.1x + 31.3$ ($r^2 = 0.956$); OPN248–264, $y = 7.8x + 30.3$ ($r^2 = 0.982$); OPN290–301, $y = 46.8x + 68.3$ ($r^2 = 0.961$). All gradients were significantly greater than zero ($P < 0.05$).

Table 2 Inhibitory potencies of synthetic peptides tested in the *de novo* constant-composition assay

The inhibition constants were measured on a mass basis [min/(μg/ml)] and then converted into a molar basis (min/μM) using the molecular masses obtained from MS analysis.

	Inhibitory potency	
Peptide	[min/(μg/ml)]	(min/μM)
OPN7–17	13.7	19.7
OPN41–52	50.1	73.5
OPN248–264	7.8	16.3
OPN290–301	46.8	72.2
OPN290–301S*	29.5	45.6
OPN290–301NP	4.3	5.2

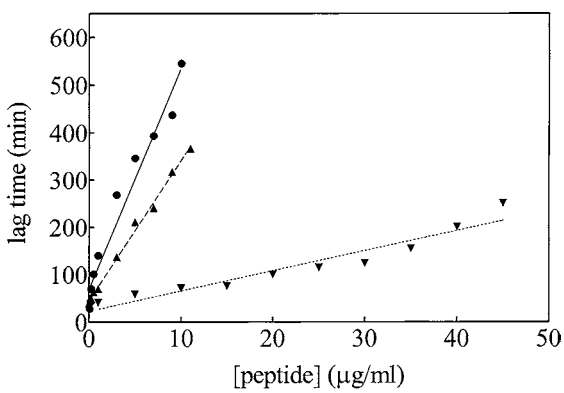

Figure 3 Determination of inhibition constants for OPN290–301 and its scrambled (OPN290–301S*) and non-phosphorylated (OPN290–301NP) versions

Solid line, OPN290–301 ($y = 46.8x + 68.3$; $r^2 = 0.961$); broken line, OPN290–301S* ($y = 29.5x + 46.1$; $r^2 = 0.995$); dotted line, OPN290–301NP ($y = 4.3x + 22.7$; $r^2 = 0.920$). All gradients were significantly greater than zero ($P < 0.05$).

time, but did not significantly affect subsequent HA growth. Qualitatively similar curves were obtained for the other peptides (results not shown).

The time required for initial formation of crystals (nucleation lag time) was plotted against peptide concentration. An approximately linear relationship was found for all peptides (Figure 2). The slopes of these plots of lag time against concentration were used as a measure of the inhibition constant for the peptides [min/(μg/ml) or min/μM] (Table 2). OPN7–17 and OPN248–264 are weaker inhibitors (inhibition rates of 19.7 and 16.3 min/μM respectively) than OPN41–52 and OPN290–301 (inhibition rates of 73.5 and 72.2 min/μM respectively).

Since phosphorylation of OPN has been shown previously to be important for its inhibitory effect on HA, we tested the inhibitory activity of the non-phosphorylated counterpart to OPN290–301 (OPN290–301NP). Compared with OPN290–301, this peptide exhibited a marked reduction in its rate of inhibition (72.2 min/μM compared with 5.2 min/μM respectively) (Figure 3; Table 2). Using different assay conditions, we have found that a non-phosphorylated version of OPN41–52 is approx. 50-fold less potent than the corresponding phosphopeptide (results not shown).

To test whether the sequences of OPN phosphopeptides, rather than their compositions, play an important role in the inhibition of

HA formation, a 'scrambled' version of OPN290–301 (OPN290–301S*) was synthesized and its ability to inhibit the formation of HA was assayed. The results from the constant composition titrations showed that the inhibitory potency of this peptide was less than that of OPN290–301 (45.6 min/μM and 72.2 min/μM respectively) (Figure 3; Table 2).

In comparison, the HA-inhibiting potency of native rat bone OPN has been shown to be similar to those of phosphopeptides OPN41–52 and OPN290–301 when expressed in mass terms, but relatively much greater when expressed in molar terms [27]. Recombinant OPN expressed in bacterial cells, and therefore not phosphorylated, has no HA-inhibiting activity at concentrations as high as 20 μg/ml.

DISCUSSION

To assay the effects of synthetic OPN phosphopeptides on HA formation, a constant-composition autotitration system was used. In this assay, HA forms spontaneously in a supersaturated calcium phosphate solution in which the calcium, phosphate and hydroxyl ion concentrations are controlled by the addition of those ions in the proportions found in HA (5:3:1). The constant-composition method was devised by Nancollas [28] and has been used by him and others to study the growth of HA or calcium oxalate seed crystals. The system described in the present paper involves *de novo* HA formation and thereby allows us to study effects on crystal nucleation.

In this assay, inhibitors cause dose-dependent increases in nucleation lag time. By plotting lag time against inhibitor concentration, we have demonstrated an empirical linear relationship between these qualities.

Although the bone isoform of OPN has not been completely characterized, *in vitro* studies performed on this protein show that it binds HA crystals with high affinity, and can potently inhibit the formation of HA [21,29]. As described above (see the Introduction), phosphorylation of OPN is required for its crystal-inhibiting activity. The only published analysis of phosphorylation sites in native bone OPN positively identified ten serine residues that were variably phosphorylated, but did not analyse a large central region within the molecule [11]. Our choice of peptides was based on the finding that these known phosphoserines occur in four clusters of two to three residues each.

Each of the synthetic OPN phosphopeptides used in the present study exhibits inhibitory activity, suggesting that OPN has multiple HA-inhibiting sequences. This finding is in agreement with the studies of Giachelli and co-workers, who showed that enzymically phosphorylated recombinant OPN peptides corresponding to the N-terminal and C-terminal halves of the protein, and containing similar amounts of phosphate, are of similar potencies in inhibiting the calcification of vascular smooth muscle cell cultures [25].

The two most inhibitory peptides are OPN41–52, containing phosphoserines at residues 46 and 47, and OPN290–301, containing phosphoserines 295–297. Since the weakly inhibiting OPN7–17 and the strongly inhibiting OPN41–52 each contain two adjacent phosphoserines, the differences in potency of the peptides may be related to the number of closely spaced acidic residues contained within the molecules. An examination of the sequences of OPN7–17 (EFG**pSpS**EEKAHY) and OPN 41–52 (PQNSV**pSpS**EETDD) (where pS represents phosphoserine, also indicated in bold) reveals that the latter contains more acidic (and fewer basic) residues, and that these are closer together. A role for aspartic acid and glutamic acid, lesser than, but complementary to, that of phosphoserine, is supported by our observation that the non-phosphorylated version of OPN290–301 retained some HA-inhibiting activity. The apparent importance of negative-charge density is supported by the decreased ability of OPN290–301 to inhibit HA formation seen when acidic residues within the molecule are separated from each other. These conclusions on the relative importance of phosphate groups, carboxylate groups and negative-charge density are in general agreement with previous studies on the inhibition of HA formation by proline-rich proteins [30] statherin [31] and model compounds [32].

In a study by Hoyer and co-workers [33], four peptides derived from human OPN and containing a sequence of contiguous aspartic acid residues were tested in both phosphorylated and non-phosphorylated forms for their ability to inhibit calcium oxalate crystal formation in a seeded-growth assay. Phosphorylation of peptides was important to their ability to inhibit calcium oxalate growth, as incorporation of phosphates on these peptides increased their inhibitory potencies by one to three orders of magnitude [33]. As the phosphorylation sites within human OPN are not known, however, the physiological relevance of this finding is questionable.

The present study provides some clues to the mechanism by which OPN inhibits crystal formation. The requirement for phosphorylation of the protein is in agreement with the suggestion that phosphate groups of OPN may adsorb to the surface of HA by replacing orthophosphate ions within the crystal lattice, thus sterically preventing further deposition of lattice ions [34]. Our finding that acidic amino acids appear to contribute to the HA-inhibiting activity of OPN phosphopeptides is consistent with the proposal that such residues may act to produce an electrostatic repulsion of inorganic phosphate ions once the protein is adsorbed to the surface of the crystal [35].

One implication of our finding that phosphorylated sequences in OPN show significant differences with respect to their importance in HA inhibition is that some phosphate groups may be added to the protein to mediate other functions. It has been shown that phosphorylation of OPN is required to support osteoclastic bone resorption [36]. Also, macrophage migration and activation are dependent on the interaction of phosphorylated OPN with its receptors [37]. Thus the high number of phosphorylation sites on OPN may be a function of the protein's multiple phosphate-dependent functions.

In conclusion, studies on the effects of OPN phosphopeptides on HA formation have identified two high-potency crystal-inhibiting sequences (residues 41–52 and 290–301). Serine phosphorylation, acidic amino acids and the order (or spacing) of both types of residues have been implicated as factors in the HA-inhibiting potencies of OPN sequences. Understanding how anionic proteins and peptides function to inhibit calcification processes will be crucial to the development of inhibitors to combat such conditions as atherosclerosis, urolithiasis and dental calculus.

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