Control of Osteopontin Signaling and Function by Post-Translational Phosphorylation and Protein Folding

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Abstract Osteopontin (OPN) plays roles in a variety of cellular processes from bone resorption and extracellular matrix (ECM) remodeling to immune cell activation and inhibition of apoptosis. Because it binds receptors (integrins, CD44 variants) typically engaged by ECM molecules, OPN acts as a ''soluble'' ECM molecule. A persistent theme throughout the characterization of how OPN functions has been the importance of phosphorylation. The source of the OPN used in specific experiments and the location of modified sites is an increasingly important consideration for OPN research. We review briefly some of the ways OPN impacts on the biology of mammalian systems with an emphasis on the importance of serine phosphorylation in modulating its signaling ability. We describe experiments that support the hypothesis that differences in the post-translational phosphorylation of OPN expressed by different cell types regulate how it impacts on target cells. Analyses of OPN's potential secondary structure reveal a possible beta-sheet conformation that offers an interpretation of certain experimental observations, specifically the effect of thrombin cleavage; it is consistent with an interaction between the C-terminal region of the protein and the central integrin-binding RGD sequence. J. Cell. Biochem. 102: 912–924, 2007. © 2007 Wiley-Liss, Inc.

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Osteopontin (OPN, also known as Eta-1, early T cell activation gene 1, and spp1, secreted phosphoprotein 1) is predominantly a secreted protein expressed by many tissues and cell types and found in all body fluids; there is also an intracellular form (iOPN) [Zohar et al., 1997]. The secreted protein is substantially post-translationally modified by O-glycosylation, sulfation, and serine/threonine phosphorylation,

which is very heterogeneous and can vary according to its cellular origin. OPN exists both as a soluble cytokine and an immobilized protein adsorbed to calcified matrices. Recent reviews provide considerable background information that supplements this review [Giachelli and Steitz, 2000; Sodek et al., 2000; Denhardt et al., 2001; Qin et al., 2004; Rittling and Chambers, 2004; Standal et al., 2004; Rangaswami et al., 2006; Singh et al., 2007]. Because of space constraints, we cite mostly recent reports. OPN is synthesized by, or found in, numerous tissues including the mammary gland, kidney, brain, bone, smooth muscle, and immune organs. The protein is secreted into body fluids (bile, urine, semen, sweat, and milk) by the epithelia lining ducts that connect to the exterior. OPN expression is up-regulated in numerous pathological situations (e.g., cancer, inflammation, and ischemia) and in response to injury or infection, in part due to its increased expression by activated macrophages and T-lymphocytes. OPN's function in many tissues is to promote cell adhesion and to facilitate cell migration or survival via interactions with integrins and CD44 variants; it is also a cytokine that activates signal transduction pathways similar to those activated by the extracellular matrix (ECM).

OPN is a member of a small family of proteins named the SIBLINGs (Small Integrin-Binding Ligand, N-linked Glycosylation), which are prominent in mineralized tissues [Fisher and Fedarko, 2003].

OSTEOPONTIN PROTEIN SEQUENCE AND SECONDARY STRUCTURE

The molecular mass of murine OPN (amino acid sequence only) is approximately 32 kDa; however, when analyzed via SDS–PAGE, OPN's apparent molecular weight can range from 45 to 75 kDa. This is due to both glycosylation and the high and variable negative charge resulting from the preponderance of acidic amino acids and the multiple serine phosphorylations. Recent determinations using matrix-assisted laser desorption ionization and time-of-flight (MALDI-TOF) mass spectroscopy resulted in masses of 34.9 and 35.9 for OPN produced by cultured murine fibroblast and osteoblast cell lines [Christensen et al., 2007], and 37.6 kDa for rat bone OPN [Keykhosravani et al., 2005].

The functional domains of OPN are well conserved among species. The central integrin attachment motif GRDGS is completely conserved and a high degree of conservation also exists in the neighboring thrombin cleavage site and cryptic integrin attachment motif "SVVYGLR" ("SLAYGLR" in mouse), which becomes accessible upon cleavage of OPN by thrombin. The mineral binding poly-aspartate region is also conserved, although the overall number of consecutive aspartic acid residues varies. Many of the phosphorylated and glycosylated sites are well conserved. In addition, OPN can be crosslinked to itself or other proteins by transglutamination utilizing two highly conserved glutamine residues [Sørensen et al., 1994]. Other predicted motifs include a potential (EF) calcium-binding site as well as two putative heparin-binding sites.

An investigation of OPN structure in dilute aqueous solution by nuclear magnetic resonance revealed that OPN exists as an open, flexible molecule largely devoid of secondary structure [Fisher et al., 2001]. This is not surprising given the high density of negative charges throughout the protein and the paucity of hydrophobic segments. A study by attenuated total reflection infrared spectroscopy of the highly phosphorylated bone and milk OPN in the presence and absence of hydroxyapatite or calcium ions also indicated a mostly random coil conformation, although binding of OPN to hydroxyapatite slightly increased the β-sheet percentage [Gericke et al., 2005]. Interestingly, computer algorithms consistently predict the existence of some secondary structure (short α helices and potential β strand structures). We propose, as shown in Figure 1, that the sequences supporting the two major beta strands interact as shown and, further, that this interaction (possibly strengthened when OPN is associated with mineral) has important consequences for how OPN signals cells. As noted above, when OPN is cleaved by thrombin an additional integrin binding site is revealed. The thrombin cleavage site (RS), located seven amino acids C-terminal to the RGD motif, is conserved in all sequenced species. Cleavage at this site would destabilize the proposed beta sheet formation by breaking the covalent connection between the N- and C-terminal portions of OPN. Similarly, phosphorylation of the serine in the LKFRISHEL sequence [Christensen et al., 2005] may disrupt the β-sheet structure, not only freeing the VVYGLR sequence but also abrogating a conjectured interaction of the C-terminal region with the RGD sequence [Kazanecki, 2007]. This is discussed further at the end of this prospect. OPN is also a substrate for the matrix metalloproteinases MMP-3 (stromelysin-1) and MMP-7 (matrilysin) [Agnihotri et al., 2001]. Cleavage by these MMPs occurs at a limited number of sites, one of which is at the GL sequence immediately preceding the thrombin cleavage site at RS. As with thrombin cleavage, the fragmented OPN exhibited an enhanced ability to stimulate cell adhesion and migration.

RECEPTORS

The primary receptors for OPN are those integrins that bind to the RGD motif. The widely expressed $\alpha_v\beta_3$ integrin was established early on as a primary receptor for OPN. The OPN-$\alpha_v\beta_3$ interaction is essential for osteoclast migration and resorption, as well as smooth muscle cell migration and adhesion. Additional RGD-binding integrins, $\alpha_v\beta_1$ and $\alpha_v\beta_5$, were later determined to be used by cells to bind to OPN. The binding of all three of these integrins is enhanced by Mg^{2+} or Mn^{2+}, but not by Ca^{2+}, which inhibits the OPN-$\alpha_v\beta_3$ interaction [Hu
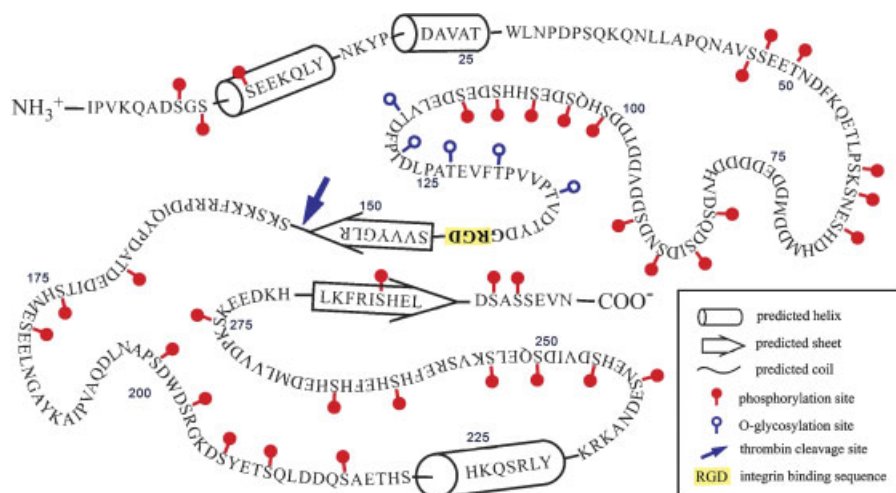
Fig. 1. A model for human osteopontin based upon secondary structure prediction by APSSP2 analysis [Raghava, 2002] (http://www.imtech.res.in/raghava/apssp2). In this method a PSIBLAST neural network and multiple sequence alignment is determined, then a modified example-based learning technique is used to predict secondary structure; those results are combined based on reliability scores to generate a final structure prediction. The secondary structure predicted from this algorithm is highly consistent with results obtained from other prediction algorithms (3-D Jigsaw http://www.bmm.icnet.uk/~3djigsaw/ [Bates et al., 2001], Porter http://distill.ucd.ie/porter/ [Pollastri and McLysaght, 2005], Jalview http://www.jalview.org/ [Clamp et al., 2004], Jnet http://www.compbio.dundee.ac.uk/ ~www-jpred/ [Cuff and Barton, 2000]). Two predicted helices have been omitted due to the internal presence of destabilizing phosphorylated serines. These include DEDITSHMESH beginning at Asp 170 and KVSRE beginning at Lys 252. Also not illustrated are small beta strands at LVTD (Leu 116) and KF (Lys

157). The tertiary structure is hypothesized to minimize the free energy of the beta strands and to explain possible interactions of the C-terminal portion of OPN with the central RGDSVVYGLR integrin binding region. We thank Drs. P. Bradley, A. Doig, D. Eramian, A. Golovanov, S. Rajan, and J. Reed for comments on the proposed model, which is highly speculative. For example it is not clear that bringing the two beta strands together offsets the concomitant entropic loss; much depends on the microenvironment of the protein and interacting partners. The phosphoserine (partly faded out) in the LKFRISHEL beta strand could be stabilizing or destabilizing depending on the three-dimensional environment. With regard to the effect of serine phosphorylation on alpha helices, Dr. Doig offers the following thoughts: Ser10— unclear—stabilizing as this is at an N-cap, but it is i,i+3 to a Glu which would be repulsive; Ser11—stabilizing as it is at N1 and i,i+3 to a Lys which would form a strong salt-bridge [Andrew et al., 2002]. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

et al., 1995b]. Other integrins ($\alpha_5\beta_1$ and $\alpha_8\beta_1$) have also been reported to bind OPN in vitro in an RGD-dependent manner, and more recently $\alpha_v\beta_6$ [Yokosaki et al., 2005]. Notably, certain integrins ($\alpha_5\beta_1$ and $\alpha_9\beta_1$) bound more effectively to the N-terminal thrombin cleavage fragment than to intact recombinant OPN through a cryptic integrin recognition site that lies at the C-terminal end of the N-terminal thrombin fragment (Fig. 1) and comprises the amino acids SVVYGLR [Yokosaki et al., 1999, 2005]. This cryptic integrin recognition sequence that is exposed upon cleavage by thrombin is also used by integrins $\alpha_9\beta_1$ and $\alpha_4\beta_1$ for adhesion [Barry et al., 2000]. OPN is also cleaved in this area by MMP-3 or MMP-7 (at the GL in the above sequence) [Agnihotri et al., 2001], and recombinant OPN cleaved by MMP-3 eliminated the binding of the $\alpha_5\beta_1$ and $\alpha_9\beta_1$ integrins [Yokosaki et al., 2005]. Additional sequences important for integrin binding have been reported; for exam-

ple, peptides containing amino acids 132–146 of the human protein supported cell adhesion via integrin $\alpha_4\beta_1$ [Barry et al., 2000] and mutation of the two aspartic acid residues immediately upstream of the RGD site inhibited adhesion of $\alpha_v\beta_5$ or $\alpha_v\beta_6$ [Yokosaki et al., 2005].

The hyaluronan receptor CD44 has been identified as a receptor for OPN [Weber et al., 1996], but many of the details of this interaction have yet to be elucidated. There are a number of versions of CD44 due to alternative splicing of several "variant" exons into the membrane proximal extracellular region of the protein. The standard version of the protein, CD44s, consists of a core set of exons found in all variants. Weber et al. [1996] first reported that a CD44 species expressing the variant exons 7–10 was able to bind OPN at a site C-terminal to the central RGD motif. Katagiri et al. [1999] demonstrated that the standard form of CD44 was not able to bind OPN, but that certain

variant isoforms of CD44 (those specifically containing v6–v7) were able to bind independently each of the two OPN fragments generated by thrombin. This interaction seemed also to require binding of the β1 integrin in an RGD-independent manner. The interaction of CD44 with OPN has been implicated in the migration of macrophages and tumor cells. A feedback loop may exist as some research has shown that OPN increased expression of CD44, primarily using cancer cell lines such as the breast cancer cell line 21NT [Khan et al., 2005], the liver carcinoma cell line HepG2 [Gao et al., 2003], melanoma cells [Samanna et al., 2006], and macrophages [Marroquin et al., 2004].

TRANSGLUTAMINASE CROSSLINKING

OPN has also been observed to be a substrate for tissue transglutaminase. Two glutamines (Gln-34 and Gln-36), which are conserved in those OPN molecules whose sequence has been determined, have been demonstrated to be substrates for transglutaminase cross-linking to unidentified lysine residues [Sørensen et al., 1994]. Transglutaminase-mediated cross-linking between OPN and fibronectin has been reported, as well as the generation of OPN multimers, the latter of which increases OPN's collagen-binding properties. The bone protein osteocalcin inhibits transglutaminase cross-linking of OPN. These studies are highly suggestive of a role for transglutaminase cross-linking in mediating the covalent incorporation of OPN into extracellular matrices, particularly that of bone. Indeed, recent studies have identified high-molecular weight cross-linked OPN complexes in pathological situations, usually involving calcification. High-molecular weight OPN complexes were observed in calcified arteries of matrix Gla protein-deficient mice [Kaartinen et al., 2006]. Transglutaminase cross-linked OPN can augment the formation of calcium pyrophosphate dihydrate crystals commonly found in osteoarthritic joint tissues in an in vitro model using cultured chondrocytes [Rosenthal et al., 2007].

POST-TRANSLATIONAL MODIFICATIONS OF OPN

The locations of residues that potentially can be phosphorylated in OPN isolated from both bovine and human milk [Sørensen et al., 1995; Christensen et al., 2005] and from rat bone [Keykhosravani et al., 2005] have been determined. Bovine milk OPN contains 27 phosphoserine and 1 phosphothreonine residues. All but two of these modifications were shown to be in the recognition motif for mammary gland casein kinase (Ser/Thr-X-Glu/Ser(P)/Asp) with the other two found in the sequence Ser-X-X-Glu/Ser(P), which is recognized by casein kinase II. The human milk protein contained an even larger number of phosphorylations, a total of 34 phosphoserines and two phosphothreonines [Christensen et al., 2005]. The majority of phosphorylated residues (29) were located in mammary gland casein kinase motifs. Six residues were located in casein kinase II motifs and one phosphoserine was in a sequence not corresponding to either recognition motif. Other enzymes have been shown to also phosphorylate OPN in vitro [Salih et al., 1996]. The phosphorylation patterns of both milk OPNs revealed that the phosphorylated residues were located in clusters of three to five residues separated by larger strings of unmodified amino acids. Milk OPN is more highly phosphorylated than bone OPN, perhaps because the harsh methods required for purification of OPN from bone results in the random loss of some phosphates. The expression of phosphatases such as alkaline phosphatase and tartrate-resistant acid phosphatase (TRAP) by osteoblasts and osteoclasts, respectively, as well as extracellular kinases such as ectokinase may significantly alter the phosphorylation state of OPN after secretion in vivo.

Rat bone OPN possesses 4 O-linked glycosylations and no N-linked glycosylations [Keykhosravani et al., 2005]. Bovine mammary gland OPN contains 3 O-glycosylations but no N-glycosylations [Sørensen et al., 1995]. Similar results were obtained for human milk OPN, which has 5 O-glycosylations and no N-glycosylations [Christensen et al., 2005]. In all studies the O-linked glycosylations contained sialic acid residues. N-linked glycosylation has been described for OPN produced by normal rat kidney cells and for human bone OPN [Masuda et al., 2000]. Shanmugam et al. [1997] reported that sialylation of OPN was reduced in transformed cells compared to non-transformed counterparts and, further, that this reduction in sialic acid content led to less receptor-mediated localization of OPN to the cell surface.

PHYSIOLOGICAL FUNCTIONS OF OPN: IMPORTANCE OF PHOSPHORYLATION

In Bone

OPN is one of the more abundant non-collagenous proteins in bone and is localized to cell-matrix and matrix–matrix interfaces [McKee and Nanci, 1996]. The protein serves as an attachment protein linking cells to the bone mineral via its hydroxyapatite-binding poly-aspartate region and various receptor-binding attachment motifs. Many in vitro studies (discussed below) have also suggested that OPN is a regulator of crystal growth and nucleation. OPN has been demonstrated to have a critical role in osteoclast function. Early studies indicated that osteoclasts are able to attach to and migrate on OPN-coated surfaces via the $\alpha_v\beta_3$ integrin receptor. Hu et al. [1995a] reported that this interaction was inhibited by Ca^{2+}, suggesting a mechanism of regulation dependent on the fact that resorbing osteoclasts greatly increase the local concentration of calcium ions.

The importance of OPN's function in bone was not revealed until the development of the OPN knock-out (KO) mouse. Surprisingly, the OPN-KO mice developed normally and had morphologically normal bones, including at the interfaces where OPN is typically localized, although there was some osteopetrosis with age. Later studies have identified differences in the ultrastructure of the bones, such as increased mineral crystallinity [Boskey et al., 2002], possibly translating into a difference in nanomechanical properties [Kavukcuoglu et al., 2007]. Much research has uncovered a major role for OPN in stress-induced bone remodeling, with most studies focusing on stresses that induce bone resorption by osteoclasts. Yoshitake et al. [1999] demonstrated that OPN-KO mice are deficient in ovariectomy-induced bone remodeling. Microcomputed tomography analysis of trabecular bone volume after ovariectomy revealed an approximate 60% reduction in wild-type mice compared to about a 10% reduction in OPN-KO mice. Similarly, bone loss due to reduced mechanical stress using a hind-limb-unloading model was also impaired in the OPN-KO mice [Ishijima et al., 2002]. Parathyroid-induced and high-phosphate load-induced bone resorption also do not occur to the same extent in OPN-KO mice compared to their wild-type counterparts [Ihara et al., 2001; Koyama et al., 2006].

In vitro studies using osteoclasts isolated from OPN-KO mice have revealed that osteoclast motility is impaired in the absence of OPN and that both $\alpha_v\beta_3$ and CD44 are involved [Chellaiah and Hruska, 2003]. The hypo-motility of OPN-KO osteoclasts is due in part to the decreased expression of surface CD44; exogenous OPN stimulated CD44 expression and partially restored bone resorption [Chellaiah et al., 2003]. From these and other studies it is generally accepted that OPN facilitates osteoclast migration to sites of resorption and is necessary for proper resorption and bone turnover. Intracellular OPN also has a role in osteoclast motility, fusion, and resorption, and may be responsible for some of the consequences of OPN deficiency [Suzuki et al., 2002].

Some of OPN's effects on osteoclast function require phosphorylation of the molecule, and osteoblasts at different stages of differentiation have been shown to alter OPN PTMs, including phosphorylation and sulfation [Nagata et al., 1989; Sodek et al., 1995]. Phosphorylation of OPN by casein kinase II increased osteoclast (but not osteoblast) adhesion [Katayama et al., 1998], and dephosphorylation of OPN by the osteoclast-expressed TRAP eliminated osteoclast binding in vitro [Ek-Rylander et al., 1994]. It remains to be determined whether such dephosphorylation has regulatory significance in vivo. Highly phosphorylated milk OPN stimulates in vitro bone resorption to a greater extent than unphosphorylated recombinant OPN, and two OPNs differing in their levels of phosphorylation also showed similar differences, with the more phosphorylated form supporting more in vitro bone resorption [Razzouk et al., 2002].

In addition to its effects on the cells in bone, OPN is also a regulator of crystal growth, including bone hydroxyapatite (HA). In a gelatin gel diffusion system, OPN at concentrations greater than 25 µg/ml inhibited both HA formation and crystal growth in a dose-dependent manner. Partial enzymatic dephosphorylation reduced the inhibitory activity. Similarly, using an autotitration system to buffer metastable solutions, Hunter et al. [1996] found that OPN inhibited HA formation, and again enzymatic dephosphorylation reduced the effect. In a steady state agarose gel system, nucleation of HA was unaffected by OPN. The importance of phosphorylation in OPN's regulation of HA formation was demonstrated

by Pampena et al. [2004], who used OPN phosphopeptides to show that specific phosphorylated sequences had the most influence, and by Gericke et al. [2005], who compared OPN from several sources. OPN isolated from rat bone inhibited HA formation and seeded growth, whereas recombinant OPN and dephosphorylated OPN had much less of an effect. In contrast, highly phosphorylated milk OPN promoted HA formation, as did mixtures of OPN and osteocalcin. This last result highlights the confusion regarding OPN and crystal regulation. Generally considered to be a negative regulator of crystal growth, especially in fluids such as urine and milk, OPN has also been shown to induce crystal growth under certain conditions. For example, OPN crosslinked to agarose beads promoted HA formation whereas OPN adsorbed to these beads did not [Ito et al., 2004].

Role in the Kidney

OPN, abundantly synthesized by kidney epithelial cells, has been identified as a mediator of urinary stone formation. The protein is a component of kidney stones and its expression is up-regulated in stone-formers [McKee et al., 1995]. OPN has been shown to inhibit the in vitro growth of calcium oxalate (CaOx) crystals and the aggregation of HA crystals, which are also present in a significant fraction of renal stones [Beshensky et al., 2001]. Seemingly at odds with this report are experiments suggesting that OPN facilitates the attachment of CaOx crystals to renal cells [Yasui et al., 2002] and that immobilized OPN increases crystal aggregation—OPN adhering to the surface of collagen granules caused an increase in CaOx crystal adherence and aggregation [Umekawa et al., 2001; Konya et al., 2003]. Further complicating matters is the finding that phosphorylation of OPN also plays a role. Phosphorylated peptides were much more effective at inhibiting CaOx crystal growth than non-phosphorylated peptides [Hoyer et al., 2001]. Because of its affinity for calcium ions, OPN in solution can buffer Ca^{2+}, thereby inhibiting crystal formation, but when immobilized on a surface it can bind Ca^{2+} and stimulate crystal adhesion and aggregation in vitro.

Role in the Vasculature

OPN is expressed by vascular smooth muscle cells and has been shown to increase cell proliferation, adhesion, and spreading; it is chemotactic for these cells, in part mediated by the $\alpha_v\beta_3$ integrin. A component of atherosclerotic plaques, OPN has been shown to inhibit the calcification of smooth muscle cells in vitro [Speer et al., 2005]. An in vivo study in which glutaraldehyde-fixed aortic valve leaflets were subcutaneously implanted into OPN-KO and wild-type mice showed four to fivefold greater calcification in the OPN-KO mice. Interestingly, this calcification could be mitigated by recombinant histidine-tagged OPN that had been suitably phosphorylated [Ohri et al., 2005]. Post-translational modifications also have a role; maximum inhibition of calcification was achieved when the protein was phosphorylated.

In another study, native phosphorylated OPN inhibited calcification of human smooth muscle cells in culture, whereas recombinant (unphosphorylated) or enzymatically dephosphorylated OPN had no effect [Jono et al., 2000]. In spite of these results, the precise role of OPN in atherosclerotic plaque formation remains unclear. Chiba et al. [2002] generated transgenic mice in which hematopoietic cells were engineered to express OPN, and these mice had significantly larger atherosclerotic lesions when fed atherogenic diets. The authors observed high numbers of activated macrophages and determined that the OPN in these lesions was produced by the infiltrating macrophages.

Role in Immune Cells

OPN is expressed by several immune cell types, particularly in pathological situations. These include macrophages, T-cells, B-cells, NK cells, and platelets. OPN expression is increased in response to cellular injury, attracting and supporting the infiltration of macrophages and T-lymphocytes into sites of inflammation and infection [Giachelli et al., 1998]. Besides serving as a chemoattractant for macrophages, OPN has also been associated with other macrophage functions. For example, wound repair following skin incisions is impaired in OPN-KO mice, with reduced levels of tissue debridement, reduced organization of matrix and collagen fibrillogenesis. These results could be attributed to abnormal macrophage function, possibly the absence of intracellular OPN, as increased levels of inactive, resting macrophages were observed. OPN has also been

shown to reduce nitric oxide production by kidney epithelial cells and macrophages activated by interferon-γ and LPS, thereby reducing the cytolytic activity of macrophages toward tumor cells.

OPN has a role in cell-mediated and granulomatous responses [O'Regan and Berman, 2000]. It induces cell migration and binds to activated leukocytes and macrophages, causing changes in cytokine production, modulating the balance between Th1 and Th2 responses. OPN enhances Th1 cytokine (IFNγ, TNF) levels and inhibits Th2 cytokine (IL-4, IL-10) levels. Ashkar et al. [2000] showed that OPN enhanced IL-12 production by peritoneal macrophages and inhibited IL-10 production. Interestingly, these effects were mediated by different receptors, as the IL-12 response was blocked by a GRGDS peptide or an antibody to integrin subunit β_3, while the IL-10 response was blocked by an anti-CD44 antibody, but not the β_3 antibody. Also important was the observation that phosphorylation of OPN was needed for IL-12 induction, suggesting that phosphorylation has a role in regulating integrin recognition of or binding to OPN. Resistance to infection by certain intracellular pathogens is augmented by OPN secreted by activated T cells; the secreted OPN recruits and activates macrophages in a phosphorylation-dependent manner [Weber et al., 2002].

Wang et al. [2007] have recently discovered yet another role for OPN in mammalian systems physiology—that it is involved in the organism's response to some forms of stress. Normal, wild type mice subjected to the stress of hind limb unloading (or physical restraint) exhibit, after 2–4 days, substantial atrophy of the spleen and thymus. This immune organ involution, mediated by elevated corticosteroid levels, does not occur in OPN-KO mice. Apoptosis of splenocytes and thymocytes is the major cause of organ atrophy, but just how OPN promotes this process is not known.

Role in Malignancies

One of the early characterizations of OPN was as "a secreted transformation-associated phosphoprotein" whose expression was up-regulated in transformed cells. Elevated OPN expression has since been documented in a wide variety of cancers; it is associated with increased metastatic potential and poor prognosis [Rittling and Chambers, 2004; Wai and Kuo, 2004;

El-Tanani et al., 2006]. Expression of OPN antisense RNA in ras-transformed NIH3T3 fibroblasts reduced the tumorigenicity of the cells in vivo. Another study using a rat mammary epithelial line transfected with the human OPN gene increased the ability of these cells to metastasize. OPN expression has been correlated with disease progression and decreased survival in lung, breast, gastric, prostate, ovarian, and uterine cervical cancer, as well as head and neck squamous cell carcinomas. The source (tumor cells vs. infiltrating macrophages) of OPN in tumors remains controversial and may depend on the type and stage of malignancy; OPN produced by the two cell types may also be functionally different [Rittling et al., 2002].

Tumor-derived OPN is likely less phosphorylated. This is supported by early experiments by Nemir et al. [1989] using normal rat kidney cells. These cells produced both a phosphorylated (pp69) and a non-phosphorylated form (np69) of OPN; however when the cells were treated with vanadyl sulfate, which inhibits phosphatases and causes transformation, the expression of np69 was significantly increased and pp69 decreased. OPN produced by ras-transformed fibroblasts is significantly less phosphorylated than OPN produced by osteoblasts and possibly non-transformed fibroblasts (see below) [Christensen et al., 2007; Kazanecki et al., 2007]. We propose that the tumor-produced, less phosphorylated OPN may be more effective at promoting cancer progression, either by increasing anchorage-independence and metastasis, or protecting the cells from the immune response and apoptosis, whereas a more highly modified macrophage-produced OPN would serve as an effective chemoattractant and activate T-lymphocytes to attack the tumor cells. One distinguishing factor is proposed to be differences in phosphorylation, which may affect receptor interaction and signaling in a direct or indirect manner, possibly via an interaction with CD44 variants. The latter seems much more likely as there is a large body of data connecting certain CD44 variants and OPN with tumor progression. Many studies have linked CD44 and OPN expression in tumor cells and associated the two with augmented cell migration. OPN increased the expression of CD44v6 in liver carcinoma cells [Gao et al., 2003] and a human breast cancer cell line, which also exhibited

increased CD44-dependent migration [Khan et al., 2005]. *Ras*-transformation of mouse fibroblasts up-regulated both OPN and CD44 in an autocrine manner and increased invasion in an in vitro assay [Teramoto et al., 2005].

Many of OPN's previously mentioned functions contribute to its role in cancer progression. OPN's ability to stimulate migration clearly contributes to the metastatic ability of tumor cells. OPN decreases nitric oxide expression and cytotoxicity of macrophages toward tumor cells, and it promotes cell survival in a number of paradigms [Malyankar et al., 2000; Khan et al., 2002; Lee et al., 2007]. Although the contribution of PTMs to OPN's role in transformation and tumor progression has not been elucidated, a study by Crawford et al. [1998] suggested that tumor-produced OPN and host-produced OPN had distinct functions. Host-produced OPN was shown to be a more effective macrophage chemoattractant, whereas tumor-produced OPN contributed to the growth and survival of metastases.

FUNCTIONAL CONSEQUENCES OF POST-TRANSLATIONAL PHOSPHORYLATION

Post-translational modifications of OPN purified from various species have been characterized, although they have been from only two sources—milk and bone, and OPN from these sources is typically used experimentally as native OPN. As a result, little is known of the differences in PTMs of OPN expressed by other cell types and whether potential cell-type specific differences can alter OPN's functions. It is possible that OPN expressed by different cell types differs in PTM status and that these differences modulate OPN's function or the cellular response to OPN. This hypothesis stemmed from the observations that OPN's PTM state was different in proliferating versus mineralizing osteoblasts [Kubota et al., 1989; Nagata et al., 1989; Kasugai et al., 1991], as well as normal versus transformed kidney cells [Nemir et al., 1989], and that vitamin D3 treatment altered the apparent pI of OPN [Safran et al., 1998], likely by suppressing serine phosphorylation. Also, as noted above, OPN produced by tumor cells seemed to be functionally different in comparison with OPN produced by macrophages [Crawford et al., 1998]. This is a prime example of a situation where OPN from multiple sources can coexist.

In tumors, OPN can be expressed by the tumor cells themselves, by the surrounding host tissue, and by infiltrating macrophages and lymphocytes. The action of OPN on target cells differs and can be contradictory—does OPN increase survival and metastasis or help attract macrophages? Does OPN increase the activation of infiltrating immune cells or decrease their nitric oxide production and cytotoxicity? We suggest that both differences in the PTM state of the OPN produced by these various cell types and variations in the target cell receptor repertoire are important in determining the outcome of OPN signaling, modulating the degree of response in a given cell type.

In further support of our hypothesis, experiments with anti-OPN monoclonal antibodies suggested that the OPN produced by the pre-osteoblast MC3T3-E1 cell line differed from that produced by another murine cell line—the *ras*-transformed cell line 275-3-2 [Wu et al., 2000; Kazanecki et al., 2007]. This strongly suggests that there are differences in the post-translational modifications of OPN expressed by the cell lines tested. In collaboration with Esben Sørensen (University of Aarhus, Denmark), the sites of PTMs of OPN expressed by the MC3T3 osteoblast (ObOPN) and 275-3-2 fibroblast (FbOPN) cell lines were identified [Christensen et al., 2007]. There was a dramatic difference in the degree of phosphorylation of OPN between the two lines. Analysis of proteolytic fragments of OPN identified ~21 phosphates distributed over 27 potential sites of phosphorylation for the ObOPN, but only ~4 phosphates distributed over 16 potential sites for the FbOPN. The degree of modification of the ObOPN agrees well with earlier characterizations of PTMs of OPN from rat bone and from bovine and human milk with 27 potential sites of phosphorylation compared to 29, 28, and 36, respectively [Sørensen et al., 1995; Christensen et al., 2005; Keykhosravani et al., 2005]. The milk OPN was determined in both cases to be almost completely modified, whereas the rat bone OPN was determined to contain an average of 10–11 phosphates [Keykhosravani et al., 2005]. Both murine OPNs displayed heterogeneity in their phosphorylation, with many more potential sites of phosphorylation identified compared to the average number of phosphates removed by alkaline phosphatase [Christensen et al., 2007].

The observed differences in phosphorylation of the two characterized murine OPNs translated into a functional difference in the degree of cell adhesion they supported, which differed depending on the cell line used [Christensen et al., 2007]. Human breast cancer MDA-MB-435 cells showed a much greater adhesion to the less phosphorylated FbOPN than to the more phosphorylated ObOPN, which supported only slight cell adhesion. The *ras*-transformed fibroblast 275-3-2 cell line [Wu et al., 2000] in contrast displayed greater binding to the ObOPN compared to the FbOPN. One explanation for the observed differences in adhesion of the two cell lines to the two OPN forms would be a difference in receptor repertoire expression by the cell lines. However, adhesion of both cell lines were almost completely inhibited by the addition of an RGD peptide, indicating that adhesion is primarily RGD-integrin mediated.

There are few sites of phosphorylation in proximity to the RGD site, and the closest observed site of differential phosphorylation between the two forms of OPN described above is 24 amino acids away. Some data exist suggesting that phosphorylation is able to affect RGD-dependent adhesion. Katayama et al. [1998] showed that phosphorylation of OPN by casein kinase II increased the adhesion of osteoclasts but not osteoblasts to recombinant rat OPN. This attachment was also RGD-dependent and was completely abolished by 1 mM GRGDS peptide. Also, an antibody to the integrin β_3 subunit, but not a CD44-blocking antibody, blocked the increase in adhesion to phosphorylated OPN. Ashkar et al. [2000] showed that exposure of mouse peritoneal macrophages to native, but not unmodified, OPN increased IL-12 production in an RGD-dependent manner. Weber et al. [2002] showed that phosphorylation of OPN was required for RGD-dependent cell spreading of a murine monocytic cell line. These studies support the argument that differences in phosphorylation are regulating RGD-dependent integrin attachment to OPN.

How might this occur? The reports by Ashkar et al. [2000] and Weber et al. [2002] describe a 10-kDa fragment (NK10) of OPN isolated after protease digestion that retained cell attachment ability. Analysis of the fragment determined that the N-terminal sequence was QETLPSN and that it appeared to extend to the thrombin cleavage site, thus containing the RGDSVVYGLR sequence at its C-terminus. Dephosphorylation of the fragment (which contained 5 mol of phosphate) reduced its ability to support cell attachment. This sequence contains 10 sites of potential phosphorylation in ObOPN and 5 sites in FbOPN. Differences in this region may explain the observed differences in cell adhesion supported by the OPN forms. OPN may contain one of more as yet unidentified "synergy" sites that may help regulate integrin binding to the RGD site, similar to those found in fibronectin [Clark et al., 2003].

A recent publication offers an alternative explanation of the regulation of RGD-binding integrins by specific phosphorylations. Lee et al. [2007] demonstrated that an OPN-CD44v interaction is able to increase integrin adhesion by activation of integrins via inside-out signaling, resulting in increased cell survival. Exposure of the AZ521 gastric cancer cells to OPN, but not an RGE-mutant OPN, activated their β_1 integrins and stimulated their attachment to fibronectin. The activation of β_1 integrins was blocked by anti-CD44v6 antibody pretreatment. The activation was dependent on src-kinase signaling, as the src-kinase family inhibitor PP2 eliminated the effect. The region of OPN that binds to CD44v is controversial; although several putative CD44v binding sites have been reported, none has been independently confirmed. This leaves open the possibility that the OPN-CD44v interaction could be regulated by phosphorylation of OPN. Thus, phosphorylation of OPN could indirectly affect RGD binding by regulating integrin activation through inside-out signaling initiated by engagement of the CD44v receptors with OPN. A feedback loop may also exist as an interaction with OPN and α_v integrins was shown to increase CD44 expression and MMP-2 activity [Samanna et al., 2006].

Some of our recent data suggest another possible method of regulation. We have shown that two monoclonal antibodies that recognize the extreme C-terminal region of the molecule are able to inhibit MDA-MB-435 breast cancer cell adhesion to recombinant human OPN [Kazanecki et al., 2007]. The adhesion of these cells in this system was also completely inhibited by the $\alpha_v\beta_3$-blocking antibody LM609, suggesting that the binding of the monoclonal antibodies to the extreme C-terminal region of OPN adversely affected $\alpha_v\beta_3$ binding to the
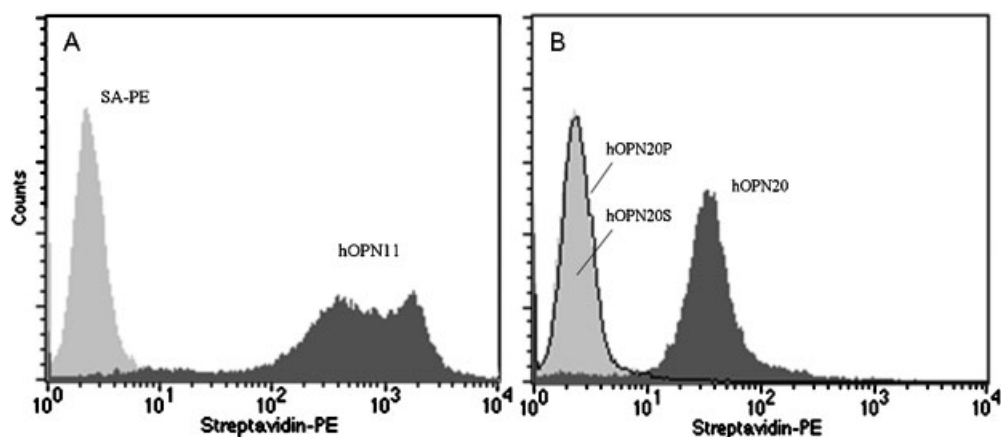
Fig. 2. Binding of both a central peptide and a C-terminal peptide to MDA-MB-435 cells. Human breast cancer MDA-MB-435 cells (2.5×10^5) were trypsinized, washed, and incubated with the indicated biotinylated peptides at 50 µM for 15 min at room temperature. The cells were washed twice with phosphate-buffered saline and incubated with 0.5 µg/ml streptavidin-phycoerythrin (SA-PE, eBioscience) for 15 min at room temperature. The cells were again washed three times, fixed in 1% paraformaldehyde in PBS, and analyzed by flow cytometry using a FACSCalibur instrument (BD Biosciences). **A**: Negative control with cells and SA-PE only (light grey), and a positive control employing the peptide hOPN11: SVVYGLRSKSKKFRRPDIGG-Biotin (dark grey) corresponding to amino acids 146–163 of human OPN. **B**: hOPN20: HLKFRISHELDSASSEVNGG-Biotin (dark grey) corresponding to amino acids 281–298 of human OPN; hOPN20 scrambled (hOPN20S): LDEHSSAISRSFEKVLNHGG-Biotin (light grey); hOPN20 phosphorylated (hOPN20P): HLKFRIpSHELDpSApS-SEVNGG-Biotin (black line). We thank Larry Steinman (Stanford University) for the generous gift of these peptides. Data shown are representative of three independent experiments.

RGD site. The experiment shown in Figure 2, suggests that a synthetic peptide (hOPN20), corresponding to the C-terminal 18 amino acids is bound by the MDA-MB-435 cells; neither a phosphorylated version (hOPN20P) nor a version with a scrambled sequence (hOPN20S) was able to bind. This C-terminal region is highly conserved among mammalian species and may be the region of CD44 binding and signaling observed above by Lee and colleagues. We hypothesize that there is an association between this region, part of which is predicted to adopt a β-sheet conformation, and the β-sheet structure immediately downstream of the RGD site, bringing these regions in proximity to each other (Fig. 1) thereby facilitating a synergistic interaction between the integrin and putative C-terminal binding receptors. Such a structure may also explain the observations of Katagiri et al. [1999], who found that CD44 binding required the β1 integrin, consistent with an association between the CD44 and integrin receptors. This structure could be regulated by phosphorylation (the S in LKFRISHEL), buffer conditions, or association with mineralized matrices (and tissue culture plastic), all of which may modulate the RGD-integrin interaction.

The simplified models above are an attempt to explain observations primarily regarding the adhesion of cells to immobilized OPN. Clearly the receptor repertoire expressed by a given cell type has a major role in determining the biological response, however, it is also likely that immobilized OPN recruits a different subset of receptors for adhesion or migration than when OPN is presented as a soluble cytokine (possibly because of differences in the ability of the proposed β-sheet structure to form). Differences in phosphorylation and the presence of various OPN fragments resulting from thrombin or MMP cleavage contribute to the complexity of OPN-receptor binding and downstream signaling.

In summary, we propose a model in which a β-strand adjoining the RGD domain associates with the β-strand at the extreme C-terminal region of OPN to form a beta sheet structure (Fig. 1). This structure would facilitate an interaction between integrin receptors binding the RGD domain and the putative receptor (CD44v?) binding the C-terminus; interaction with the SVVYGLR domain would be inhibited. Another possibility is that this beta sheet structure contributes some specificity enabling only certain RGD-binding integrins (i.e., β_1 integrins) to engage OPN or interact with the C-terminal-binding receptor. This structure, as well as the receptor binding the C-terminus

would be regulated by phosphorylation of the C-terminal region. This model is proposed to provide a mechanism by which cell-specific differences in post-translational modifications of OPN contribute to the protein's wide range of sometimes-contradictory functions.

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