Cell-type specific post-translational modifications of mouse osteopontin are associated with different adhesive properties

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Running title: Osteopontin isoforms with different adhesive properties

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Osteopontin (OPN) is a highly modified integrin-binding protein found in all body fluids. Expression of OPN is strongly correlated with poor prognosis in many different human cancers, suggesting an important, but poorly understood role for this protein in tumorigenesis and metastasis. The protein exists in a number of different isoforms differing in the degree of post-translational modifications (PTM) which are likely to exhibit different functional properties. This study examines for the first time the PTMs of OPN from transformed cells and the effects of these modifications on cell biology. We have characterized the complete phosphorylation and glycosylation pattern of OPN made by murine ras-transformed fibroblasts (FbOPN) and differentiating osteoblasts (ObOPN) by a combination of mass spectrometric (MS) analyses and Edman degradation. MS analysis showed masses of 34.9 kDa and 35.9 kDa for FbOPN and ObOPN, respectively, while enzymatic dephosphorylation, sequence- and mass analyses, demonstrated that FbOPN contained approximately four phosphate groups distributed over 16 potential phosphorylation sites, whereas ObOPN contained approximately 21 phosphate groups distributed over 27 sites. Five residues were O-glycosylated in both isoforms. These residues were fully modified in FbOPN whereas one site was partially glycosylated in ObOPN. While both forms of OPN mediated robust integrin-mediated adhesion of mouse ras-transformed fibroblasts, the less phosphorylated FbOPN mediated binding of human MDA-MD-435 tumor cells almost six-fold more than the heavy phosphorylated ObOPN. These results strongly support the hypothesis that the degree of phosphorylation of OPN produced by different cell types can regulate its function.

Osteopontin (OPN) is a highly phosphorylated glycoprotein comprising approximately 300 amino acid residues. The protein was first purified from the mineralized matrix of bovine bone (1). However, the presence of OPN is not limited to mineralized tissues but extends to a variety of tissues, cell types and physiological fluids, including blood, urine and milk (2). The amino acid sequence of OPN is rich in acidic amino acids and contains an integrin binding Arg-Gly-Asp (RGD)-sequence. OPN is a pleiotropic protein involved in a variety of cellular processes such as migration, adhesion and signalling (2,3). OPN is a key molecule in bone remodelling and functions as an inhibitor of ectopic calcification by inhibiting the formation of hydroxyapatite and calcium oxalate (4-6). Furthermore, OPN is implicated in diverse biological processes including tumorigenesis, metastasis, cytokine production, wound healing, autoimmune disease, and stroke (3,7-10). OPN has recently been demonstrated to be required for mucosal protection in acute inflammatory colitis (11).

OPN-integrin interaction controls many aspects of cell behavior including cell attachment, migration, chemotaxis, and immune modulation in various cell types (2,12). The α5β1, αvβ5, αvβ1, αβ1, αβ3 and αvβ3 integrins recognize OPN through the conserved RGD-sequence (2,13); whereas the integrins αβ1 and αvβ1 bind OPN independent of the...
RGD-sequence via the motif SVVYGLR (in human OPN) that is exposed by thrombin cleavage of the protein (14,15). OPN is also a ligand for certain variants of the CD44 receptor, specifically those containing the v6-v7 regions (16,17). CD44 is expressed on cells in normal and malignant tissues and its interaction with OPN has been implicated in both immune responses and bone remodeling (7,18). Activities mediated by the CD44 receptor have been associated with residues in both the N- and C-terminal parts of OPN (12).

It is well established that OPN function is highly dependent on posttranslational modifications (PTM) and significant regulation of the processes involving OPN are mediated through phosphorylation (19). The interaction between OPN and some receptors are dependent on the PTM state of the protein. For instance, OPN promotes RGD-dependent osteoclast attachment provided that the protein is phosphorylated. Phosphorylation of recombinant OPN increases osteoclast attachment in vitro (20) whereas partial dephosphorylation of bovine OPN by tartrate-resistant acid phosphatase abolished the ability to mediate osteoclast attachment (21). Similarly, OPN can stimulate bone resorption only if it is properly phosphorylated (22). OPN has cytokine-like properties and can influence the production of cytokines by interaction with surface receptors on immune cells. Phosphorylation of OPN is required for integrin binding and subsequent induction of IL-12 expression in macrophages (7) and for mediating their activation and spreading (23). The protein has also been shown to promote trophoblastic cell migration in a process dependent on the level of phosphorylation of the protein (24). In addition, the importance of OPN phosphorylation in mineralization became evident from studies of dephosphorylated OPN which had lost the ability to inhibit hydroxyapatite formation (5,25). Further emphasizing the importance of the phosphorylations, it was recently shown that highly phosphorylated milk OPN containing approximately 28 phosphorylations promoted hydroxyapatite formation and growth, whereas bone OPN modified by only 13 phosphates inhibited formation (26). Similar results have been obtained in other studies, showing that phosphorylation is crucial for OPN inhibition of calcium oxalate crystallization in urine (27) and calcification of vascular smooth muscle cells (28).

OPN is encoded by a single copy gene but exists in a number of different isoforms which mainly differ in the extent of PTM. Normal rat kidney cells secrete both phosphorylated and non-phosphorylated variants of OPN which also vary in glycosylation patterns and in their ability to associate with fibronectin (29). Likewise, differentiating rat osteoblasts produce two forms of OPN as seen in SDS-PAGE gels; a 55-kDa form that contains little phosphorylation and a highly phosphorylated 44-kDa form (30). The extent of phosphorylation of OPN in osteoblast and epidermal cells is responsive to hormonal influences such as 1,25-dihydroxyvitamin D3 (31,32) and oncogene-transfected Rat-1 cells switch from the synthesis of sialylated to non-sialylated OPN upon phorbol-ester treatment (33).

The only OPN isoforms that have been thoroughly characterized with regard to the PTM pattern are those from bovine and human milk and rat bone. The bovine milk OPN contains 27 phosphoseryl residues and one phosphothreonine (34). Up to 34 phosphoserines and two phosphothreonines were identified in the human counterpart (35). Studies of OPN purified from rat bone revealed up to 29 potential phosphorylation sites, however in this isoform the average level of phosphorylation was estimated to be only approximately 10-11 phosphates per OPN molecule (36). Phosphorylations in OPN are predominantly located on serines in the recognition motifs of the golgi kinase/mammary gland casein kinase (S/T-X-E/S(P)/D) (37,38) and/or casein kinase II (S-X-X-E/S(P)/D) (37,39). In addition to phosphorylation, the characterized OPN isoforms all contain glycosylations. Bovine and human milk OPN contain three and five O-glycosylated residues, while four glycans have been identified in the sequence of rat bone OPN (34-36).

Here, we have purified and extensively characterized two isoforms of OPN expressed by 275-3-2 murine ras-transformed fibroblasts (FbOPN) and differentiating immortalized MC3T3 osteoblasts (ObOPN). This is the first comparative characterization of OPN isoforms expressed by cells from the same species and it is the first study to characterize the PTMs of OPN produced by
transformed cells. Major differences in the degree of phosphorylation of these proteins were found, and these correlated with differences in biological activity, confirming that the function of OPN produced by different cell types is distinct.

**EXPERIMENTAL PROCEDURES**

**Materials** – Sequencing grade modified trypsin was from Promega (Madison, WI), thermolysin, Percoll, bovine serum albumin (BSA), bovine alkaline phosphatase (ALP) and fibronectin were from Sigma (St. Louis, MO) and endoproteinase Asp-N was from Roche Diagnostics (Penzberg, Germany). The µRPC (narrow-bore reverse-phase chromatography) C2/C18 PC 2.1/10 and the Superdex 75 PC 3.2/30 columns were from Amersham Pharmacia Biotech (Uppsala, Sweden). Reagents used for sequencing were purchased from Applied Biosystems (Warrington, UK). 2,5-dihydroxybenzoic acid were from LaserBio Labs (Sophia-Antipolis Cedex, France). Peptide-N-Glycosidase F (PNGase F) kit was obtained from New England Biolabs (Beverly, MA). GRGDNP and GRADSP peptides were from BIOMOL International LP (Plymouth Meeting, PA). All other chemicals used were of analytical grade.

**Cell lines and culture** - The highly mineralizing murine osteoblast MC3T3-E1 subclone 4 cell line (40) was a kind gift from Dr. R. Franceschi (University of Michigan) and was cultured in alpha-minimal essential medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 50 U/ml penicillin, 50 µg/ml streptomycin and 2 mM glutamine. The 275-3-2 cells were used to make conditioned medium when approximately 80% confluent for purification of FbOPN.

**Purification of OPN** - OPN was purified from medium conditioned by MC3T3-E1 (ObOPN) and 275-3-2 murine ras-transformed fibroblasts (FbOPN). Cell lines were grown as described above and conditioned medium was generated by incubating the cells in serum-free medium overnight. OPN was affinity-purified using 2A1 monoclonal antibody (42) coupled to protein G-Agarose beads (Pierce, Rockford, IL). Approximately 50 ml of conditioned medium was incubated with 1 ml of antibody-coupled beads at 4 °C overnight with end-over-end rotation. The beads were gently pelleted, washed with phosphate-buffered saline and packed into 2 ml disposable columns (Pierce). OPN was eluted with 100 mM glycine, 500 mM NaCl, pH 2.5 and immediately neutralized. Fractions were analyzed by SDS-PAGE, and proteins visualized by non-ammoniacal silver staining or by western blotting with 2A1 antibody. Positive fractions were pooled, desalted on PD-10 columns (GE Healthcare Bio-Sciences, Piscataway, NJ) and lyophilized.

**Analysis of OPN** – Native and dephosphorylated FbOPN and ObOPN were analyzed by MS using a Voyager DE-PRO MALDI-TOF mass spectrometer (Applied Biosystems). Samples for MS analyses were prepared by mixing the sample with a saturated solution of 2,5-dihydroxybenzoic acid and 2 mM glutamine (all from Invitrogen). For differentiation, cells were grown until confluent, then switched to medium containing 10 µg/ml ascorbic acid and 10 mM β-glycerophosphate (both from Sigma) for an additional 10-12 days before generating conditioned medium for ObOPN purification. The 275-3-2 murine ras-transformed fibroblast cell line (41) and the MDA-MB-435 human breast cancer cell line (American type culture collection) were maintained in Dulbecco’s modified Eagle’s Medium (Mediatech Inc., Herndon, VA) supplemented with 10% fetal bovine serum, 50 U/ml penicillin, 50 µg/ml streptomycin and 2 mM glutamine. The 275-3-2 cells were used to make conditioned medium when approximately 80% confluent for purification of FbOPN.

**Generation and separation of peptides** - OPN was digested with trypsin using an enzyme to substrate ratio of 1:30 (w/w) in 0.1 M ammonium bicarbonate at 37 °C for 6 h. Tryptic peptides were separated by RP-HPLC on a µRPC C2/C18 PC 2.1/10 column connected to a Pharmacia SMART system. Separation was carried out in 0.1% TFA (buffer A) and eluted with a gradient of 60% acetonitrile in 0.1% TFA (buffer B) developed over 54 min (0-9
min, 0% B; 9-49 min, 0-50% B; 49-54 min, 50-100% B) at a flow rate of 0.15 ml/min. The peptides were detected in the effluent by measuring the absorbance at 214 nm. A large fragment not susceptible to trypsin cleavage (Gln^{35}/Gln^{54}-Arg^{128}) was separated from the tryptic peptides by gel filtration on a Superdex 75 PC 3.2/30 column. The column was equilibrated with 0.1 M ammonium bicarbonate and operated at a flow of 0.05 ml/min. The fractions containing the fragment in question were pooled, lyophilized and further digested with thermolysin in 0.1 M pyridine-acetate, 5 mM CaCl_{2}, pH 6.5, at 56 °C for 6 h. The resulting peptides were separated by RP-HPLC as described for the tryptic digest. Fraction Fb-Th1 and Ob-Th1 (Fig. 3 C-D) from the thermolysin digests were further digested with endoproteinase Asp-N (2 µg) in 50 mM sodium phosphate buffer, pH 8.0, at 37 °C for 18 h.

Characterization of peptides - Peptides were characterized by mass spectrometric and amino acid sequence analyses. All MS spectra were obtained in both positive reflector-ion and positive linear-ion mode as described above. The theoretical peptide masses and sequence coverage were calculated using the GPMAW program (Lighthouse Data, Odense, Denmark). Amino acid sequence analyses were performed on an Applied Biosystems PROCISE HT protein sequencer with on-line identification of PTH-derivatives. Glycosylated serine/threonine residues were identified by the lack of a PTH-derivate in the cycles where these amino acids are modified.

Enzymatic deglycosylation - N-linked deglycosylation was performed as described by the manufacturer using the PNGase F deglycosylation kit. OPN (15 µg) was incubated with 500 U PNGase F at 37 °C for 20 h. The reaction products from the enzymatic deglycosylations were analyzed by SDS-PAGE.

Cell adhesion assays - Flat-bottom 96-well tissue culture treated polystyrene microtiter plates (Corning, NY) were coated with 100 µl OPNs (10 µg/ml) or fibronectin (2.5 µg/ml) in phosphate-buffered saline at 4 °C overnight and then blocked with 1% BSA. MDA-MB-435 and 275-3-2 ras-transformed fibroblast cells were trypsinized, then washed twice and re-suspended in Dulbecco’s modified Eagle’s Medium containing 1 mg/ml BSA. Cells were pre-incubated with 100 µM GRGDNP or GRADSP control peptides for 30 min at 37 °C. 5x10^4 cells were then added to coated wells and adhered for 1 h (275-3-2) or 18 h as described (43) (MDA-MB-435) at 37 °C in a humidified atmosphere with 5% CO_{2}. Non-adhered cells were removed by washing twice with 75 µl Percoll (73% Percoll, 0.9% NaCl) and adherent cells were fixed with 50 µl fixative (10% glutaraldehyde in Percoll). Fixed cells were stained with 100 µl 0.1% crystal violet and solubilized in 50 µl 0.5% Triton X-100 before reading at 570 nm in a MRX Revelation Reader (Thermo Labsystems, MA).

RESULTS

Molecular masses – OPN was purified from medium conditioned by the mouse MC3T3-E1 and 275-3-2 ras-transformed fibroblast cells as described and their purity analyzed by SDS-PAGE and silver staining (Fig. 1). There is a clear difference in the migration during SDS-PAGE of the two OPN isoforms suggesting that they are differently modified by PTMs. More exact estimation of the average masses of the OPN isoforms was determined by linear MALDI-TOF MS showing molecular weights of approximately 34.9 kDa and 35.9 kDa for FbOPN and ObOPN, respectively (Fig. 2). The MS spectra show broad peaks which suggest that both OPNs are heterogeneously modified. To estimate the total number of phosphate groups present, FbOPN and ObOPN were treated with ALP. The molecular weight of dephosphorylated FbOPN was 34.6 kDa corresponding to a loss of approximately four phosphate groups (Fig. 2A). Phosphatase treatment of ObOPN reduced the mass to 34.2 kDa corresponding to a loss of approximately 21 phosphorylations (Fig. 2B). Parallel control experiments with bovine OPN (data not shown) showed that the dephosphorylation reaction is complete. Subtraction of the observed average mass of the dephosphorylated OPNs from the theoretical mass of the mouse OPN polypeptide (30746 Da) leaves approximately 3.9 kDa and 3.5 kDa for glycosylations on FbOPN and ObOPN, respectively, suggesting that FbOPN contains slightly more carbohydrates than ObOPN.
Phosphorylations - FbOPN and ObOPN were digested with trypsin and the resulting peptides separated by RP-HPLC (Fig. 3A and B). A large acidic fragment of OPN (Gln54-Gln54-Arg128) was not susceptible to tryptic cleavage and had to be isolated from the tryptic digests of FbOPN and ObOPN by gel filtration (data not shown). The purified fragments were further digested with thermolysin and the resulting peptides separated by RP-HPLC (Fig. 3C and D).

All fractions from the RP-HPLC separations of tryptic and thermolytic peptides were analyzed by MALDI-TOF MS (Table 1). Detection of phosphorylated peptides and peptide mapping of the OPN sequence were performed as previously described (35). In total, 16 sites of phosphorylation were identified in the sequence of FbOPN and 27 sites were identified in ObOPN. Several of the phosphopeptides contained more serines/threonines than the observed number of phosphorylations. In these situations the phosphate groups were assigned to residues fitting the target sequence of the golgi kinase/mammary gland casein kinase (S/T-X-E/S(P)/D) based on the localization of phosphorylated residues in other OPN isoforms (34-36). Data from the peptide phosphorylation analysis of the two proteins are summarized in Table 1 and the resulting map of modifications is shown in Fig. 4.

Characterization of the proteins revealed that most peptides exist in several states of phosphorylation. The only phosphoresidues identified as fully phosphorylated were Ser8, Ser10 and Ser11 in ObOPN (Table 1). As seen in Table 1 it is evident that the osteoblast OPN contains significantly more phosphorylations than the fibroblast analogue. Furthermore, based upon how often each residue was encountered as phosphorylated, it is noticeable that the most abundant species of a given peptide from FbOPN was unmodified or singly phosphorylated whereas the corresponding peptide in the osteoblastic counterpart is predominantly phosphorylated at all potential sites. This corresponds well with the data from the dephosphorylation studies (Fig. 2) and further emphasizes that the potential sites of phosphorylation in ObOPN are phosphorylated to a much higher degree than in FbOPN.

The RP-HPLC chromatograms (Fig. 3) show that there is a significant structural difference between the two OPNs. This is especially true for the separation of thermolytic peptides (Fig. 3C and D) which indicate that the proteins have different PTMs and perhaps also different proteolytic cleavage. For instance, the peptides starting at Ser59, Asn60, Ser61 and Ser64 are only detected in the FbOPN digest suggesting that thermolysin can not cleave ObOPN in this region (Table 1). This could be explained by the heterogeneous phosphorylation of these serines, and intensive phosphorylation at Ser59, Ser61 and Ser64 in ObOPN could potentially hinder thermolytic cleavage in this region.

O-glycosylations - All O-glycosylated residues in FbOPN were detected in fraction Fb-Th1 (Fig. 3C). N-terminal sequencing of this peak revealed the presence of three peptides starting at Val88, Ser99, and Ile118, respectively. Linear MALDI MS showed that all the peptides contained species with mass increments of approximately 365 Da and/or 291 Da corresponding to different amounts of N-acetylhexosamines (HexNAc), hexoses (Hex) and sialic acid (SA) (Table 2). A peptide covering I118VPTVDVPNQR128 was observed in positive reflector mode with m/z values at 1531.80, 1822.82, 2113.91 and 2478.93 (Fig. 5A). These masses correspond to Ile118-Arg128 with increasing amounts of N-acetylhexosamines (HexNAc), hexoses (Hex) and sialic acid (SA) (Table 2). The peaks at m/z 2478.93 and 2113.91 represent excess masses of 1312.28 Da and 947.26 Da from the theoretical mass of the peptide. This suggests modification by either the disialylated core 1 O-glycan SA-Hex-[SA]-HexNAc or by the more complex SA-Hex-[SA-HexHexNAc]-HexNAc structure (Table 2). The lack of a PTH-Thr at the position of Thr121 during Edman sequencing indicates that this amino acid is modified in the peptide. Furthermore, sequencing of the peptide Ser59-Arg128 showed no PTH-amino acid in the cycles corresponding to Thr107, Ser109 and Thr110 which together with the MS data in Table 2 indicates O-glycosylation at these residues.

To confirm the O-glycosylation sites and their structures the glycopeptides in fraction Fb-Th1 were further digested with endoproteinase Asp-N. This resulted in peptides cleaved N-terminal to Asp103 and Asp113 which subsequently were separated by RP-HPLC (data not shown). Edman sequencing of the peptides showed no identifiable PTH-amino acids in the cycles corresponding to Thr107, Ser109, Thr110, Thr116 and Thr121 (Table 2).
signals of the peptide D103ETVTASTQA112 were observed in MS. However, masses corresponding to Asp103-Arg128 were detected in linear MALDI MS mode with m/z values consisting of a series of peaks separated by approximately 365 Da or 291 Da, showing that the peptide contained multiple glycosylations composed of up to seven HexNAc-Hex and ten SA units, respectively. This peptide contains all the five amino acids in question (Thr107, Ser109, Thr110, Thr116 and Thr121) and the masses strongly indicate that they are all glycosylated. Furthermore, monoisotopic masses of the peptide D113TFTPIVPTVDVPNGR128 showed that this peptide exists in different glycosylation variants. The most abundant species had an excess mass of 1895.67 Da corresponding to two HexNAc-Hex and four SA units which indicates that Thr116 and Thr121 are modified by the SA-Hex-[SA]-HexNAc structure.

Fraction Ob-Th1 (Fig. 3D) contained the peptides Val88-Arg128 and Ile118-Arg128 (Table 2). It was determined that Thr121 is modified by O-glycans consisting of SA-Hex-[SA]-HexNAc or SA-Hex-[SA-Hex-HexNAc]-HexNAc moieties and that the peptide Val88-Arg128 contains multiple HexNAc, Hex and SA units (Table 2). Furthermore, the peak Ob-Th2 (Fig. 3D) was only observed in the RP-HPLC analysis of thermolytic ObOPN peptides and not seen in the corresponding chromatogram for FbOPN. This fraction contained Val106-Arg128 as determined by MS and sequencing. Linear MS of Ob-Th2 showed a series of m/z values at 6181.98 Da, 5891.57 Da, 5600.66 Da and 5309.40 Da (Fig. 5B). These masses correspond to the peptide V106TASTQADTFPTIPVPTVDVPNGR128 containing four HexNAc-Hex moieties with varying amounts of SA. Amino acid sequencing suggested modifications on Ser109, Thr107, Thr110 and Thr121 which in combination with the MS data indicate O-glycosylations on these residues.

As with FbOPN, the glycopeptides from Ob-Th1 (Val88-Arg128 and Ile118-Arg128), were further digested with endoproteinase Asp-N. A peptide starting at Asp103 was detected by N-terminal sequencing with no identifiable PTH-amino acid in the cycles corresponding to Thr107, Ser109, Thr110. Sequencing and MS analysis of the peptide Asp113-Arg128 supported that Thr116 and Thr121 is glycosylated as the corresponding peptide in FbOPN (Table 2).

N-glycosylation - The mouse OPN sequence contains a single asparagine (Asn62) in a putative N-glycosylation motif. MS analysis of peptides containing this residue did not show glycosylation in either of the two proteins. This observation was supported by N-terminal sequencing of the large tryptic peptide isolated by gel filtration, which clearly showed a PTH-Asn in the cycle corresponding to Asn62. In addition, incubation of either of the proteins with PNGase F did not result in altered migration in SDS/PAGE (data not shown).

In summary these data show that Thr107, Ser109, Thr110, Thr116 and Thr121 are glycosylated in FbOPN. The absence of any trace of PTH-amino acids in these positions indicates that they were fully glycosylated. The corresponding residues were also found to be glycosylated in ObOPN, but with heterogeneity at Thr107 as this residue was observed both with and without glycans. The heterogeneity at Thr107 on ObOPN could account for the excess mass observed on dephosphorylated FbOPN compared to dephosphorylated ObOPN (Fig. 2). The masses of the glycopeptides in both proteins show that each glycosylated amino acid can be modified by various glycan structures. Though in most cases they consist of SA-Hex-[SA]-HexNAc units and to a lesser extent of SA-Hex-[SA-Hex-HexNAc]-HexNAc units. The characteristic MALDI-induced fragmentation pattern that reveals the presence of phosphorylations in a peptide (44) was not observed during analyses of the peptides Asp103-Arg128, Asp113-Arg128, Ile118-Arg128 and Val106-Arg128, strongly indicating that these residues do not contain phosphorylations.

Adhesion assays – To assess the biological consequences of these different patterns of phosphorylation, we test the ability of FbOPN and ObOPN to mediate cell adhesion. The 275-3-2 cells, transformed mouse embryonic fibroblasts, are able to adhere to both FbOPN and ObOPN: adhesion of these cells to FbOPN is slightly less (30% lower) than to ObOPN (Fig. 6A). These cells adhere to both proteins at greater than 50% of the maximal adhesion (to fibronectin). ObOPN, on the other hand supports minimal cell adhesion, less than 10% of...
that to fibronectin, and 6-fold lower than adhesion of these same cells to FbOPN (Fig. 6B). In every case, the addition of RGD peptide reduced adhesion to OPN to control (BSA) levels. An RAD peptide was ineffective in blocking adhesion. These results suggest that both cell lines adhere to OPN through integrins that bind RGD, thus excluding CD44 and the integrins $\alpha_4\beta_1$ and $\alpha_9\beta_1$. Together, these results support the hypothesis that integrin-mediated binding of some cell types to OPN is modulated by the degree of phosphorylation of the protein.

DISCUSSION

We present here the first direct comparison of OPNs expressed by different cell types originating from the same species, and the first comprehensive analysis of PTMs of OPN associated with transformed cells. We have identified 27 and 16 sites of phosphorylation in ObOPN and FbOPN, respectively. However, the difference in phosphorylation among the proteins is even more pronounced since ObOPN on average contains approximately 21 phosphate groups compared to an average of only four phosphorylations in FbOPN. The presence of only four moles of phosphate per OPN molecule makes FbOPN the least phosphorylated OPN isoform characterized yet. Many experiments have demonstrated the tumorigenic and metastatic abilities of ras-transformed fibroblasts, and ras family proto-oncogenes are one of the most widely mutated in tumors. The FbOPN characterized in this study produced by ras-transformed fibroblasts can be hypothesized to be similar to tumor-produced OPN and cause similar functional differences.

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The amino acid sequence of murine OPN contains a single potential N-glycosylation site (Asn$^{107}$). Analysis of thermolytic peptides covering this residue (Table 1) as well as treatment with PNGase F indicates that neither FbOPN nor ObOPN are N-glycosylated. The absence of N-glycosylations is consistent with data on milk and rat bone OPN which also lack N-glycosylations (34-36). OPN is a member of the SIBLING (Small Integrin-Binding LIgand N-linked Glycoprotein) family of glycoprophosphoproteins (19). However, the presence of N-linked glycans in OPN is debatable and data unambiguously showing this modification in OPN remains to be presented.

The differences in PTMs of FbOPN and ObOPN translated into functionally distinct proteins exhibiting dissimilar adhesion of 275-3-2 and MDA-MB-435 cells (Fig. 6). FbOPN mediated adhesion of both cell lines with almost similar efficiency. In contrast, ObOPN behaved very differently with each cell line, as it mediated strong binding of the 275-3-2 cells and very weak binding of the MDA-MB-435 cells. In fact, adhesion of the human breast cancer cells supported by ObOPN was six-fold lower than the binding of these cells to FbOPN. The significant difference between the results for each cell line with ObOPN as substrate indicates that the receptor(s) on the 275-3-2 cells mediating the binding to ObOPN is not present on the MBA-MB-435 cells, at least not in the same configuration.

To investigate which receptors mediate the binding between either of the two cell lines and OPN we examined the effect of an RGD peptide in the adhesion assay. As shown in Fig. 6A, the ability of the 275-3-2 cell line to adhere to either the FbOPN or ObOPN could be reduced to BSA control levels by addition of this peptide, but not an RAD-containing control. Similarly, the adhesion supported by FbOPN to the MDA-MB-435 cells was completely inhibited when treated with the RGD-peptide. The adhesion of the MDA-MB-435 cells to ObOPN was not significantly above the negative BSA control in any case (Fig. 6B). The ability of the RGD peptide but not the RAD control to block all observed adhesion suggests that OPN binding is integrin-mediated on both the 275-3-2 and MDA-
MB-435 cells. These data are supported by previous experiments showing that MDA-MB-435 cells are unable to adhere to RGD-mutant recombinant (non-phosphorylated) OPN (43).

These data suggest that OPN-mediated cell adhesion is a complex event that is influenced by the PTMs on OPN. For example, all binding of the MDA-MB-435 cells to FbOPN is via integrins, and these integrins cannot recognize ObOPN, perhaps due to conformational changes. Cellular interactions of OPN are complicated by the multiple integrins that can act as receptors for OPN. Involvement of the α4β1 and α9β1 integrins in this study can be excluded, since they only bind the N-terminal part of the protein resulting from thrombin cleavage (14,15), but several other RGD-binding integrins could be mediating the binding to OPN we observed. Further work is required to understand which integrins are involved.

In summary, FbOPN produced by ras-transformed fibroblasts contains on average approximately four phosphate groups whereas ObOPN produced by immortalized osteoblasts contains approximately 21 phosphate groups. The phosphorylations are distributed on 16 and 27 sites in FbOPN and ObOPN, respectively. Both proteins are O-glycosylated in a region N-terminal to the integrin-binding RGD sequence. FbOPN contains five fully substituted O-glycosylation sites, one of which is only partially substituted in the ObOPN counterpart. Both OPNs contain a single O-glycosylated serine, a novel type of modification in OPN. The fact that different cell types produce diversely modified OPN with different cell binding properties indicates that PTMs are very important in the regulation of OPN function.

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FOOTNOTES

*This work was supported in part by NIH grant # DK067685 (SRR), the Danish Dairy Board (ESS) and the National Science Foundation IGERT grant #DGE 0333196 (CK). The research in the Denhardt laboratory was supported in part by a Busch Biomedical Research Award, the National Multiple Sclerosis Society, and a grant from the Rutgers Technology Commercialization Fund.

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***The abbreviations used are: OPN, osteopontin; PTM, posttranslational modification; FbOPN, osteopontin from 275-3-2 murine ras-transformed fibroblast cells; ObOPN, osteopontin from MC3T3-E1 cells; RGD, Arg-Gly-Asp; BSA, bovine serum albumin; ALP, bovine alkaline phosphatase; µRPC, narrow-bore reverse-phase chromatography; PNGase F, Peptide-N-Glycosidase F; TFA, trifluoroacetic acid; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; PTH, phenylthiohydantoin; HexNAc, N-acetylhexosamines; Hex, hexoses; SA, sialic acid; Th, thermolysin

Acknowledgements - We thank Tajneen Natasha for help with OPN purification and Dr. R. Franceschi at University of Michigan (USA) for the MC3T3-E1 subclone 4 osteoblasts.

FIGURE LEGENDS

Fig. 1. SDS-PAGE of affinity-purified FbOPN and ObOPN. Samples were separated on a 12% tris-glycine gel by SDS-PAGE, and silver stained using the non-ammoniacal procedure. Lane 1: Conditioned medium from 275-3-2 ras-transformed fibroblasts. Lane 2: FbOPN purified from 2A1-coupled protein G-Agarose column. Lane 3: Conditioned medium from differentiating MC3T3-E1 subclone 4 osteoblasts. Lane 4: ObOPN purified from 2A1-coupled protein G-Agarose column. The bands at approximately 21-26 kDa in lane 2 and 4 represent C-terminal fragments of OPN.

Fig. 2. MALDI-TOF mass spectrometric analysis of native and dephosphorylated FbOPN and ObOPN. (A) The average mass peaks at approximately 34.9 kDa (black) and 34.6 kDa (grey) represent FbOPN before and after treatment with ALP, respectively (B) The average mass peaks at approximately 35.9 kDa (black) and 34.2 kDa (grey) represent ObOPN before and after treatment ALP, respectively.

Fig. 3. Reverse-phase HPLC separation of trypsin and thermolysin digests of OPN. Peptides were separated on a µRPC C2/C18 PC 2.1/10 column operated by a Pharmacia SMART system. Separation was carried out in 0.1% TFA and peptides eluted with a gradient of 60% (trypsin) or 80% (thermolysin) acetonitrile in 0.1% TFA (dotted line) at a flow rate of 0.15 ml/min. The peptides were detected in the effluent by measuring the absorbance at 214 nm (solid line). (A) RP-HPLC of tryptic peptides from FbOPN (B) RP-HPLC of tryptic peptides from ObOPN (C) RP-HPLC of thermolytic peptides from the FbOPN fragment Gln35/Gln54-Arg128 (D) RP-HPLC of thermolytic peptides from the ObOPN fragment Gln35/Gln54-Arg128.

Fig. 4. Localization of posttranslational modifications in FbOPN and ObOPN. Phosphorylations are indicated with P’s. Glycosylations are indicated with filled diamonds. The half filled diamond represents a variable glycosylated site in ObOPN.

Fig. 5. MALDI-TOF MS of O-glycopeptides from OPN. (A) MS of Fb-Th1 (from Fig. 3C). The monoisotopic mass at m/z 2113.91 corresponds to the peptide $^{135}$VPTVDVPNGR$^{128}$ with the indicated
glycan unit attached at Thr^{121}. The masses at $m/z$ 2478.93 (+1 HexNAc-Hex), 1822.82 (-1 SA) and 1531.80 (-2 SA) represent other glycosylation variants of the peptide. The unnumbered peaks in the spectrum represent unresolved masses from metastable decomposition of the glycans. (B) MS of Ob-Th2 (from Fig. 3D). The protonated average mass at $m/z$ 6181.98 corresponds to the peptide V^{106}TASTQADTFIPTVDPNGR^{128} containing four HexNAc-Hex units and eight SA glycans. The masses at $m/z$ 5891.57, 5600.66 and 5309.40 show mass differences of approximately 291 Da corresponding to variations in the amount of SA units attached to the peptide (Table 2).

Fig. 6. Adhesion of 275-3-2 and MDA-MB-435 cells to OPN. Adhesion of 275-3-2 (A) and MDA-MB-435 (B) cells to surfaces coated with the specified proteins. OPN isoforms were coated at 10 µg/ml. Cells were pre-incubated in the presence or absence of 100 µM GRGDNP or GRADSP peptides. Cell adhesion to positive control fibronectin was set to 100% and 1% BSA was used as negative control. The percent of attached cells was measured as described in Experimental Procedures. Bars show mean values for four wells per protein ± S.D, n.s. indicate non-significance and * means that P < 0.01 in Student’s t-test. Data shown are representative of three independent experiments for each cell line.

Fig. 7. Comparison of modification sites in FbOPN, ObOPN, rat bone (36), human (35) and bovine milk (34) isoforms of OPN. Phosphorylations and glycosylations are highlighted in black and grey, respectively. The underlined sequence in rat bone OPN has not been analyzed and introduced gaps are indicated by broken lines.
Table 1: Characterization of phosphorylated peptides

| Peptide* | S/T in MGCK or CKII motif | FbOPN | ObOPN |
|----------|---------------------------|-------|-------|
| V5-K34   | T, S, S10, S11            | 1/2/3 | 3     |
| L15-K34  |                           | 0     | 0     |
| Q35-K53  | S45, S46                  | 0     | 2     |
| A43-D51  | S45, S46                  | 1/2   | 2     |
| V44-D51  | S45, S46                  | 0/1/2 | 2/1   |
| Q54-H67  | T, S60, S61, S64          | 0/1/2 | 3/2   |
| Q54-H81  | T, S60, S61, S64          | 0     | 3     |
| T56-H67  | T, S60, S61, S64          | 0/1/2 | 3/2/1 |
| T56-H81  | T, S60, S61, S64          | 0     | 3/2/1 |
| S56-H67  | T, S60, S61, S64          | 0     | n.o.  |
| S56-H81  | T, S60, S61, S64          | 1/0   | n.o.  |
| N56-H67  | S14, S15                  | 0     | n.o.  |
| S56-H71  | S14, S15                  | 0     | n.o.  |
| S57-H11  | S14, S15                  | 0     | n.o.  |
| M58-H81  |                           | 0     | 0     |
| M58-S47  | S14, S15                  | 0     | 1/2/0 |
| A59-S7   | S14, S15                  | 0     | 1/0   |
| A59-T105 | S45, S46                  | 0     | n.o.  |
| V48-S64  | S45, S46                  | 0/1/2 | 2     |
| V48-H81  | S45, S46                  | 0/1/2 | n.o.  |
| V48-T105 | S45, S46                  | 0     | n.o.  |
| H17-T205 | S45, S46                  | 0     | 2     |
| G22-R137 | S146, S154                | 0     | 0     |
| S164-K163| S146, S154                | 0/1   | 1/0/2 |
| S164-R191| S146, S154                | 0/1   | n.o.  |
| S164-R208| S146, S154                | 0/1   | n.o.  |
| E168-R208| S146, S154                | 1/2/0 | 2/3   |
| G192-R208| S146, S154                | 0     | 2     |
| S164-R208| S146, S154                | 0     | 1     |
| T209-K232| S212, S215, S218, S231    | 2/3/1 | 3/2/1 |
| T209-K241| S212, S215, S218, S231    | n.o.  | 4     |
| A233-K241| S231, S234                | 0     | 1/0   |
| F256-K246| S231, S234                | 0     | 0     |
| D272-K  | S231, S234                | 0     | 0     |
| F278-N    | S267, S272, S273, S274    | 0     | 3/2   |
| I280-N    | S267, S272, S273, S274    | 0     | 2/1   |

* Peptides from the tryptic and thermolytic (Gln^35->Gln^34->Arg^128) digests of FbOPN and ObOPN observed by MS.

** Number of phosphates shows the different phosphorylation variants of each individual peptide observed by MS listed in order of abundance. Peptide abundance was quantitatively based upon peak size and the intensity of signal in MS. n.o.: not observed.
Table 2: Characterization of glycosylated peptides

| Peptide a | Sequence b | Observed MW c | Difference d | Modification e | Glycan structures f |
|-----------|------------|---------------|--------------|---------------|--------------------|
| Fb-Th1    | V^{i,v}R^{ii} | 9208.00^a 9202.20^a | 4890.54 4812.74 | (HexNAc-Hex)_{5,9} | HexHexNAcThrSer | HexNAcThrSer |
|           | VDSDESDESHK-  | 7598.70 7306.80 | 4447.39 4155.49 | (HexNAc-Hex)_{5,9} | HexHexNAcThrSer | HexNAcThrSer |
|           | I^{i,v}R^{ii} | 2478.93 2113.91 | 1312.28 947.26 | (HexNAc-Hex)_{5,9} | HexHexNAcThrSer | HexNAcThrSer |
|           | IVP*VDPVRG-  | 1822.82 1531.80 | 656.17 365.15 | (HexNAc-Hex)_{5,9} | HexHexNAcThrSer | HexNAcThrSer |
| Fb-Th1+Asp-N | D^{i,i}R^{ii} | 8204.50 7548.90 | 5471.55 4815.95 | (HexNAc-Hex)_{5,9} | HexHexNAcThrSer | HexNAcThrSer |
|           | DETV*A**QAADF- | 7474.10 | 4741.15 | (HexNAc-Hex)_{5,9} | HexHexNAcThrSer | HexNAcThrSer |
| Ob-Th1    | V^{i,v}R^{ii} | 9606.50 9523.10 | 5217.04 5133.64 | (HexNAc-Hex)_{5,9} | HexHexNAcThrSer | HexNAcThrSer |
|           | VD*DK*- | 2480.10 2114.96 | 1313.45 948.31 | (HexNAc-Hex)_{5,9} | HexHexNAcThrSer | HexNAcThrSer |
| Ob-Th2    | V^{i,i}R^{ii} | 6181.98 5891.57 | 3794.34 3503.93 | (HexNAc-Hex)_{5,9} | HexHexNAcThrSer | HexNAcThrSer |
|           | VTD**QADF*PIVP*V- | 5600.66 5309.40 | 3213.02 2921.76 | (HexNAc-Hex)_{5,9} | HexHexNAcThrSer | HexNAcThrSer |
| Ob-Th1+Asp-N | D^{i,i}R^{ii} | 7182.70 | 4449.75 | (HexNAc-Hex)_{5,9} | HexHexNAcThrSer | HexNAcThrSer |
|           | DETV*A**QA- | 4356.00 3988.40 | 2627.07 2363.56 | (HexNAc-Hex)_{5,9} | HexHexNAcThrSer | HexNAcThrSer |

Table 2
Amino acid sequence of peptides in fractions Fb-Th1, Ob-Th1 and Ob-Th2 (Fig. 3C and 3D) and peptides resulting from endoproteinase asp-N digestion of Fb-Th1 and Ob-Th1. Peptides were identified by sequence and MALDI-TOF MS analysis. In parentheses the MW of the unmodified peptide.

Result of the N-terminal sequence analysis. (-) denotes that the peptide has not been sequenced to the end. (*) denotes that no PTH-derivative was identified which indicates the presence of a modified residue.

Molecular mass determined by MALDI-TOF MS. If nothing else is mentioned all masses are monoisotopic.

Difference between observed and calculated mass.

The glycan composition attached to the peptide.

The two dominating O-glycan structures observed are SA-Hex-[SA]-HexNAc and SA-Hex-[SA-Hex-HexNAc]-HexNAc.

Molecular average masses determined by MALDI-TOF MS in linear mode.

Table 2
Figure 2

A. FbOPN

+ ALP: 34.6 kDa - ALP: 34.9 kDa

B. ObOPN

+ ALP: 34.2 kDa - ALP: 35.9 kDa
Figure 3
Figure 4

Ob  LPVKVTDSGSEEEKLYSLHPDPIATWLVPDPSQKNLAPQNAVSEEEKD  
     P P P P P P P P P P P P P 50
Fb  LPVKVTDSGSEEEKLYSLHPDPIATWLVPDPSQKNLAPQNAVSEEEKD  
     P P P P P P P P P P P P P

Ob  DFKQETLPSNSNESHDHMDNDDDDDDDDDGDHAESDVSDESHSD  
     P P P P P P P P 100
Fb  DFKQETLPSNSNESHDHMDNDDDDDDDDDGDHAESDVSDESHSD  
     P P P P P P P P

Ob  ESDETVTASTQADTFIVPTVDPNGRGLRKSRSFQVSDEQY  
     P P P P 150
Fb  ESDETVTASTQADTFIVPTVDPNGRGLRKSRSFQVSDEQY  
     P P P P

Ob  PDATDELDSTSHMSGESKESLDPVIPVAQLMSDSQDDNNGKSHESQLD  
     P P P P 200
Fb  PDATDELDSTSHMSGESKESLDPVIPVAQLMSDSQDDNNGKSHESQLD  
     P P P P

Ob  EPSLTHRLHSEQSADQSDVIDSQASSKASLEHQSHKHSKDKLV  
     P P P P 250
Fb  EPSLTHRLHSEQSADQSDVIDSQASSKASLEHQSHKHSKDKLV  
     P P P P

Ob  LDPKSKEDDRYKLFRISHELESSSEVN  
     P P 278
Fb  LDPKSKEDDRYKLFRISHELESSSEVN  
     P P
Figure 5
Figure 6
Cell-type specific post-translational modifications of mouse osteopontin are associated with different adhesive properties
Brian Christensen, Christian C. Kazanecki, Torben E. Petersen, Susan R. Rittling, David T. Denhardt and Esben S. Sørensen

J. Biol. Chem. published online May 11, 2007

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