C-terminal Modification of Osteopontin Inhibits Interaction with the $\alpha_\nu \beta_3$-Integrin*

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Brian Christensen‡, Eva Kläning‡, Mette S. Nielsen†, Mikkel H. Andersen§, and Esben S. Sørensen∗†

From the ‡Protein Chemistry Laboratory and §Protein Core Facility, Department of Molecular Biology and Genetics, Aarhus University, DK-8000 Aarhus, Denmark

Osteopontin (OPN) is a multifunctional phosphorylated glycoprotein containing an integrin binding sequence Arg-Gly-Asp (RGD) through which it interacts with several integrin receptors, such as the $\alpha_\nu \beta_3$-integrin. OPN exists in many different isoforms differing in phosphorylation status that are likely to interact differently with integrins. The C-terminal region of OPN is particularly well conserved among mammalian species, which suggests an important functional role of this region. In this study, we show that modification of the extreme C terminus of OPN plays an important regulatory role for the interaction with the $\alpha_\nu \beta_3$-integrin. It is demonstrated that highly phosphorylated OPN has a much reduced capability to promote cell adhesion via the $\alpha_\nu \beta_3$-integrin compared with lesser phosphorylated forms. The cell attachment promoted by highly phosphorylated OPN could be greatly increased by both dephosphorylation and proteolytic removal of the C terminus. Using recombinantly expressed OPN containing a tag in the N or C terminus, it is shown that a modification in the C-terminal part significantly reduces the adhesion of cells to OPN via the $\alpha_\nu \beta_3$-integrin, whereas modification of the N terminus does not influence the binding. The inhibited binding of the $\alpha_\nu \beta_3$-integrin to OPN could be restored by proteolytic removal of the C terminus by thrombin and plasmin. These data illustrate a novel mechanism regulating the interaction of OPN and the $\alpha_\nu \beta_3$-integrin by modification of the highly conserved C-terminal region of the protein.

Osteopontin (OPN)‡ is a highly acidic phosphorylated glycoprotein containing an integrin binding Arg-Gly-Asp (RGD) sequence. OPN is a multifunctional protein, and it has been implicated in bioactivities including tumorigenesis, immune responses, bone remodeling, wound healing, and inhibition of ectopic calcification (1–3). A wide variety of cell types express OPN, and the protein is found both as an immobilized extra-cellular matrix molecule in e.g. bone and as a secreted protein in body fluids, such as milk, urine, and blood (4–7). Nuclear magnetic resonance studies have shown that OPN has an open flexible conformation largely devoid of secondary structure (8). Other biophysical studies have also consistently demonstrated that OPN is an intrinsically disordered protein; however; binding of OPN to hydroxyapatite slightly increased the $\beta$-sheet percentage (9), a transient intramolecular interaction between the N and C terminus has been suggested (10), and recently it was shown that quail OPN contains some local segments with secondary structure (11). Many of the versatile functions exhibited by OPN are dependent on interactions between the protein and integrin receptors. The $\alpha_\nu \beta_1$, $\alpha_\nu \beta_2$, $\alpha_\nu \beta_3$, $\alpha_\nu \beta_5$, and $\alpha_\nu \beta_5$-integrins bind OPN through the conserved RGD sequence (12, 13), whereas the $\alpha_\nu \beta_1$, and $\alpha_\nu \beta_3$-integrins bind the cryptic non-RGD motif Ser-Val-Val-Tyr-Gly-Leu-Arg (SVVYGLR) (in human OPN) (14, 15), and recently the monocyte $\alpha_\nu \beta_3$-integrin receptor was shown to interact with the highly acidic parts of OPN (16).

OPN is extensively altered through posttranslational modifications, such as phosphorylation, glycosylation, sulfation, and proteolytic processing which significantly influence the function of the protein (5, 13, 17). The proteases thrombin, matrix metalloprotease-3 and -9, plasmin, and cathepsin D cleave OPN close to the RGD sequence, which in all cases generates N-terminal fragments containing the integrin binding RGD sequence (4, 18, 19). These N-terminal fragments have shown greater capability to mediate RGD-dependent cell attachment than the full-length protein presumably due to a more exposed integrin binding sequence (5, 13, 20).

OPN is very heterogeneously phosphorylated; and although a similar number of potential phosphorylation sites have been identified in OPN from different sources, the degree of phosphorylation varies a lot depending on the origin of the protein (1, 13). The most phosphorylated form is found in milk where OPN has been shown to contain ~25–30 phosphate groups depending on the species (21, 22). In contrast, OPN from urine and bone is only decorated by ~8 and ~10 phosphorylations, respectively (6, 23). Further emphasizing the cell type-specific phosphorylation of OPN, a comparison of OPN produced by two different murine cell types showed that ras-transformed fibroblasts expressed a variant containing only four phosphate groups whereas osteoblast-derived OPN contained an average of 21 phosphorylations (24).

The widely expressed $\alpha_\nu \beta_3$-integrin is a well characterized receptor for OPN in processes such as cell adhesion, migration, and bone resorption (18, 19, 25, 26). However, the regulation of this interaction has not been clearly described. In one study, cell adhesion to highly phosphorylated OPN via the $\alpha_\nu \beta_3$-integrin is significantly increased after proteolytic cleavage of OPN (19).
In another study, no difference in adhesion via this receptor between full-length and matrix metalloprotease and thrombin-cleaved bacterial produced OPN was observed (12), indicating that exposure of the RGD sequence after cleavage is not necessary for binding to the α5β3-integrin. These seemingly conflicting results could likely be ascribed to the difference in phosphorylation of the OPNs used in the studies. The significance of the phosphorylations in cell binding was substantiated in a direct comparison of two well characterized full-length murine OPNs. Here, a less phosphorylated isoform containing ~4 phosphates supported greater adhesion of the α5β3-integrin expressing melanoma cell line MDA-MB-435 than a more phosphorylated (24)

This suggests that a high degree of phosphorylation can reduce the capability of the α5β3-integrin to interact with OPN. The phosphorylation sites in OPN are located in clusters of three to five sites with stretches of unphosphorylated residues in between (21, 22). In low phosphorylated OPN variants, e.g. urinary and bone OPN, only few of the potential sites are actually phosphorylated (6, 23, 24), whereas most of the phosphorylation sites are occupied in highly phosphorylated forms, like milk OPN (21, 22). This leaves open the possibility that phosphorylation of specific clusters in OPN can influence the binding to the α5β3-integrin.

The extreme C-terminal region of OPN is highly conserved among mammalian species (see Fig. 1) and contains four serines (in human OPN) which constitute potential phosphorylation sites. The high degree of amino acid conservation could indicate an important functional role of this part of OPN as is the case for other highly conserved elements like the integrin binding sequences and sites of posttranslational modification. In support of this, it has recently been shown that monoclonal antibodies recognizing the extreme C terminus inhibited MDA-MB-435 cell adhesion to unphosphorylated recombinant OPN (27). Furthermore, a synthetic unmodified peptide corresponding to the C-terminal 18 amino acids was able to bind these cells, whereas neither a phosphorylated nor a scrambled variant had this effect (13). This suggests that phosphorylation of the extreme C-terminal region of OPN influences its cell binding capabilities.

In the present study we show that phosphorylation and other modification of the highly conserved C-terminal region of OPN inhibits its interaction with the α5β3-integrin. Adhesion mediated by C-terminally modified OPN was increased significantly after thrombin and plasmin cleavage, whereas cell attachment promoted by OPN not modified at the C terminus was only slightly increased by proteolytic removal of the C terminus. This indicates that the increase in adhesive properties is a result of removal of the modified C terminus rather than improved exposure of the RGD sequence. Collectively, these results point toward a novel mechanism by which the interaction between OPN and the α5β3-integrin can be regulated by modification of the extreme C-terminal region of the protein.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Culture**—The MDA-MB-435 human melanoma cell line (a kind gift from Dr. David T. Denhardt, Rutgers University, New Brunswick, NJ), human embryonic kidney 293T cells, Moαv cells (from Mark H. Ginsberg), and the embryonic monkey kidney cell line MA104 were all maintained in Dulbecco’s modified Eagle’s medium (DMEM) with Glutamax (Invitrogen) supplemented with 10% fetal bovine serum, and 1% antibiotics (penicillin/streptomycin). Moαv is a subclone of M21 melanoma cells and has previously been shown to express high levels of the α5β3-integrin (28).

**Plasmid Construction**—For generation of C-terminally tagged full-length OPN (rOPN-C), human cDNA encoding residues 1-314 of OPN (the first 16 residues is the signal peptide which is not included in Figs. 1 and 2) was amplified by PCR using the forward primer 5’-GCTGGATCATCACCATACTCAACGAGGATC-3’ which included a BamHI restriction site (underlined) and the first 5 amino acids of the signal peptide. The reverse primer was 5’-CTCGACTCAAGCTTTGATTTGCAGTG-3’, which contained a HindIII restriction site (underlined) and the last 5 amino acids of the signal peptide. The amplified fragments were cloned into the BamHI/HindIII site of pEXPR-IBA42 (IBA BioTAGnology), which contained a HindIII restriction site and the last 5 amino acids of the signal peptide. The amplified fragments were cloned into the BamHI/HindIII sites of pcDNA3.1/Myc-His(-)A (Invitrogen). These constructs encoded OPN residues 1–298 (full-length) and the fragment consisting of residues 1–228, both followed immediately by residues KLGP, the myc epitope (EQKLISEEDL), residues NSAVD and H82, increasing the molecular mass by 2.9 kDa.

For generation of N-terminally tagged full-length OPN, the residues 1–298 of OPN (without the signal peptide) were amplified by PCR using the forward primer 5’-GCTAGCAGAGGATCGCATCACCACAATCCACACGAGGATCAGTAAAAACGCCTGATCTCG-3’, which include an NheI restriction site (underlined), a H82 tag (italic), and the first 8 residues of the secreted OPN sequence (1’PVKQADS). The reverse primer was 5’-GACCTCGAGCTACTAATTTGATCCAGAAGATGC-3’, which include a restriction site for XhoI (underlined), two stop codons (italic), and the last 7 C-terminal residues of OPN. The amplified fragment was cloned into the NheI/XhoI sites of pEXPR-IBA42 (IBA BioTAGnology). The pEXPR-IBA42 vector contains the BM40 signal sequence resulting in secretion of the construct. This construct encoded residues ASRGS, H82, and the residues GG, increasing the mass with 1.4 kDa, followed by the full-length OPN sequence (residues 1–298).

Plasmids were propagated in Escherichia coli DH5α cells, and all constructs were verified by sequence analysis. Plasmid DNA for transfection was prepared using a NucleoSpin Plasmid QuickPure kit (Macherey-Nagel).

**Protein Expression and Purification**—Human embryonic kidney 293T cells were plated on 10-cm tissue culture dishes and were transfected 18 h later by calcium phosphate co-precipitation (29) using 7.5 μg of plasmid DNA. After a further 48 h, the supernatants were harvested and replaced by serum-free medium (293 SFM II; Invitrogen). Serum-free medium was harvested and changed three times at 48-h intervals.
His-tagged recombinant OPN was purified from serum-free medium (~200 ml) using a metal-chelate affinity column (1 ml; Qiagen) charged with nickel ions. Bound protein was eluted with 250 mM imidazole in phosphate-buffered saline (PBS). The fractions containing OPN were identified by Western blotting and desalted on a PD10 column in 25 mM ammonium bicarbonate at 37 °C for 1 h.

Full-length and N-terminal OPN were purified from human milk and urine as described (6, 19). All purified proteins were analyzed by N-terminal sequencing, MALDI-TOF MS (matrix-assisted laser desorption ionization time-of-flight mass spectrometry), and SDS-PAGE with Coomassie Brilliant Blue visualization. The amount of purified OPN was determined with a BCA protein assay kit (Pierce). OPN was digested with thrombin (30 milliunits/µg OPN) (Sigma) and plasmnin (0.2 milliunit/µg OPN) (Roche Applied Science) in 0.1 M ammonium bicarbonate at 37 °C for 1 h.

Dephosphorylation of OPN—For dephosphorylation, the different OPNs were incubated with bovine alkaline phosphatase (ALP) (20 milliunits/µg OPN) (Sigma) in 10 mM ammonium bicarbonate (pH 8.5) overnight at 37 °C. Native and dephosphorylated OPNs were analyzed by MS in positive linear ion mode using a Voyager DE-PRO MALDI-TOF mass spectrometer (Applied Biosystems) before and after ALP treatment. The phosphate content on mOPN was also analyzed with a phosphoprotein phosphate estimation assay kit (Pierce). Finally, to ensure that ALP-treated mOPN was free of phosphates, it was digested with trypsin (Warthington Biochemical) using an enzyme to substrate ratio of 1:30 (w/w) in 20 mM ammonium bicarbonate at 37 °C for 6 h. Tryptic peptides were separated on a µRPC C2/C18 PC 2.1/10 column (narrow-bore reversed-phase chromatography; GE Healthcare) connected to a GE Healthcare SMART system. Separation was carried out in 0.1% trifluoroacetic acid (buffer A) and eluted with a gradient of 60% acetonitrile in 0.1% trifluoroacetic acid (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 and analyzed for phosphorylations by MS.

For adhesion assays, 100 µg of milk and urinary OPN were incubated with ALP as described above. Dephosphorylated OPN was separated from ALP and buffer components by reversed-phase HPLC as described for separation of tryptic mOPN peptides.

Carbohydrate Analyses—For removal of simple O-linked glycans, OPN was incubated with O-glycanase (0.1 milliunit/µg OPN) and sialidase (0.35 milliunit/µg OPN) (both from Prozyme) in 50 mM sodium phosphate (pH 7.0) overnight at 37 °C. The deglycosylation was analyzed by MS before and after treatment with the enzymes.

Full-length milk OPN was additionally hydrolyzed in 2 M trifluoroacetic acid for 4 h at 100 °C under oxygen-free conditions. α-1-Rhamnose was added as internal standard. For quantification of the more stable amino sugars, N-acetylgalactosamine (GalNAc) and N-acetylglucosamine (GlcNAc), an additional OPN sample was hydrolyzed in 4 M HCl for 6 h at 110 °C. For sialic acid analysis, the protein was hydrolyzed in 0.1 M HCl for 60 min at 80 °C. Monosaccharides were separated by high pH anion-exchange chromatography using a CarboPac PA1 column (4 × 250 mm) (Dionex) and monitored by pulsed electrochemical detection. The neutral monosaccharides were eluted isocratically with 16 mM NaOH, whereas sialic acid was eluted with 100 mM NaOH and 150 mM sodium acetate. Standard monosaccharide mixtures of α-L-fucose, d-galactosamine, d-glucosamine, d-galactose, d-glucose (all 0.1 mM), d-mannose (0.5 mM), α-1-rhamnose (0.2 mM), and N-acetylenuraminic acid (0.02 mM) (all from Sigma) were used. Linearity was observed between the injected amounts of monosaccharide standards and the peak areas, and hence the amounts of the individual monosaccharides in the samples were deduced from standard curves.

Cell Adhesion Assays—Flat-bottom 96-well tissue culture-treated polystyrene microtiter plates (Corning) were coated overnight at 4 °C with OPN diluted in PBS to the appropriate concentrations and then blocked with 1% bovine serum albumin (BSA). The cells were trypsinized, then washed twice and resuspended in DMEM containing 1 mg/ml BSA. For blocking of integrin function, cells were preincubated for 30 min at 37 °C with a neutralizing antibody against αvβ3 (clone LM609; Chemicon) or an IgG1 isotype control mAb (Sigma) (both at 5 µg/ml), or with 100 µM GRGDNP or GRADSP peptides (Enzo Life Science). Subsequently, cells (5 × 10^4) were added to coated wells and incubated for 1.5 h at 37 °C in a humidified atmosphere with 5% CO2. Nonadhered cells were removed by washing twice with 75 µl of Percoll (Sigma) (73% Percoll, 0.9% NaCl), and adherent cells were fixed with 50 µl of fixative (10% glutaraldehyde in Percoll). Fixed cells were stained with 100 µl of 0.5% toluidine blue and solubilized in 50 µl of 0.5% Triton X-100 before reading at 630 nm using a Microplate Autoreader EL 311 (BioTek).

Analysis of OPN Coating—The 96-well plates used in the adhesion assays were coated overnight at 4 °C with OPN diluted in PBS in the same concentration range as used for the adhesion assays. Subsequently, the wells were blocked with 1% BSA in PBS for 1 h at room temperature. All washes and dilutions after this were performed in PBS-Tween (10 mM disodium phosphate anhydrate (pH 7.2), 150 mM NaCl, 0.05% (v/v) Tween 20). The coated OPNs were detected by addition of 0.5 µg of the monoclonal mouse anti-human OPN antibodies 2A1, 1H3, and 3D9 (27) (a kind gift from Dr. David T. Denhardt) followed by incubation with hors eradish peroxidase-conjugated polyclonal goat anti-mouse IgG (Dako) (diluted 1:1000). Both antibodies were incubated for 1 h at 37 °C. Color development was obtained with TMB-one substrate (Kem-En-Tec), and the reaction was stopped by addition of 0.2 M H₂SO₄. Color intensity was measured at 450 nm using an ELISA reader (BioTek).

Statistical Analysis—Statistical analysis of data was done by Student’s t test. Difference was considered to be statistically significant at p < 0.05.

RESULTS

Purification, Expression, and Characterization of Human OPNs—The human melanoma cell line MDA-MB-435 has previously shown a much greater adhesion to a less phosphorylated murine OPN variant (4 phosphates on average) from ras-transformed fibroblasts than to a more phosphorylated mouse OPN
form secreted by osteoblasts (21 phosphates on average) (24).

To analyze the role of OPN modification in cell adhesion further, several different human OPN forms were either purified from natural sources or expressed recombinantly in mammalian cells. Full-length OPN (mOPN) and a naturally occurring N-terminal fragment (nmOPN) (Ile1-Lys154; Fig. 1) (19), were purified from milk and the urinary OPN isoform (uOPN) was purified from urine. Full-length recombinant human OPN with either an N- or a C-terminal tag (rOPN-N and rOPN-C) was produced in HEK293T cells together with a C-terminally tagged fragment consisting of amino acids 1–228 (rOPN-228C; see Fig. 1). Fig. 2 shows a schematic overview of the different OPN variants, and the evaluation of their purity by SDS-PAGE. The N-terminal fragment from milk migrates and stains as a broad indistinct band in SDS-PAGE (Fig. 2, lane 3). This pattern has also been observed in a previous study, although, the purified nmOPN was shown to be a well defined homogenous molecule when analyzed by MS and RP-HPLC (19). Similarly, below the dominating band representing rOPN-N (Fig. 2, lane 5) some faint bands are present. These represents fragments of OPN; however, RP-HPLC, MS, and Western blotting analyses (data not shown) showed that these fragments of rOPN-N only constituted very minor proportions of the protein in the sample.

The degree of phosphorylation of the different OPNs was estimated by MS before and after treatment with bovine ALP (Table 1). The two native full-length OPNs differed in the level of phosphorylation, as mOPN contained ~26 phosphates whereas uOPN contained ~8 phosphates. The N-terminal fragment, nmOPN, showed an excess mass corresponding to ~13 phosphates. None of the recombinant proteins was phosphorylated in any significant degree. To confirm that ALP treatment resulted in complete dephosphorylation, mOPN was also analyzed by use of a phosphoprotein phosphate estimation kit. This revealed a total of 25 phosphate groups in mOPN corresponding well with the number of phosphorylations estimated by MS. Finally, MS analysis of tryptic peptides from ALP-treated mOPN showed that none of these contained phosphate groups (data not shown).

The excess mass between the dephosphorylated proteins and the calculated mass of the amino acid residues indicated that all the OPNs were O-glycosylated. The surplus of 3–4 kDa for uOPN and the recombinant proteins suggested that the glycans on these proteins are composed of similar monosaccharides, whereas the excess mass of 8–9 kDa for mOPN and nmOPN indicated that human milk OPN is decorated by larger glycan structures. The glycans on uOPN, rOPN-N, rOPN-C, and rOPN-228C were of the disialylated core-1 O-glycan sialic acid-
galactose-[sialic acid]-GalNAc type, as the enzymes sialidase and O-glycanase were able to remove carbohydrates from these OPNs (data not shown). In contrast, the milk OPNs were unaffected by treatment with these enzymes. Instead, the carbohydrate composition of mOPN was determined by mild acid hydrolysis followed by high pH anion-exchange chromatography separation of the resulting monosaccharides. The hydrolysates showed the presence of the five monosaccharides GalNAc, GlcNAc, galactose, sialic acid, and fucose (Table 2). Thus, human milk OPN is most likely decorated by glycans composed of the GalNAc-galactose-[GlcNAc] core-2 structure extended by fucosylated, and to lesser degree sialylated, poly-N-acetyllactosamine units.

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![Figure 2. Structure and purity of OPN variants.](image)

**TABLE 1**

| Protein   | Theoretical mass (kDa) | Observed mass (kDa) before ALP treatment | Mass after ALP treatment (kDa) | Number of phosphates | Mass difference (Da) |
|-----------|------------------------|------------------------------------------|-------------------------------|----------------------|----------------------|
| mOPN      | 33.7                    | 45.3                                     | 43.2                          | ~25–26              | 9.5                  |
| uOPN      | 33.7                    | 37.7                                     | 37.1                          | ~7–8                | 3.4                  |
| nmOPN     | 17.1                    | 26.5                                     | 25.5                          | ~12–13              | 8.4                  |
| rOPN-N    | 35.1                    | 38.5                                     | 38.5                          | ~0                  | 3.4                  |
| rOPN-C    | 36.6                    | 39.8                                     | 39.8                          | ~0                  | 3.2                  |
| rOPN-228C | 28.4                    | 32.5                                     | 32.5                          | ~0                  | 4.1                  |

**TABLE 2**

Carbohydrate composition of mOPN

Monosaccharide amounts were determined as described under “Experimental Procedures.”

| Carbohydrate composition | mol monosaccharide/mol mOPN |
|--------------------------|-----------------------------|
| GalNAc                   | 4.8                         |
| GlcNAc                   | 16.5                        |
| Galactose                | 20.7                        |
| Sialic acid              | 4.1                         |
| Fucose                   | 15.3                        |
| Mannose                  | ND*                         |

*ND, not detected.

**Adhesion of MDA-MB-435 Cells to OPN**—The adhesion of the MDA-MB-435 cells to high and low phosphorylated OPN was investigated by comparing their binding to mOPN, nmOPN, and uOPN (Fig. 3A). The number of cells increased dose-dependently on all three OPNs at low coating concentrations (0–50 nM), and the binding was saturated at high OPN concentrations (100–200 nM). At binding saturation, similar numbers of MDA-MB-435 cells were adhering to uOPN and nmOPN, whereas the adhesion promoted by highly phosphorylated mOPN was approximately three times lower. The different adhesion pattern could be the result of altered cell surface receptor recognition of the different OPNs. However, in all cases the binding of the cells to OPN was RGD-dependent and via the $\alpha_\text{v}\beta_3$ integrin, as RGD peptides and a blocking antibody against $\alpha_\text{v}\beta_3$ reduced the adhesion to a level similar to the negative BSA control, whereas RAD control peptides or an isotype IgG had no effect on the adhesion (Fig. 3B).

The orientation and exposure of functional sites on the various fragments and differently phosphorylated OPNs are important parameters when using assays involving binding...
to surfaces. To compare the exposure of the C terminus after binding to the plates, the three C-terminal recognizing monoclonal antibodies 2A1 (epitope, P190VA192), 1H3 (epitope, S218AETHSH224), and 3D9 (epitope, K283FRISHELDSASSEVN298) (Fig. 1) were tested for recognition of the different proteins. 2A1 and 1H3 recognized all OPNs equally well at a coating concentration of 100 nM, whereas 3D9 did not detect native mOPN or rOPN-228C (Fig. 3C). The recognition pattern of 3D9 was expected as this antibody does not bind the extreme C terminus of heavily phosphorylated OPN (27) and as rOPN-228C lacks the C terminus. Similar results were obtained with coating concentration of 1 nM, 10 nM, and 25 nM (data not shown). This indicates that the different OPNs bind to the surface in a comparable manner with similar orientation and exposure of the C terminus. Therefore, the different adhesion patterns could not be ascribed to differently binding of the OPNs to the plastic plates.

A high degree of phosphorylation of full-length OPN considerably reduced the ability of the protein to interact with the αvβ3-integrin. The phosphates responsible for this inhibition seemed to be located in the C terminus, as nmOPN lacking the highly phosphorylated C terminus exhibited adhesion at the same level as low phosphorylated uOPN. The monoclonal antibody 3D9 recognized uOPN but not mOPN (Fig. 3C), illustrating that the extreme C terminus is unphosphorylated or modestly phosphorylated in uOPN and highly phosphorylated in mOPN. Following ALP treatment 3D9 recognized mOPN, indicating an unmodified C terminus. If phosphorylation of the C terminus inhibited the interaction between mOPN and the αvβ3-integrin, then phosphatase treatment should increase its adhesive properties. Indeed, dephosphorylated mOPN promoted significantly higher adhesion of the MDA-MB-435 cells than its phosphorylated counterpart (Fig. 3D). On the contrary, no difference was observed in the binding of the cells to uOPN before and after dephosphorylation.

To examine further the influence of modification of the extreme C terminus in cell adhesion, the three recombinant OPNs (rOPN-N, rOPN-C, and rOPN-228C) with either an N- or C-terminal tag were compared in adhesion assays. A dose-response curve showed that maximal MDA-MB-435 binding to all of the recombinant proteins was obtained at a coating concentration of 100 nM (data not shown). At this coating concentration, rOPN-C displayed approximately one third of the bind-

![FIGURE 3. Cell adhesion of MDA-MB-435 cells to native human OPN. A, wells of microtiter plates coated with human milk OPN (■), N-terminal milk OPN (◼), or urinary OPN (○) in different concentrations. B, wells of microtiter plates coated with OPN at a concentration of 100 nM. Cells were preincubated in the presence or absence of 5 μg/ml of an antibody against the αvβ3-integrin or an isotype IgG control, or with or without 100 μM GRGDNP (RGD) or GRADSHP (RAD) peptide. 1% BSA was used as negative control. For all experiments data are expressed as mean ± S.D. (error bars; n = 3). C, microtiter plates coated with 100 nM mOPN, mOPN + ALP, uOPN, rOPN-N, rOPN-C, and rOPN-228C were analyzed for coating efficiency and orientation of the proteins by detection with the monoclonal antibodies 2A1, 1H3, and 3D9. D, adhesion of MDA-MB-435 cells to surfaces coated with 100 nM uOPN and mOPN ± ALP. The data are expressed as mean ± S.D. (n = 3). n.s., nonsignificant; *, p < 0.05 (Student’s t test).](https://www.jbc.org/content/287/6/3793)
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FIGURE 4. Cell adhesion of MDA-MB-435 cells to recombinant human OPN and proteolytically cleaved OPN. Wells of microtiter plates were coated with OPN at a concentration of 100 nM. The data are expressed as mean ± S.D. (error bars; n = 3). A, adhesion to rOPN-N (full-length, N-terminal tag), rOPN-C (full-length, C-terminal tag), and rOPN-228C (fragment 1–228, C-terminal tag) after preincubation of the cells in the presence or absence of 5 μg/ml of an antibody against the αvβ3 integrin or an isotype IgG control, or preincubation with or without 100 μM GRGDNP (RGD) or GRADSP (RAD) peptide. 1% BSA was used as negative control. B, adhesion to mOPN, uOPN, rOPN-N, rOPN-C, and rOPN-228C before and after cleavage by thrombin and plasmin.

If modification of the C terminus can inhibit the adhesion mediated by OPN, it could be expected that separation of the N- and C-terminal parts of OPN by proteolytic cleavage would result in an increase in cell adhesion. Proteolytic cleavage of OPN by thrombin generates an N-terminal fragment (Ile1-Lys154, and in contrast to thrombin, plasmin completely degrades the C terminus (19). The adhesion mediated by mOPN and rOPN-C increased ~5-fold after thrombin and plasmin cleavage. On the contrary, cleavage of uOPN, rOPN-N, and rOPN-228C increased the adhesion to a much lesser degree (Fig. 4B). The cell binding to plasmin- and thrombin-cleaved OPN was also neutralized by both RGD-containing peptides and a blocking antibody against the αvβ3 integrin (data not shown).

Adhesion of Moαv and MA104 Cells to OPN—The effect of C-terminal modification on the interaction between OPN and the αvβ3 integrin was further examined using Moαv cells. The Moαv cells are a subgroup of human melanoma cells and have previously been shown to express high levels of the αvβ3 integrin (28). As shown for MDA-MB-435 cells, adhesion of these cells to OPN were also completely RGD-dependent and via the αvβ3 integrin (Fig. 5A). Furthermore, the Moαv cells bound to uOPN and rOPN-N with similar efficiency, which was approximately two times greater than the binding to mOPN and rOPN-C (Fig. 5B). Next, MA104 embryonic monkey kidney cells were investigated to see whether the tendency also applied to cells that were not of melanoma origin. Adhesion of the kidney cells was inhibited ~75% by the αvβ3-integrin-blocking antibody, and addition of RGD-containing peptides reduced the binding to a level comparable with the negative BSA control (Fig. 5C). This showed that the αvβ3 integrin is the principal OPN receptor on the MA104 cells. The kidney cells exhibited approximately ~25–40% higher adhesion to uOPN and rOPN-N compared with mOPN and rOPN-C, respectively (Fig. 5D). Thus, the interaction between OPN and other types of αvβ3 integrin-expressing cells was also mitigated by high phosphorylation of the C terminus or the presence of a tag at the C-terminal Asn298.

DISCUSSION

In the present study modification of the C terminus in terms of both phosphorylation and a covalently attached tag to Asn298 was demonstrated to significantly reduce the adhesion of different cell lines to human OPN via the αvβ3 integrin. This indicates a potential new mechanism for regulation of the interaction between OPN and integrins. The alignment shown in Fig. 1 illustrates that the extreme C-terminal region of OPN is highly conserved among mammalian species. Several functional important motifs like phosphorylation sites, glycosylation sites, the RGD sequence, and the cryptic integrin binding motif SVVYGLR are highly conserved. The conservation of the C terminus suggests that this region may also be important for the function of OPN.

OPN is O-glycosylated at 3–5 conserved threonines in an unphosphorylated region to the N-terminal side of the RGD sequence (6, 21–24). All the OPN isoforms used in the present study were also observed to be O-glycosylated. The carbohy-
drates on the recombinant OPNs and uOPN consisted of a disialylated GalNAc-galactose core. This glycan structure has previously been described in uOPN and in other isoforms of the protein from bone and fibroblasts (6, 23, 24). The excess mass after dephosphorylation and the carbohydrate composition analysis of human milk OPN showed that this isoform is glycosylated differently from the other OPNs. The presence of large fucosylated N-acetyllactosamine units on mOPN shown here has also been demonstrated in another study (30). The difference in glycan structure between the OPNs is nevertheless not likely to cause the observed functional differences because milk OPN lacking the C terminus (nmOPN and proteolytically cleaved mOPN) but still containing the large O-glycan structures promoted strong cell attachment; furthermore, rOPN-C decorated by the small glycans exhibited only minor cell adhesion.

The significant reduction of α<sub>v</sub>β<sub>3</sub>-integrin-mediated cell adhesion to C-terminally modified OPN was consistent using two different melanoma cell lines. The tendency also applied to other cell types as seen for the MA104 kidney cells. The difference between the OPNs was not as pronounced for the kidney cells as for the melanoma cells. This could be explained by the presence of several OPN-binding integrins on the MA104 cells, as complete inhibition of adhesion was not obtained using anti-α<sub>v</sub>β<sub>3</sub>-integrin antibodies. In both melanoma cell types, complete inhibition of cell adhesion was obtained when the α<sub>v</sub>β<sub>3</sub>-integrin was blocked.

The adhesion mediated by highly phosphorylated OPN via the α<sub>v</sub>β<sub>3</sub>-integrin on the MDA-MB-435 melanoma cells was negligible and increased after dephosphorylation; however, previous studies have shown that partial enzymatic dephosphorylation of OPN from bovine milk and bone results in decreased α<sub>v</sub>β<sub>3</sub>-integrin-dependent adhesion of osteoclasts (25, 31). Similarly, in vitro phosphorylation of recombinant (nonphosphorylated) rat OPN increased the number of osteoclasts adhering to OPN, although only 1–2 phosphates were incorporated by the kinase (32). These studies all used in vitro enzymatic treatment to either add or remove phosphates without characterization of the resulting products. In addition, comparison of results across different experimental set-ups using different OPN species varying in amino acid sequence can also be difficult. In the present study thoroughly characterized human

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**FIGURE 5.** Adhesion of Mo<sub>αvβ3</sub> melanoma and MA104 kidney cells to human OPN. Adhesion of Mo<sub>αvβ3</sub> (A and B) and MA104 kidney (C and D) cells to surfaces coated with 100 nM specified proteins with (A–C) or without (B–D) preincubation of the cells in the presence or absence of 5 μg/ml of an antibody against the α<sub>v</sub>β<sub>3</sub>-integrin or an isotype IgG control, or with or without 100 μM GRGDNP (RGD) or GRADSP (RAD) peptide is shown. For all experiments data are expressed as mean ± S.D. (error bars; n = 3).
OPN forms were utilized, thereby making a direct comparison in functionality more straightforward. Consistent with the present study, a direct comparison of two well characterized murine OPNs showed that a less phosphorylated isoform containing ~4 phosphates supported much higher adhesion of the MDA-MB-435 cells, also used in this study, than a more phosphorylated form containing ~21 phosphates (24). Monoclonal antibodies directed against the extreme C-terminal region of OPN were able to inhibit OPN-mediated cell adhesion (27). These findings are in agreement with the present study and support our conclusion that modification of the C terminus of OPN can influence the RGD-mediated interaction with the $\alpha_5\beta_3$-integrin.

The mechanism by which phosphorylation of the C terminus can regulate the integrin-mediated cell binding of OPN is not clear. It has been demonstrated that OPN can mediate inside-out signaling through the CD44 receptor resulting in increased RGD-dependent integrin adhesion (33). An unidentified region in the C-terminal half of OPN has been implicated as the CD44 binding site (34); hence, the extreme C terminus of OPN could potentially contain the CD44 binding site. If this were the case, phosphorylation of the C terminus could affect CD44 binding and thereby indirectly regulate RGD-dependent integrin activation. However, both OPN containing (uOPN and rOPN-N) as well as lacking (nmOPN and rOPN-228C) the C terminus promoted strong cell attachment, which indicates that involvement of CD44 inside-out signaling is not responsible for cell binding. OPN is considered to be an intrinsically disordered protein; however, the protein has been shown to exhibit a long range intramolecular interaction between the N- and C-terminal regions (10), and recently it was demonstrated that OPN consists of several segments, e.g. the integrin binding region, that display local secondary structure (11). In addition, $\beta$-sheet conformations of the region immediately after the RGD sequence and of the C-terminal part of OPN have been predicted to bring these two regions in proximity of each other (13). Phosphorylation of the C terminus could potentially have an influence on the suggested structural elements in the vicinity of the RGD sequence and thereby influence the integrin binding properties of the OPN.

The adhesion mediated by mOPN and rOPN-C increased 4–5-fold after thrombin and plasmin cleavage. This illustrates that the inhibition of OPN functionality by C-terminal modification can be eliminated by removal of the C terminus after cleavage close to the RGD sequence. The increased cell binding after proteolytic cleavage cannot only be ascribed to exposure of the RGD sequence because only small increases in adhesion after thrombin and plasmin cleavage of uOPN, rOPN-N, and rOPN-228C were observed. Furthermore, involvement of integrins recognizing the SVVYGLR motif exposed after cleavage of the OPNs is also not likely because all adhesion to both cleaved and uncleaved OPN was neutralized by RGD-containing peptides and antibodies against the $\alpha_5\beta_3$-integrin.

Adhesion of various cell types via the $\alpha_5\beta_3$-integrin to different full-length OPN forms has in some cases been demonstrated to be very negligible and increases considerably after protease cleavage (18–20), whereas other reports have observed either strong adhesion to full-length OPN or no difference between cleaved and uncleaved OPN (12, 26, 27). The novel mechanism observed in this study for melanoma and kidney cells, in which phosphorylation of the C terminus can inhibit the interaction between OPN and the $\alpha_5\beta_3$-integrin, might offer a possible explanation for these seemingly conflicting observations. However, whether the described mechanism also is true for adhesion to osteoclasts which are the classical cells that bind OPN via the $\alpha_5\beta_3$-integrin remains to be investigated. Different structural requirements for binding of several integrins to OPN have been demonstrated (12, 35). Further studies are needed to clarify whether modification of the C terminus affects the interaction between OPN and other integrins than $\alpha_5\beta_3$.

In summary, we have shown that modification of the extreme C terminus can regulate OPN interaction with the widely expressed $\alpha_5\beta_3$-integrin. The presented data indicate that enhanced adhesion mediated by OPN via this integrin after proteolytic cleavage probably is the result of removal of an inhibitory part of the protein, e.g. the C terminus rather than better exposure of the RGD sequence. This adds a new mechanism to the in vivo control of this multifunctional protein.

REFERENCES

1. Anborgh, P. H., Mutrie, J. C., Tuck, A. B., and Chambers, A. F. (2011) Pre-and post-translational regulation of osteopontin in cancer. J. Cell Commun. Signal. 5, 111–122
2. Frenzel, D. F., and Weiss, J. M. (2011) Osteopontin and allergic disease: pathophysiology and implications for diagnostics and therapy. Expert Rev. Clin. Immunol. 7, 93–109
3. Lund, S. A., Giachelli, C. M., and Scatena, M. (2009) The role of osteopontin in inflammatory processes. J. Cell Commun. Signal. 3, 311–322
4. Senger, D. R., Perruzzi, C. A., Gracey, C. F., Papadopoulos, A., and Tenen, D. G. (1988) Secreted phosphoproteins associated with neoplastic transformation: close homology with plasma proteins cleaved during blood coagulation. Cancer Res. 48, 5770–5774
5. Sodek, J., Ganss, B., and McKee, M. D. (2000) Osteopontin. Crit. Rev. Oral Biol. Med. 11, 279–303
6. Christensen, B., Petersen, T. E., and Sørensen, E. S. (2008) Post-translational modification and proteolytic processing of urinary osteopontin. Biochem. J. 411, 53–61
7. Schack, L., Lange, A., Kelsen, J., Agnholt, J., Christensen, B., Petersen, T. E., and Sørensen, E. S. (2009) Considerable variation in the concentration of osteopontin in human milk, bovine milk, and infant formulas. J. Dairy Sci. 92, 5378–5385
8. Fisher, L. W., Torchia, D. A., Fohr, B., Young, M. F., and Fedarko, N. S. (2001) Flexible structures of SIBLING proteins, bone sialoprotein, and osteopontin. Biochem. Biophys. Res. Commun. 280, 460–465
9. Gericke, A., Qin, C., Spévak, L., Fujimoto, Y., Butler, W. T., Sørensen, E. S., and Boskey, A. L. (2005) Importance of phosphorylation for osteopontin regulation of biomineralization. Calcif. Tissue Int. 77, 45–54
10. Yamaguchi, Y., Hanashima, S., Yagi, H., Takahashi, Y., Sasakawa, H., Kurihara, E., Ichiyama, A., Kon, S., Uede, T., and Kato, K. (2010) NMR characterization of intramolecular interaction of osteopontin, an intrinsically disordered protein with cryptic integrin-binding motifs. Biochem. Biophys. Res. Commun. 393, 489–491
11. Platek, G., Schedlbauer, A., Chemelli, A., Ozdowy, P., Coudry, V., Auer, R., Kontaxis, G., Hartl, M., Miles, A. J., Wallace, B. A., Glatter, O., Bister, K., and Konrat, R. (2011) The metastasis-associated extracellular matrix protein osteopontin forms transient structure in ligand interaction sites. Biochemistry 50, 6113–6124
12. Yokosaki, Y., Tanaka, K., Higashikawa, F., Yamashita, K., and Eboshita, A. (2005) Distinct structural requirements for binding of the integrins $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_v\beta_3$ to osteopontin. Matrix Biol. 24, 418–427
13. Kazanecki, C. C., Uziak, D. J., and Denhardt, D. T. (2007) Control of...
osteopontin signaling and function by post-translational phosphorylation and protein folding. J. Cell. Biochem. 102, 912–924
14. Bayless, K. J., and Davis, G. E. (2001) Identification of dual α5β1 integrin binding sites within a 38-amino acid domain in the N-terminal thrombin fragment of human osteopontin. J. Biol. Chem. 276, 13483–13489
15. Yokosaki, Y., Matsuura, N., Sasaki, T., Murakami, I., Schneider, H., Higashiyama, S., Saitoh, Y., Yamakido, M., Taooka, Y., and Sheppard, D. (1999) The integrin α5β1 binds to a novel recognition sequence (SV-VYGLR) in the thrombin-cleaved amino-terminal fragment of osteopontin. J. Biol. Chem. 274, 36328–36334
16. Schack, L., Stupilionis, R., Christensen, B., Kofod-Olsen, E., Skov Sørensen, U. B., Vorup-Jensen, T., Sørensen, E. S., and Hollósberg, P. (2009) Osteopontin enhances phagocytosis through a novel osteopontin receptor, the α5β1 integrin. J. Immunol. 182, 6943–6950
17. Qin, C., Baba, O., and Butler, W. T. (2004) Post-translational modifications of sibling proteins and their roles in osteogenesis and dentinogenesis. Crit. Rev. Oral Biol. Med. 15, 126–136
18. Agnihotri, R., Crawford, H. C., Haro, H., Matrisian, L. M., Havrda, M. C., and Liaw, L. (1994) Adhesive properties of osteopontin: regulation by a naturally occurring thrombin cleavage in close proximity to the GRGDS cell-binding domain. Mol. Biol. Cell 5, 565–574
19. Christensen, B., Schack, L., Klæning, E., and Sørensen, E. S. (2010) Osteopontin is cleaved at multiple sites close to its integrin-binding motifs in milk and is a novel substrate for cathepsin and clathrin D. J. Biol. Chem. 285, 7929–7937
20. Senger, D. R., Perruzzi, C. A., Papadopoulos-Sergiou, A., and Van de Water, L. (1994) Adhesive properties of osteopontin: regulation by a naturally occurring thrombin cleavage in close proximity to the GRGDS cell-binding domain. Mol. Biol. Cell 5, 565–574
21. Sørensen, E. S., Højrup, P., and Petersen, T. E. (1995) Post-translational modifications of bovine osteopontin: identification of 28 phosphorylation and 3 O-glycosylation sites. Protein Sci. 4, 2040–2049
22. Christensen, B., Nielsen, M. S., Haselmann, K. F., Petersen, T. E., and Sørensen, E. S. (2005) Post-translationally modified residues of native human osteopontin are located in clusters: identification of 36 phosphorylation and 5 O-glycosylation sites and their biological implications. Biochem. J. 390, 285–292
23. Keykhosravani, M., Doherty-Kirby, A., Zhang, C., Brewer, D., Goldberg, H. A., Hunter, G. K., and Lajoie, G. (2005) Comprehensive identification of post-translational modifications of rat bone osteopontin by mass spectrometry. Biochemistry 44, 6990–7003
24. Christensen, B., Kazanecki, C. C., Petersen, T. E., Rittling, S. R., Denhardt, D. T., and Sørensen, E. S. (2007) Cell type-specific post-translational modifications of mouse osteopontin are associated with different adhesive properties. J. Biol. Chem. 282, 19463–19472
25. Ek-Rylander, B., and Andersson, G. (2010) Osteoclast migration on phosphorylated osteopontin is regulated by endogenous tartrate-resistant acid phosphatase. Exp. Cell Res. 316, 443–451
26. Ross, F. P., Chappel, J., Alvarez, J. L., Sander, D., Butler, W. T., Farach-Carson, M. C., Mintz, K. A., Robey, P. G., Teitelbaum, S. L., and Cheresh, D. A. (1993) Interactions between the bone matrix proteins osteopontin and bone sialoprotein and the osteoclast integrin αvβ3 potentiate bone resorption. J. Biol. Chem. 268, 9901–9907
27. Kazanecki, C. C., Kowalski, A. J., Ding, T., Rittling, S. R., and Denhardt, D. T. (2007) Characterization of anti-osteopontin monoclonal antibodies: binding sensitivity to post-translational modifications. J. Cell. Biochem. 102, 925–935
28. Chen, Y. P., O’Toole, T. E., Leong, L., Liu, B. Q., Diaz-Gonzalez, F., and Ginsberg, M. H. (1995) β3-Integrin-mediated fibrin clot retraction by nucleated cells: differing behavior of αvβ3 and αvβ5. Blood 86, 2606–2615
29. Pear, W. S., Nolan, G. P., Scott, M. L., and Baltimore, D. (1993) Production of high titer helper-free retroviruses by transient transfection. Proc. Natl. Acad. Sci. U.S.A. 90, 8392–8396
30. Miura, Y., Kato, K., Takegawa, Y., Kurogochi, M., Furukawa, J., Shinohara, Y., Nagahori, N., Amano, M., Hinou, H., and Nishimura, S. (2010) Glyco-blotting-assisted O-glycomics: amonium carbamate allows for highly efficient O-glycan release from glycoproteins. Anal. Chem. 82, 10021–10029
31. Ek-Rylander, B., Flores, M., Wendel, M., Heinegård, D., and Andersson, G. (1994) Diphosphorylation of osteopontin and bone sialoprotein by osteoclastic tartrate-resistant acid phosphatase: modulation of osteoclast adhesion in vitro. J. Biol. Chem. 269, 14853–14856
32. Katayama, Y., House, C. M., Udagawa, N., Kazama, J. J., McFarland, R. J., Martin, T. J., and Findlay, D. M. (1998) Casein kinase 2 phosphorylation of recombinant rat osteopontin enhances adhesion of osteoclasts but not osteoblasts. J. Cell. Physiol. 176, 179–187
33. Lee, J. L., Wang, M. J., Sudhir, P. R., Chen, G. D., Chi, C. W., and Chen, J. Y. (2007) Osteopontin promotes integrin activation through outside-in and inside-out mechanisms: OPN-CD44V interaction enhances survival in gastrointestinal cancer cells. Cancer Res. 67, 2089–2097
34. Weber, G. F., Zawaideh, S., Hikita, S., Kumar, V. A., Cantor, H., and Ashkar, S. (2002) Phosphorylation-dependent interaction of osteopontin with its receptors regulates macrophage migration and activation. J. Leukocyte Biol. 72, 752–761
35. Ito, K., Kon, S., Nakayama, Y., Kurotaki, D., Saito, Y., Kanayama, M., Kimura, C., Diao, H., Morimoto, J., Matsu, Y., and Uede, T. (2009) The differential amino acid requirement within osteopontin in αv and α5 integrin-mediated cell binding and migration. Matrix Biol. 28, 11–19