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ARTICLES

Characterization of Anti-Osteopontin Monoclonal Antibodies: Binding Sensitivity to Post-Translational Modifications

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Abstract Osteopontin (OPN) is primarily a secreted phosphoglycoprotein found in a variety of tissues and body fluids. It has a wide range of reported functions, many of which are affected by the degree of post-translational modification (PTM) of the protein. These PTMs include phosphorylation, glycosylation, and cross-linking by transglutaminase. Here we describe the generation of unique monoclonal antibodies raised against recombinant OPN utilizing the OPN knockout mouse. The antibodies exhibit differential binding to OPN produced by different cell lines from the same species, as well to the multiple OPN forms in human urine. Most of the antibodies generated are able to recognize OPN produced by *ras*-transformed mouse fibroblasts, however only one antibody recognizes the more phosphorylated protein produced by the differentiating pre-osteoblast murine cell line MC3T3E1. Using a novel biopanning procedure combining T7 phage gene fragment display and protein G precipitation, we have epitope-mapped these antibodies. Several of the antibodies bind to regions of the OPN molecule that are phosphorylated, and one binds the region of OPN that is glycosylated. Using phosphorylated and non-phosphorylated peptides, we show that the binding of two antibodies to the C-terminal end of OPN is inhibited by phosphorylation of this region. In addition, these two antibodies are able to inhibit cell adhesion to recombinant and weakly modified OPN. The antibodies described herein may prove useful in determining the presence of modifications at specific sites and for identifying structural forms of OPN. Also, the sensitivity of these antibodies to PTMs suggests that caution must be taken when choosing anti-OPN monoclonal antibodies to detect this highly modified protein. J. Cell. Biochem. 102: 925–935, 2007.

Key words: gene fragment display; phage display; epitope mapping; T7 phage; phosphorylation; cell adhesion; protein G

The secreted phosphoglycoprotein osteopontin (OPN) is often considered to be a cell attachment protein due to its ability to bind to a number of integrin receptors via its RGD domain or a cryptic SVVYGLR site that is exposed after cleavage of OPN by thrombin. However, OPN can exist as a soluble cytokine as well as a mineralized-matrix associated protein. There is now considerable evidence that OPN is a ligand for certain variants of the hyaluronate receptor CD44 [Weber et al., 1996], but this interaction may require the presence of the β1 integrin [Katagiri et al., 1999]. An intracellular form of OPN has also been discovered that colocalizes with CD44 at the inner surface of the cell membrane [Zohar et al., 1997; Zhu et al., 2004]. OPN has been associated with a variety of functions including cell adhesion, migration, and survival, as well as activation of immune cells, inhibition of nitric oxide production, and

Abbreviations used: GFD, gene fragment display; OPN, osteopontin; PTM, post-translational modification; mAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene fluoride; ELISA, enzyme-linked immunosorbent assay; GST, glutathione S-transferase; PBS, phosphate buffered saline.

Christian C. Kazanecki and Aaron J. Kowalski contributed equally to this study.

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regulation of crystal formation and growth [reviewed in Denhardt et al., 2001a,b; Sodek et al., 2006]. Interestingly, OPN expression is upregulated in a number of pathological conditions, during tumorigenesis and in response to injury or inflammation.

Post-translational modification (PTM) of OPN, notably serine phosphorylation, plays an important role in many of OPN's functions. Other modifications include glycosylation, sulfation, and sialylation. Many sites of PTMs are conserved across species analyzed; however the degree of modification of the protein varies depending on the source tissue, cell line, or differentiation state of the cells used. For example, both the bovine and human milk forms have a large number of phosphorylated residues (~28 and 32 respectively) that are located in clusters [Sørensen et al., 1995; Christensen et al., 2005]. In contrast, rat bone OPN (after purification) contains only 10–11 phosphates [Keykhosravani et al., 2005]. In all cases, the degree of phosphorylation of any individual site is variable, so that the overall phosphorylation is extremely heterogeneous.

A great deal of evidence suggests that phosphorylation of OPN plays a large role in its physiological functions. Phosphorylation of OPN has been demonstrated to be required for its ability to inhibit smooth muscle cell calcification [Jono et al., 2000], to promote migration of cancer cells [Al-Shami et al., 2005] as well as adhesion and bone resorption by osteoclasts [Ek-Rylander et al., 1994; Razzouk et al., 2002]. Evidence has also suggested that phosphorylation of OPN determines its interactions with calcium phosphate (hydroxyapatite) or oxalate crystals and subsequent regulation of crystal growth and nucleation [Hoyer et al., 2001; Pampena et al., 2003; Gericke et al., 2005]. OPN produced by different sources may have alterations in specific modifications which can affect function. Crawford et al. [1998] determined that tumor-derived OPN inhibits macrophage function and increases the survival of metastasizing cells, whereas host cell-derived OPN is a more effective macrophage chemoattractant. Glycosylation of OPN may also play a role in modulating OPNs function, but much less is known about this potential mechanism.

Recent articles have described the creation of anti-OPN monoclonal antibodies raised against full-length protein or specific peptides [Hotta et al., 1999; Kon et al., 2000, 2002]. However, the ability of these antibodies to bind OPN from multiple species (i.e., mouse and human) and tissue sources has yet to be well established. Here we describe the creation and characterization of novel mouse anti-mouse OPN and mouse anti-human OPN monoclonal antibodies employing OPN-deficient mice. We took advantage of the OPN knockout mice as naïve hosts to produce antibodies to this highly conserved protein [Declerck et al., 1995]: this approach generated highly specific antibodies that recognize conserved regions of the molecule and hence detect OPN from multiple species at functional locations. We developed a novel technique employing a protein G immunoprecipitation procedure with T7 bacteriophage gene fragment display (GFD) to rapidly determine the OPN epitope recognized by the monoclonal antibodies. Some of the described antibodies are sensitive to PTM of their epitopes and are able to inhibit cell adhesion to OPN. These antibodies may be useful in determining the degree of PTM of OPN at specific sites.

MATERIALS AND METHODS

Anti-Osteopontin Monoclonal Antibody Production

Antibodies were prepared as described by Harlow and Lane [1999] and D'Alonzo et al. [2002]. Approximately 20 μg of recombinant histidine-tagged human OPN (prepared in *E. coli* and purified using hisBind resin (Novagen, Madison WI)) or recombinant glutathione S-transferase (GST)-tagged mouse OPN (prepared in *E. coli* and purified using glutathione Sepharose-4B [GE Healthcare, Piscataway, NJ]) mixed with Freund's complete adjuvant was injected subcutaneously into female OPN knockout mice [Rittling et al., 1998]. Two and 5 weeks later the mice were boosted with an equal amount of protein in Freund's incomplete adjuvant. Three weeks later (week 8) the mice were given a final boost 3 days prior to sacrifice and splenectomy. Spleen cells were fused to SP2/0 cells and fused cells were selected with hypoxanthine–aminopterin–thymidine medium. Positive clones were identified by enzyme-linked immunosorbent assay (ELISA) and Western blotting.

Antibodies were purified from hybridoma cell culture medium that was collected after allowing the cells to grow until complete saturation in flasks (approximately 2 weeks).

The cells were pelleted and the medium filtered through a 0.45 mm filter (Millipore 0.45 mm White HAWP, 26 mm). Antibodies were purified using Immobilized Protein G (Pierce Biotech, Inc., Rockford, IL).

Human OPN Gene Fragment Library

A human OPN gene fragment library was constructed employing the Novagen T7Select Phage Display system according to the manufacturer's instructions (Novagen). Briefly, an OPN plasmid which encodes the full length human OPN molecule (OPN1b/Harpo4) [Young et al., 1990; Rollo, 1995] was digested with DNase I. DNA fragments between approximately 50–150 bp were ligated into the T7Select415-1b vector using *Eco*RI adapters. In vitro packaging reactions were performed as described in the Novagen T7Select System Manual. PCR was performed using the primers T7SelectUP (5′-GGAGCTGTCGTATTCCAGTC-3′) and T7Select Down (5′-AACCCCTCAAGACCCGTTTA-3′), which flank the T7Select-1b multicloning site. Positive clones were defined as those with PCR products >30 bp larger than products from an empty vector. The concentration of unique phage in the T7 human OPN library was determined to be 8×10^5.

Biopanning and Epitope Determination

For epitope determination, the Novagen T7Select protocol was followed, and a modified biopanning protocol was developed (see Fig. 1). Approximately 10^{12} phage were pre-incubated at room temperature in hypoxanthine–thymidine medium with 10% Protein G-agarose beads (Pierce Biotech) to remove non-specific binding phage. Antibody-containing supernatants were simultaneously mixed at room temperature for 30 min with a concentration of 10% (v/v) Protein G beads. Both mixtures were then centrifuged at ~3,000*g*. The phage-containing supernatant was added to the precipitated antibody-protein G pellet and incubated with mixing for 1 h. After incubation and multiple phosphate buffered saline (PBS) washes, the mixture was centrifuged as previously and the pellet added directly to log phase BL21 *E. coli*. The bacteria were immediately added to 3 ml of molten top-agarose and plated. Positive plaques were identified by incubating plaque lifts with the desired antibody. Positive plaques were dispersed in 10 mM EDTA, pH 8.0 and heated for 10 min at 65°C to disrupt the phage. The mixture was then clarified by centrifugation at 14,000*g* and used for PCR amplification of the insertion region with the T7Select Up and Down primers. PCR products were sequenced and OPN amino acid sequences were determined and aligned.

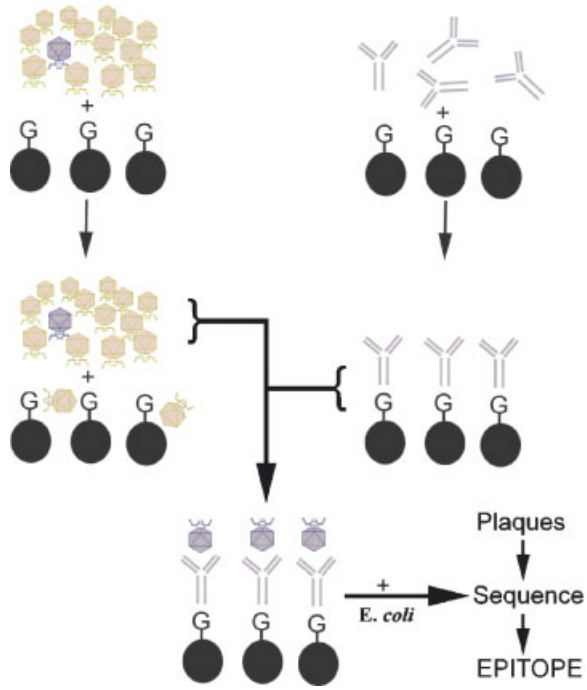

Fig. 1. Cartoon of novel biopanning protocol for antibody epitope determination using T7 phage and protein G beads. Protein G agarose beads are used to pre-clear the T7 phage library of non-specific binding phage (**left**). In a separate reaction, protein G beads are used to bind the antibody being assayed (**right**). The pre-cleared phage and the antibody-bead complexes are then incubated together to allow antibody-phage binding. The resulting complexes are washed, added directly to *E. coli* and plated for plaque formation. Positive plaques are identified by Western blotting, and the region containing the OPN insert is amplified by PCR and sequenced to determine the peptide expressed. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Cell Culture

MC3T3E1 subclone four cells (kind gift from Dr. R. Franceschi, University of Michigan) were maintained in α-MEM (Invitrogen Corp., Carlsbad, CA) with 10% FBS (Hyclone, Logan, UT), 5 μg/ml penicillin, 5 U/ml streptomycin, and 2 mM glutamine. For differentiation, cells were grown until confluent then switched to growth medium above containing 100 μg/ml ascorbic acid and 10 mM β-glycerophosphate (Sigma-Aldrich, St. Louis, MO) for an additional 10–12 days before generating conditioned medium. *Ras*-transformed fibroblasts (275-3-2), or the

parental non-transformed cells 3T3-275 [Wu et al., 2000], were maintained in DMEM (Mediatech, Inc., Herndon, VA) with 10% FBS (Hyclone), 5 μg/ml penicillin, 5 U/ml streptomycin, and 2 mM glutamine. Conditioned medium was generated from these confluent cell cultures by overnight incubation with serum-free medium.

Western Blotting

Freshly collected conditioned medium was used for Western blotting of OPN produced by cell lines. Typically 10–20 μl/lane of conditioned medium was fractionated by SDS–polyacrylamide gel electrophoresis (PAGE) with 12% gels. For purified proteins, equal amounts (typically 50 ng) in each lane were used. Protein was transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA), which were cut into strips and blotted with 1 μg/ml of purified monoclonal antibodies. Antibodies were purified from hybridoma-conditioned medium using protein G-agarose beads following the manufacturer's instructions (Pierce Biotech). Human urine was collected and dialyzed extensively against 0.1M NaCl, then concentrated approximately tenfold with Centriprep spin columns (Millipore). The equivalent of 50 μl of urine/lane was assayed by SDS–PAGE as above.

Peptide Affinity Assay

Biotin-tagged OPN peptides (kind gift from Dr. Lawrence Steinman, Stanford University) were added to Neutra-Avidin coated 96-well plates (Pierce Biotech) at 10 μg/ml. Anti-OPN monoclonal antibodies were added at 5 μg/ml and detected with a Alexafluor 594-conjugated anti-mouse IgG (Invitrogen) at 2 μg/ml. Fluorescence was detected using excitation/emission wavelengths of 584/612 nm with a Fluoroskan-Ascent fluorometer (Thermo Fisher Scientific, Inc., Waltam, MA).

Cell Adhesion Assay

Flat-bottom 96-well tissue culture-treated polystyrene microtiter plates (Corning, NY) were coated with 100 μl recombinant his-tagged human OPN [Rollo, 1995] (5 μg/ml) or fibronectin (2.5 μg/ml) in PBS at 4°C overnight and blocked with 1% BSA. MDA-MB-435 or 275-3-2 cells were trypsinized, washed, and resuspended in Dulbecco's modified Eagle's medium containing 1 mg/ml BSA. Cells (5×10^4) were added to coated wells and allowed to adhere for 3 or 3.5 h. Non-adhered cells were removed as described by Goodwin and Pauli [1995] with slight modifications. Cells were washed twice by pipetting 75μl Percoll wash solution (73% Percoll [Sigma-Aldrich], 0.9% NaCl) slowly down the sides of the wells and adherent cells were fixed by adding 50 μl fixative (10% glutaraldehyde in Percoll) in the same manner. The wash and fixative solutions were then washed from the wells with 2–3 washes of 100 μl PBS. Fixed cells were stained with 100 μl 0.1% crystal violet (25 min), washed with tap water and solubilized in 50 μl 0.5% Triton X-100 at least 1 h before reading at 570 nm in a MRX Revelation Reader (Thermo Labsystems).

RESULTS

Creation of Monoclonal Antibody Producing Hybridomas

OPN-deficient mice created in our laboratory mounted a strong immune response after immunization with recombinant OPN. Monoclonal antibody (mAb)-producing hybridoma cell lines created from multiple fusions were screened for their ability to bind OPN via ELISA. Over 1,000 clones were screened and 20+ lines were positive in our initial ELISA screen. Seven of these anti-OPN monoclonal antibodies will be detailed here and were selected based upon their performance in various immunoassays and their binding locations.

Epitope Determination

We used a GFD strategy to determine the epitopes of OPN bound by the antibodies which were positive in the initial screening [Kowalski, 2005]. We chose to employ the Novagen T7Select415 vector due to the robust nature of the T7 phage and high copy number (415) of the gene fragment on the phage surface. A human OPN expression plasmid was DNAse1 digested and fragments of 50–150 bps were cloned into the T7Select415 vector. A library of approximately 8×10^5 clones was created and amplified.

Initially, the standard manufacturer's biopanning protocol (Novagen) in which the antibodies were coated onto microtiter plates and then subjected to library panning, elution, and amplification of bound phage was employed. This method was successful for three of the monoclonal antibodies chosen for further study.

Since the highest affinity binders are the most difficult to elute, and to minimize background non-specific phage binding, we modified the biopanning protocol. In the modified protocol, selection for phage displaying antibody epitopes is performed in solution utilizing antibody-protein G agarose beads to isolate bound phage. These precipitated complexes can then be mixed directly with the host bacteria, and plated to form plaques (Fig. 1), thus avoiding the elution step. We observed over a 100-fold increase in positive binding phage and were able to epitope map antibodies that resulted in no positive clones utilizing the immobilized antibody methodology.

In total, we have mapped the epitopes of seven anti-OPN monoclonal antibodies. Interestingly, five of the seven antibodies mapped to the carboxy terminal half of the OPN molecule, and one antibody (AK1G4) mapped to the signal sequence (Fig. 2A). The antibodies AK1H3 and AK1G4 only recognize human OPN, while the other antibodies are able to bind both human and murine OPN. Figure 2B shows the results of the phage screening assay for antibody AK2A1. The screening yielded multiple peptides that were aligned to determine the minimal epitope in this case, PVA. Two of the antibodies, AK3D9 and AK7B4 recognized the same region in the extreme carboxy terminus of the molecule. The antibody AK10F6 did not yield multiple overlapping peptides from the T7 phage library screening, but yielded the same peptide multiple times.

Antibody Binding to Osteopontin

As the OPN-knockout mice were originally immunized with recombinant OPN, we hypothesized that the presence of PTMs on native OPN may prevent binding of some of the antibodies. The *ras*-transformed fibroblast cell line (275-3-2) produces abundant amounts of OPN (Fig. 3A), and this protein was recognized by all the antibodies tested (AK1G4, which

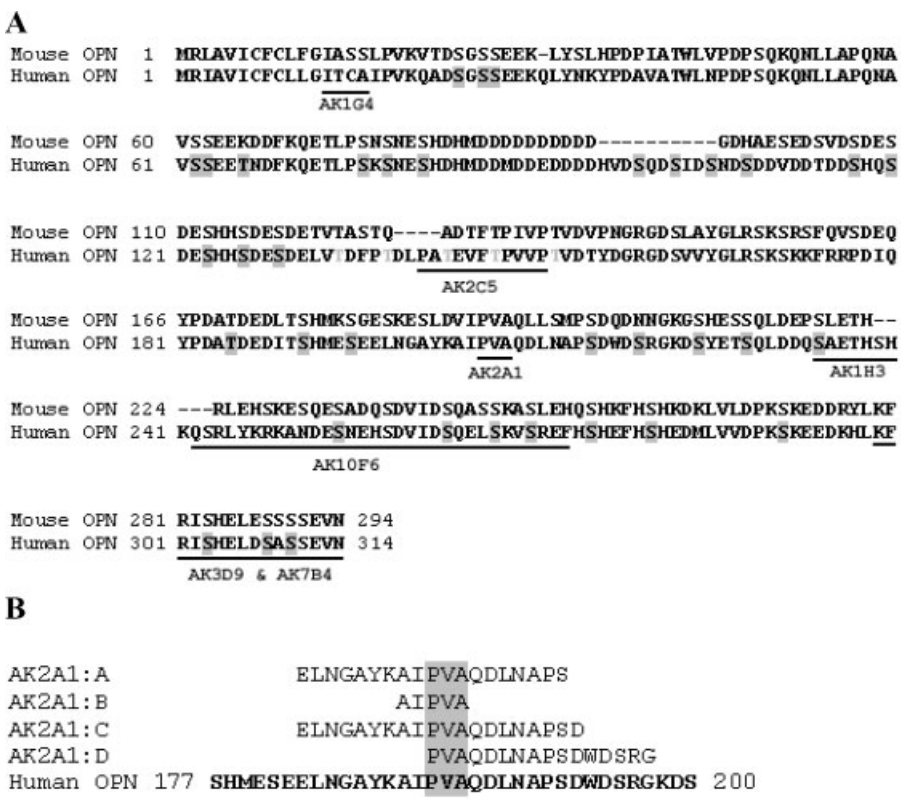

Fig. 2. Localization of monoclonal anti-OPN antibody epitopes. **A**: Alignment of the mouse and human OPN sequences showing the determined epitopes of our monoclonal antibodies. Antibody recognition sites are underlined with the antibody name below. Post-translational modifications of human OPN are taken from Christensen et al. [2005]. Phosphorylated residues are highlighted in gray. Glycosylated residues are written in gray. **B**: Peptides resulting from phage display screening demonstrating the determination of the epitope for antibody AK2A1.

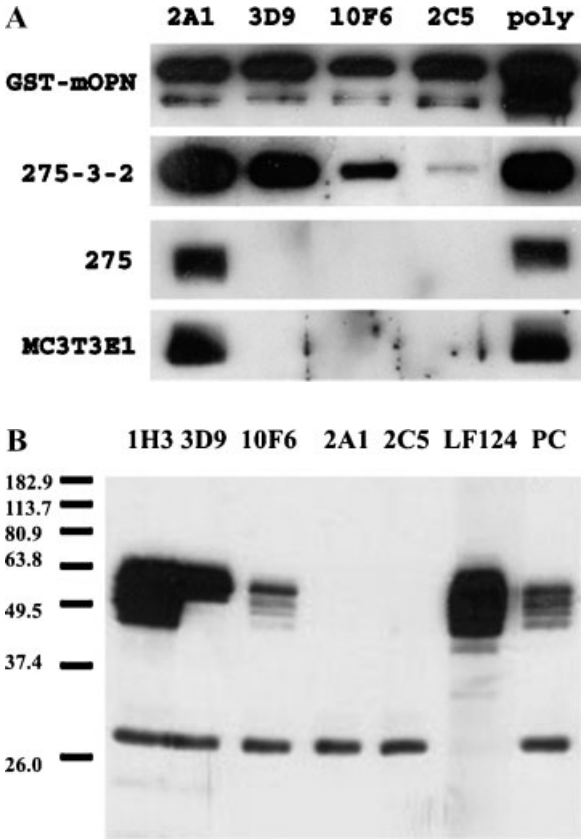

Fig. 3. Monoclonal antibody recognition of OPN. **A**: Western blotting results showing antibody recognition of murine OPN. Conditioned media (10 μl/lane) from various cell lines or 50 ng of recombinant murine OPN (GST-mOPN) were separated on 12% SDS–PAGE gels and transferred to PVDF membranes. The membranes were then cut into strips which were blotted with monoclonal antibodies at 1 μg/ml or polyclonal control at a 1:3,000 dilution (shown above each lane). 275-3-2: *ras*-transformed murine fibroblast cell line. 275: non-transformed murine fibroblast 3T3 cell line. MC3T3E1: pre-osteoblast cell line induced to differentiate for 12 days as described in Materials and Methods Section. **B**: Western blot of human urine detected with anti-OPN monoclonal antibodies. Urine was collected and dialyzed extensively against 0.1M NaCl before approximately tenfold concentration with Centriprep spin columns. Five microliters of the concentrated, dialyzed urine was assayed via SDS–PAGE and Western blotting with monoclonal antibodies AK1H3, AK3D9, AK10F6, AK2A1, and AK2C5 at 1 μg/ml. Polyclonal antibody LF124 (kindly provided by Dr. Larry Fisher, NIH) and polyclonal anti-OPN (recombinant) mouse serum were used at 1:750 and 1:3,000 respectively.

recognizes the signal sequence, and AK1H3 which does not bind murine OPN, were not tested). On the other hand, OPN from medium conditioned by differentiating MC3T3E1 pre-osteoblasts was recognized by only one of the antibodies (AK2A1). The antibodies AK2C5, AK3D9, and AK10F6 show little to no signal, even after longer exposure. The 275-3-2 cells were derived from the parental line 3T3-275 by transformation with ras^{val12} [Wu et al., 2000]. Interestingly, OPN from these non-transformed cells was similar to that from the osteoblast cells, and was only recognized by antibody AK2A1. We have recently shown that there are approximately 17 additional phosphate modifications on the MC3T3E1-produced OPN compared to the 275-3-2 *ras*-transformed fibroblast OPN [Christensen et al., 2007], which may explain the observed differences in antibody recognition.

Figure 3B shows Western blot results using the monoclonal antibodies to detect OPN present in human urine. Four closely migrating species of OPN are observed [Kleinman et al., 2004, A. Beshensky and J. Wesson personal communication] and the antibody showing the most intense binding to the four species was AK1H3. AK10F6 and two different polyclonal antibodies were able to recognize all four forms of OPN. AK3D9 binds the C-terminal region of OPN, and strongly recognized only the two higher molecular weight forms of OPN. Curiously, AK2A1 and AK2C5 showed no binding to urine OPN. This suggests that urine OPN is glycosylated (blocking AK2C5 binding), and may contain additional, possibly unique, modifications which are able to specifically inhibit AK2A1 binding. The approximately 30 kDa protein is a non-specific species cross-reacting with the goat anti-mouse secondary antibody used. From these results we hypothesized that the majority of the antibodies generated recognize sites subject to PTMs of OPN.

Antibody Binding Sensitivity to PTMs

In order to test this hypothesis, a peptide binding assay was employed. Synthetic peptides corresponding to the carboxy-terminus of the human OPN molecule (Fig. 4A), including both phosphorylated and non-phosphorylated forms, and a scrambled amino acid control peptide, were used in a modified ELISA system. After the biotinylated peptides were bound to Neutra-avidin plates, AK3D9 and AK7B4 antibody binding was determined. As shown in Figure 4B, the binding to the non-phosphorylated peptide was very strong, whereas binding to both the scrambled sequence peptide and the phosphorylated peptide was similar to background. Similar results were obtained

A

hOPN19: K-S-H-E-E-D-K-H-L-K-F-R-I-S-H-E-L-D-G-G-(CH2)-Biotin
hOPN20: H-L-K-F-R-I-S-H-E-L-D-S-A-S-S-E-V-N-G-G-(CH2)-Biotin
hOPN20S: L-D-E-H-S-S-A-I-S-R-S-F-E-K-V-L-N-H-G-G-(CH2)-Biotin
hOPN20P: H-L-K-F-R-I*p*S-H-E-L-D*p*S-A*p*S-S-E-V-N-G-G-(CH2)-Biotin

B

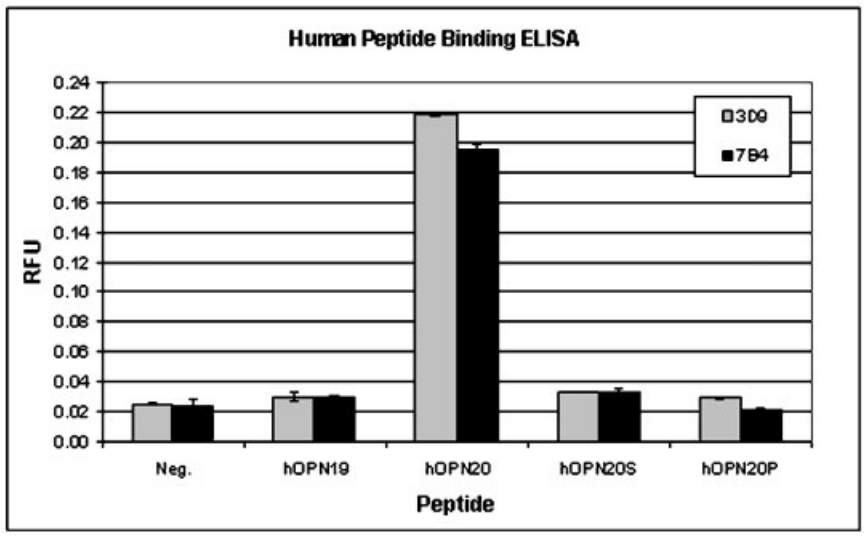

Fig. 4. Phosphorylation blocks 3D9 binding. **A**: Peptides used for the antibody-peptide binding assay kindly provided by Dr. Larry Steinman. **B**: Binding of antibodies AK3D9 and AK7B4 to synthetic human OPN peptides. The biotinylated peptides were coated onto Neutra-avidin plates at 10 μg/ml and detected with 5 μg/ml AK3D9 or AK7B4 monoclonal antibody following the manufacturer's instructions (Pierce Biotech). The secondary antibody used was Alexafluor 594 goat anti-mouse IgG (Molecular Probes) and fluorescence was detected using excitation/emission wavelengths of 584/612 nm. Data shown are representative of three independent experiments (n = 2/exp).

using phosphorylated and non-phosphorylated murine peptides (data not shown).

Figure 2A also shows the sequence of human milk OPN with PTMs noted as determined by Christensen et al. [2005]. The AK2A1 antibody is the only one whose epitope does not contain sites of PTM in the mature protein. The AK2C5 antibody binds an area that is O-glycosylated, explaining why this antibody exhibits decreased binding affinity to all forms of native OPN assayed. The remaining antibodies have been determined to bind to regions of the OPN molecule containing phosphorylations that may interfere with epitope recognition by these antibodies.

Inhibition of Cell Adhesion

Since many of the antibodies recognize the C-terminal half of the OPN molecule, where the CD44 receptor has been shown to mediate adhesion, the ability of our antibodies to inhibit the adhesion of cells to wells coated with recombinant OPN was then assessed. Antibodies were added to wells pre-coated with human recombinant his-tagged OPN (hisOPN) and allowed to bind OPN. The wells were then washed prior to adding human MDA-MB-435 breast cancer cells or 275-3-2 murine *ras*-transformed fibroblasts. The ability of the antibodies to block adhesion of these cells to hisOPN was assessed by comparing the adhesion to that in wells blocked with non-specific mouse IgG (Fig. 5). The results show that for both cell lines examined, the AK3D9 and AK7B4 antibodies are able to inhibit cell adhesion by approximately 40–50%, possibly by interfering with the CD44 receptor. Similar results were obtained with plates coated with native OPN purified from medium conditioned by *ras*-transformed fibroblasts (data not shown), which is weakly phosphorylated, containing an average of four phosphates per molecule [Christensen et al., 2007].

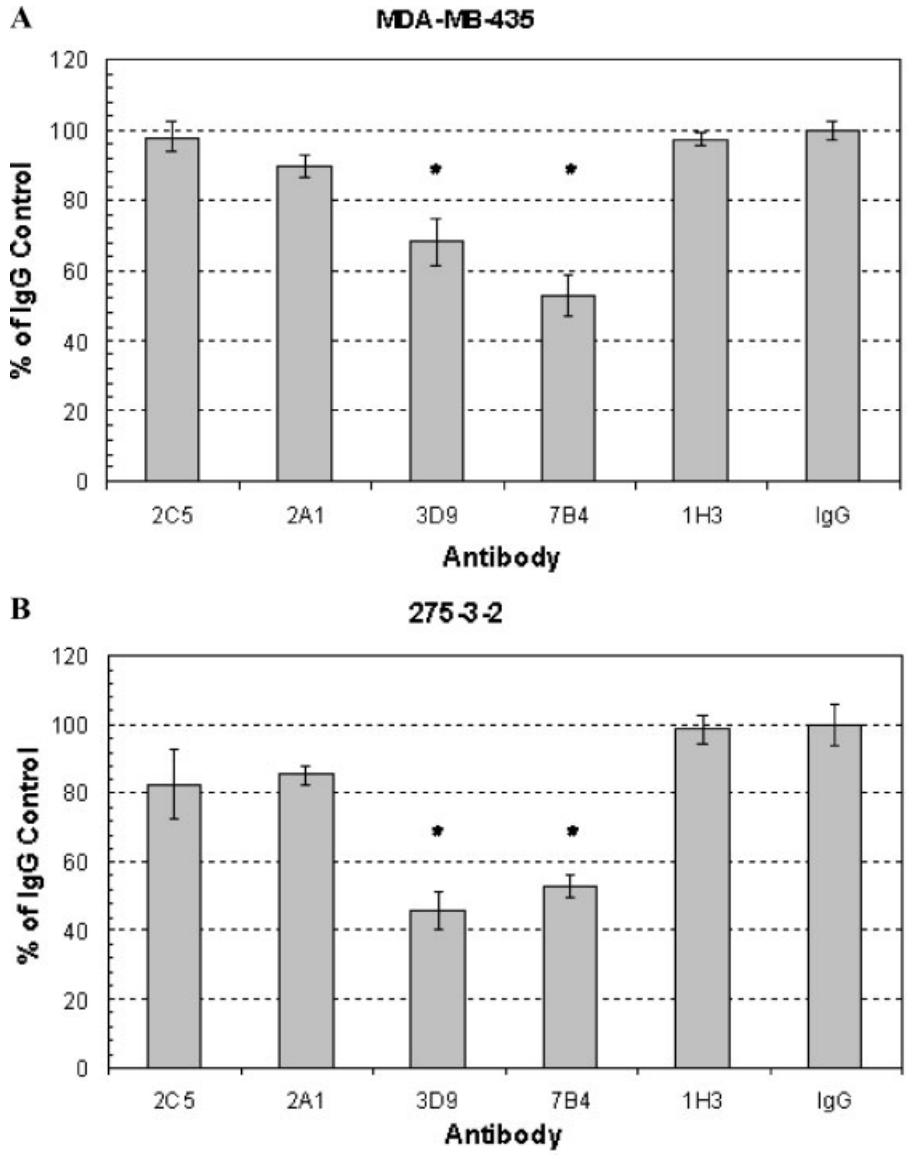

Fig. 5. Antibody inhibition of cell adhesion to recombinant human OPN. Tissue culture treated 96-well plates were coated with 150 μM human recombinant his-tagged OPN, then blocked with 1% BSA. Antibodies were then added at 125 μM and allowed to bind OPN for 2 h. The wells were then washed and 5×10^4 MDA-MB-435 (**A**) or 275-3-2 (**B**) cells were added and allowed to adhere for 3 or 3.5 h respectively. Non-adherent cells were removed by washing and adherent cells were quantitated by staining with crystal violet. Data are representative of four independent experiments for the MDA-MB-435 cell line and two independent experiments for the 275-3-2 cell line (n = 4). *$P < 0.001$ Student's *t*-test.

DISCUSSION

Phage display is a powerful technique that allows for rapid protein–protein interaction and provides a direct link between the phage phenotype and genotype [Dunn, 1996; Smothers et al., 2002]. Biopanning has the potential to enrich for a phage of interest that is rare in an initial library. However, high stringency conditions are required to enrich for phage with the highest affinities and to avoid low affinity phage and background [Smothers et al., 2002]. In many cases these elution conditions fail to release the most strongly bound phage. Some phage display vectors have incorporated protease cleavage sites to overcome this problem [Jestin et al., 2001]. This work describes a novel modification of the standard protocol for mapping linear epitopes of monoclonal antibodies using phage display. While others have combined protein G precipitation with phage display, this was not for

the purpose of epitope mapping, or to isolate antibody-bound phage [Cui et al., 2003]. The modification described here has several benefits compared to the standard microplate biopanning protocol. First, the use of protein G to precipitate antibodies allows for the use of complex mixtures such as ascites or hybridoma supernatant directly, without additional antibody purification. Second, phage can be precleared to lower non-specific binding, and antibody-phage complexes are allowed to form in solution, decreasing background. Third, since the bound phage remain infective, the protein G-antibody-phage complexes can be directly added to bacterial cultures and plated for plaque formation, thereby eliminating the troublesome elution step. Finally, this method is much faster than the standard protocol. This modified technique allowed for the identification of the epitopes of anti-OPN antibodies which were not identified using the standard microplate biopanning protocol, suggesting that this modification lends increased sensitivity to the protocol.

The antibodies described herein may be of use in determining the phosphorylation or glycosylation state of various regions of the OPN molecule. This has been demonstrated in the Western blots of conditioned medium from multiple cell lines shown in Figure 3. All of the antibodies in our panel are able to bind to OPN in medium conditioned by *ras*-transformed fibroblasts (275-3-2), however only AK2A1 is able to recognize OPN in medium conditioned by a non-transformed fibroblast line or from differentiated MC3T3E1 osteoblasts. These data suggest that the OPN produced by the overexpressing *ras*-transformed cells, is less post-translationally modified than protein made by non-*ras* transformed cells. OPN from a similar set of non-transformed and *ras*-transformed mouse NIH3T3 fibroblasts [Chambers et al., 1993] reacted similarly with the panel of antibodies as the 275 and 275-3-2 cells. OPN has been shown to be upregulated in a variety of cancers, and our data suggest that the OPN produced by certain tumors may have significantly fewer PTMs than that made by normal cells.

The antibodies described were also able to distinguish differences in OPN species found in human urine. For instance, the C-terminal antibody AK3D9 did not recognize the lower two of the four bands, suggesting that these bands are either more phosphorylated than the protein in the upper bands, or represent C-terminal truncated fragments of OPN. More work is required to distinguish these possibilities, but the ability of these antibodies to identify these subtle differences in protein structure highlights their usefulness.

Antibodies recognizing the extreme C-terminal region of the OPN molecule (AK3D9 and AK7B4) were also able to inhibit adhesion of a mouse and human cell line to recombinant human OPN. This region of OPN has not been previously implicated as having a role in cell adhesion, and may represent the binding site of the CD44 receptor to the C-terminal thrombin fragment of OPN, which has not been localized [Weber et al., 1996; Katagiri et al., 1999]. The AK3D9 binding region on OPN has been shown to be differentially phosphorylated depending on the cell line [Christensen et al., 2007], suggesting that phosphorylation of this region may have a role in regulating CD44 binding.

Uede and colleagues have developed antibodies raised against defined regions of the OPN backbone; one (1B20) recognizes the same area as the AK3D9 antibody described herein [Kon et al., 2000, 2002]. Like AK3D9, 1B20 shows similar differences in its ability to recognize the various forms of OPN contained in human urine. Interestingly, the C-terminal 1B20 antibody was demonstrated to bind OPN produced by transfected CHO cells both before and after acid-phosphatase treatment, suggesting that the C-terminal region of the OPN produced by these cells is not phosphorylated.

Recent work has suggested that different ELISA assays have variable sensitivities for plasma and urine OPN [Kon et al., 2000; Vordermark et al., 2006]. Our results suggest that some of this variability may result from the effects of PTMs on antibody reactivity, as well as changes in protein structure resulting from proteolytic cleavage. Our results provide important new reagents to begin to address this complication. Overall this study emphasizes the importance of the heterogenous nature of OPN PTMs, especially when comparing OPN produced by various sources, and stresses the need for careful selection of monoclonal antibodies used for the detection of OPN, particularly when using ELISA systems for quantitation.

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