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Osteopontin binding to the alpha 4 integrin requires highest affinity integrin conformation, but is independent of post-translational modifications of osteopontin

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ABSTRACT

Osteopontin (OPN) is a ligand for the α4ß1 integrin, but the physiological importance of this binding is not well understood. Here, we have assessed the effect of post-translational modifications on OPN binding to the α4 integrin on cultured human leukocyte cell lines and compared OPN interaction with α4 integrin to that of VCAM and fibronectin. Jurkat cells, whose α4 integrins are inherently activated, adhered to different preparations of OPN in the presence of Mn^{2+}: the EC50 of adhesion was not affected by phosphorylation or glycosylation status. Thrombin cleavage of OPN at the C-terminus of the α4 integrin-binding site also did not affect binding affinity. THP-1 cells express a low-affinity conformation of the integrin and adhered to OPN only in the presence of Mn^{2+} plus PMA or an activating antibody. This was in contrast to VCAM and fibronectin: THP-1 cells adhered to these ligands without integrin activation. Studies with ligand-induced binding site antibodies demonstrated that the SVVYGLR peptide of OPN bound to the α4 integrin with a similar affinity as the LDV peptide of fibronectin, suggesting that a high off-rate is responsible for the reduced binding of OPN to the low-affinity forms of this integrin. Together, the results suggest OPN has very low affinity for the α4 integrin on human leukocytes under physiological conditions.

1. Introduction

The α4β1 integrin is expressed on many leukocytes and is an important mediator of extravasation of leukocytes from the circulation to sites of inflammation through its binding to VCAM expressed on endothelial cells (Rose et al., 2002). The importance of this interaction in the maintenance of inflammation is illustrated by the effectiveness of natalizumab, a monoclonal antibody that blocks the α4β1 integrin, in suppressing the symptoms of multiple sclerosis by inhibiting extravasation of myelin-reactive T cells, thereby limiting the associated inflammation (Engelhardt and Briskin, 2005). The α4β1 integrin can be found in a series of activation states comprising a resting state, several intermediate states and a fully activated, unbent conformation (Chigaev et al., 2001; Chigaev and Sklar, 2012). Chemokines, such as SDF and FMLP, regulate α4 activation state, generating high-affinity binding to its ligand at sites of inflammation, where these chemokines are produced, and enhancing leukocyte tethering, adhesion and extravasation at such sites(Sanz-Rodriguez et al., 2001). Thus, the regulation of α4 integrin affinity represents an important mechanism for the regulation of inflammation.

Osteopontin (OPN) is a secreted phosphorylated glycoprotein that binds to several distinct integrins. While OPN is matrix associated in bone (McKee and Nanci, 1996), its association with the extracellular matrix in soft tissues remains controversial (Rittling et al., 2002). OPN is a high-affinity ligand for the αvβ3 and αvβ5 integrins (Hu et al., 1995a,b) and binds the α5β1 integrin (Barry et al., 2000b), all through its RGD sequence. Adjacent to the RGD, the SVVYGLR sequence of human OPN mediates binding to both α4β1 and α9β1 integrins. OPN is glycosylated in mammalian cells and is variably phosphorylated, with up to 36 phosphorylation sites identified on milk osteopontin (Christensen et al., 2005), while tumor cell-produced OPN averages only four phosphates per molecule; the degree of phosphorylation can in some cases regulate cell adhesion. Further, OPN is a substrate for thrombin and other proteases that cleave OPN just C-terminal to the

Abbreviations: OPN, osteopontin; MFI, mean fluorescence intensity; mOPN, milk osteopontin; rmOPN, recombinant mammalian OPN; rbOPN, recombinant bacterial OPN; RAA OPN, N-terminal OPN half with RGD mutated to RAA; SVV-BSA, CGGSVVYGLR peptide cross-linked to BSA; LDV-BSA, CGGGEILDVPST peptide cross-linked to BSA; FN, fibronectin; PMA, phorbol-12-myristate-13-acetate; BCECF, 2′,7′-bis-(2-Carboxyethyl)-5-(and-6)-Carboxyfluorescein; LIBS, ligand-induced binding site; BCA, bicinchoninic acid assay; CS-1, connecting segment-1 peptide of fibronectin.

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α4 binding site (Christensen et al., 2007, 2010). Osteopontin has also been implicated in the development of multiple sclerosis (Steinman, 2009), suggesting that its α4β1 interaction may be important in this disease.

The physiological role of the OPN- α4 interaction is still unclear. Since OPN is present physiologically with varying degrees of phosphorylation, we have asked if post-translational modification regulates the ability of OPN to interact with the α4 integrin on human leukocytic cell lines. Furthermore, we compared OPN binding to this integrin to the binding of the well-studied α4 ligands VCAM and fibronectin. We find that thrombin cleavage and post-translational modification do not regulate the affinity of the OPN α4–integrin interaction, but that the affinity of OPN is significantly lower than that of VCAM. Importantly, OPN only binds to the α4β1 integrin when the integrin is in its highest affinity state, and OPN at physiological concentrations cannot induce the high-affinity conformation. We conclude that while most forms of OPN can interact with the α4 integrin, any physiological function of OPN as a ligand for the α4 integrin would be limited to situations where the integrin is in the highest affinity state.

2. Results

2.1. Protein preparations

To determine whether post-translational modifications affect the binding of OPN to the α4 integrin, we selected a series of OPN forms with different modifications. Human milk OPN was used as the most highly phosphorylated form (mOPN; Christensen et al., 2005). The phosphorylation status of commercially available recombinant OPN prepared in mammalian cells (rmOPN, from R&D or Peprotech) is unknown, but it is likely a low level of phosphorylation; in addition, both these proteins are expected to be glycosylated. Bacterially produced OPN (rbOPN) is expected to be neither glycosylated nor phosphorylated. The mutated recombinant bacterially produced protein, RAA OPN, has the RGD sequence mutated to RAA and includes only the N-terminal half of the protein, terminating at the C-terminal arginine residue of the α4 binding sequence (Ito et al., 2009). To further isolate the SVVYGLR sequence, we prepared SVVYGLR peptide cross-linked to BSA (SVV-BSA) and its corollary for fibronectin EILDVPST-BSA (LDV-BSA). Full-length OPN protein was cleaved with thrombin which cuts OPN into two parts with similar molecular weight (N-terminal half = 17094; C-terminal half = 16831). The purity and migration patterns of these proteins were analyzed by SDS–PAGE (Fig. 1). Although OPN has a molecular weight of about 35,000 Da, it migrates more slowly on SDS–PAGE than expected, largely because of its low Pi, and this migration difference is exacerbated by phosphorylation. mOPN migrates more slowly than recombinant OPN made in cultured cells (rmOPN), suggesting that rmOPN is not highly phosphorylated. This difference in migration is also seen in the thrombin-cleaved forms of these molecules. Bacterially produced OPN, which is expected to be neither phosphorylated nor glycosylated, migrates at a similar position as rmOPN, further supporting a low level of phosphorylation of rmOPN. RAA OPN migrates at around 33 kDa: lower bands are degradation products, while the 66- kDa band is a contaminating bacterial protein (determined by mass spectrometry). The peptide-BSA conjugates migrate as a smear more slowly than BSA confirming the presence of multiple copies of the peptides (calculated ratio = average of 14 peptides/BSA molecule).

To confirm the glycosylation status of rmOPN, proteins were separated by gel electrophoresis and stained for glycosylations using a modified periodic acid–Schiff technique. Strong staining of mOPN was observed with weaker but clearly detectable staining of rmOPN. rbOPN as expected did not stain under these conditions (Fig. 1B).

The expression of the α4 and other OPN-binding integrins on Jurkat and THP-1 cells was confirmed by flow cytometry. Both cell lines express the α4 and α5 integrins, but no expression of αv or α9 integrins could be detected (Fig. 1B and C). Thus, the only integrin that can bind OPN through the RGD sequence on these cells is the α5 integrin.

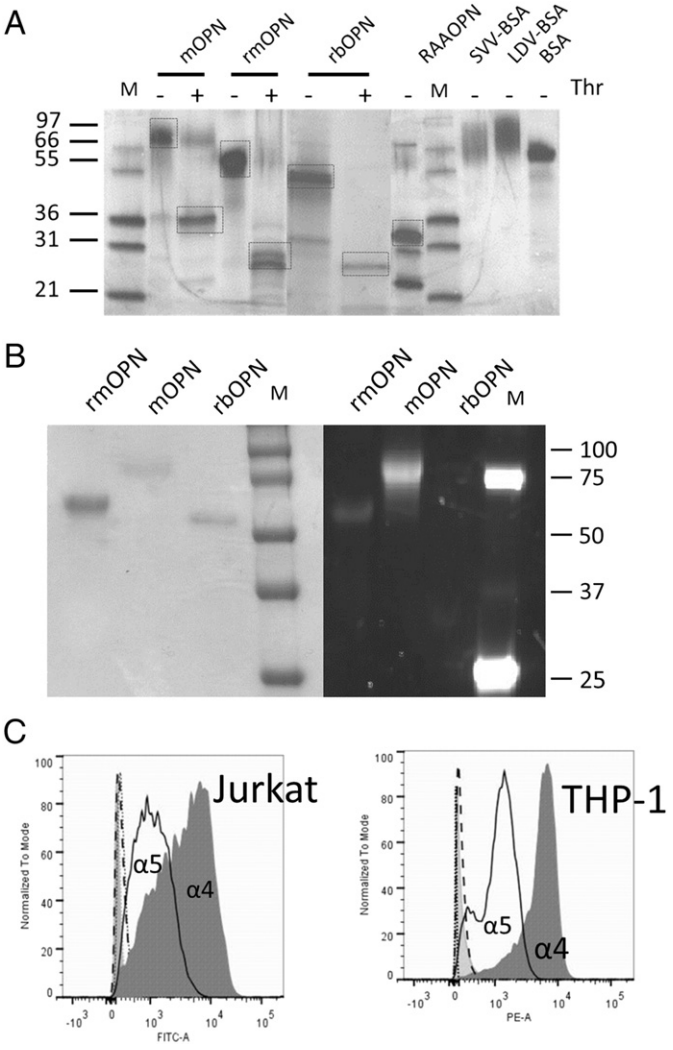

Fig. 1. (A) SDS–PAGE analysis of OPN proteins used in this work. Approximately 1 μg of each OPN preparation was separated on a gradient gel and stained with non-ammoniacal silver stain. mOPN—milk OPN, rmOPN—recombinant mammalian OPN, rbOPN—recombinant bacterial OPN, M—molecular weight marker. Proteins were cleaved with thrombin (Thr) as indicated. The OPN bands of interest in each lane are indicated by boxes. For rbOPN and RAA OPN, bands migrating below the main band are OPN fragments, while the protein migrating just above 66 kDa is a bacterial protein (determined by mass spectrometry). The lanes containing rbOPN are from a different gel. (B) Staining of OPN proteins for glycosylation. mOPN, rmOPN, and rbOPN as indicated were separated on SDS–PAGE gels and stained with Coomassie blue (left panel) or with Pro-Q Emerald stain, which stains glycoproteins (right panel). (C) FACS analysis of OPN-binding integrin expression on Jurkat and THP-1 cells. Cells were stained with antibodies to integrins αv (…), α9 –, α5 (–), α4 (dark gray shading), or isotype control, light gray shading. Secondary antibody was anti-mouse labeled with FITC (for Jurkat cells) or PE (for THP-1 cells).

2.2. α4 integrin interacts with different forms of OPN

The Jurkat T cell leukemia cell line (Schneider et al., 1977) has been previously shown to bind to recombinant N-terminal OPN, and adhesion of these cells to this form of OPN was exclusively through the α4 integrin (Pepinsky et al., 2002). Therefore, this cell line was used to determine the structural requirements for OPN binding to the α4 integrin by using a cell adhesion assay. Although Jurkat cells were reported to bind to OPN in the presence of Ca^{2+} and Mg^{2+} only (Pepinsky et al., 2002), in our hands, there was no adhesion of these cells to any of these forms of OPN unless the integrins were activated with either MnCl2 (1 mM) or PMA (50 μg/ml) or both (Fig. 2A and data not shown). This